APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1980, p. 81-84
0099-2240/01-0081/04$02.00/0

Vol. 39, No. 1

N_2O Reduction by *Vibrio succinogenes*

TADASHI YOSHINARI

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

Vibrio succinogenes grew anaerobically at the expense of formate oxidation, with nitrous oxide (N_2O) serving a terminal oxidant. N_2O was quantitatively reduced to dinitrogen (N_2). In the presence of 5×10^{-2} atm (ca. 5 kPa) of acetylene (C_2H_2), which inhibits the reduction of N_2O, growth of *V. succinogenes* was completely inhibited. Nitrate was reduced to nitrite or to ammonia, depending on the extent of availability of formate, but N_2 was not produced by reduction of nitrate. During the reduction of nitrate to ammonia, all eight electrons transported to a molecule of nitrate appeared to be coupled for energy-yielding reactions.

Vibrio succinogenes, a gram-negative anaerobe isolated from bovine rumen fluid, gains energy for growth by coupling the oxidation of hydrogen or formate to the reduction of fumarate or nitrate. When nitrate was the electron acceptor, nitrite accumulated in growing cultures, but nitrate was reduced to ammonia by resting cells (21).

We now report that *V. succinogenes* can gain energy for growth by the stoichiometric reduction of N_2O to N_2 with formate as an electron donor. However, no N_2 was produced by reduction of nitrate. These findings provide the first evidence that organisms exist which produce N_2 from an intermediate in the denitrification pathway but not from nitrate.

MATERIALS AND METHODS

Organism and growth conditions. *V. succinogenes* was provided by M. J. Wolin of this division. The organism was grown at 37°C in VSY-4 medium, modified from Kafkewitz (10): 0.4% yeast extract, 100 mM sodium formate, 25 mM potassium phosphate, and 10 mM sodium succinate. The medium was adjusted to pH 7.0 and autoclaved. Autoclaved 0.5% sodium thioglycolate was added aseptically to a final concentration of 0.05%. An alternative medium containing 0.1% yeast extract was designated VSY-1.

A 100-ml lot of VSY-4 medium was placed in a 500-ml reagent bottle, and 1 ml of inoculum, grown in the identical medium, was added. The head space was then evacuated, and N_2O was added aseptically through a sterile cotton filter, sterile air-tight valve, and a sterile 18-gauge needle plunged through the silicone rubber stopper of the bottle. Evacuation and refilling with N_2O were repeated three times.

The culture was incubated at 37°C for 18 h with agitation. The cells of early stationary phase thus obtained were used as the inoculum for the following experiments.

Preparation of experimental bottles. A 9-ml portion of VSY-4 or VSY-1 was placed in a 50-ml bottle closed with a gray butyl-rubber stopper, sealed with an aluminum cap, and autoclaved. Then 1 ml of sterile 0.5% thioglycolate was added aseptically with a hypodermic needle and syringe. The head space was flushed for 10 min with helium flowing at a rate of 300 ml/min through a sterile filter, and 0.1 ml of the inoculum was added. An appropriate amount of N_2O, H_2, or C_2H_2 was added to the head space with a hypodermic needle and syringe, replacing a portion of the volume of helium in the bottle (see Table 1). Bottles with media containing 10 or 100 mM potassium nitrate were prepared similarly with helium in the head space. Three identical samples were prepared for each treatment, and unless otherwise stated, they were incubated at 37°C for 18 h on a rotary shaker (150 rpm). Results represent average values from three replicate experiments.

Gas-chromatographic measurements of N_2, N_2O, and C_2H_2. A Gow-Mac Series 500 gas-chromatograph equipped with glass bead thermister detectors (Gow-Mac Instrument Co., Madison, N.J.) was used for gas analyses. It was operated at 50°C with helium as the carrier gas at a flow rate of 20 ml/min. For N_2 analysis, a 300-cm stainless-steel column (inner diameter, 0.2 cm) packed with molecular sieve 5A (60 to 80 mesh) was used. For N_2O and C_2H_2 determinations the same size column was packed with Porapak Q (50 to 80 mesh).

The preparation of gas standards and the analytical procedures employed have been described (23). A calibration curve for each standard gas was prepared by plotting the averages of triplicate determinations. The average coefficient of variation of both standards and samples was 2%. Reported values of gas volume are at 25°C and 1 atm (ca. 101 kPa).

Determinations of ammonia and nitrite. Dissolved ammonia (NH_3 and NH_4^+) was estimated by the nesslerization method (1), and nitrite was estimated by the modified Griess method (1).

Measurements of cell density and protein. Cell density was estimated with a spectrophotometer (Spectronic 70; Bausch & Lomb, Inc., Rochester, N.Y.) at 660 nm and a light path of 1 cm. Data presented as E_{660} in Results were calculated by subtracting the initial density at inoculation from the final density after incubation. The average coefficient of variation was 8%. Protein concentration was determined by the method of Lowry et al. (13), with bovine albumin as

the standard. An E_{660} of 0.100 corresponded to 43 μg of protein per ml, with average coefficient of variation of 5% among cells grown with either N_2O or nitrate.

RESULTS

The growth of *V. succinogenes* with N_2O or nitrate as an electron acceptor and formate or H_2 as an electron donor is shown in Table 1. Growth with N_2O and formate was comparable to that with nitrate and formate (Table 1). Growth with nitrate and formate was more than twice as much as that with nitrate and H_2. However, growth with N_2O and H_2 was approximately 1/10 the growth with N_2O and formate. Since the inoculum used for the experiment had been grown in N_2O-formate medium, it is possible that the organism may not have been adapted to using H_2 as electron donor for the reduction of the nitrogen oxides. This conjecture is supported by the fact that the E_{660} with N_2O and H_2 increased from 0.03 to 0.14 after 40 h of incubation. Cell density with N_2O and formate did not increase because the N_2O was depleted after the 18-h incubation.

In the presence of formate, reduction of N_2O to N_2 during the growth of *V. succinogenes* was quantitative (Table 2). Succinate (5 mM) stimulated growth approximately 70% on the basis of N_2O consumption. A further increase in succinate concentration did not significantly increase total growth. Although Niederman and Wolin found that succinate is essential for the growth of *V. succinogenes* when nitrate is used as an electron acceptor (14), succinate is apparently not essential when VSY-1 medium is used with N_2O.

C_2H_2, a specific inhibitor of N_2O reduction by denitrifiers (2, 5, 24), has been used successfully to measure potential and in situ rates of denitrification in soil (16, 17, 23), in lake sediments (3), and in marine sediments (2, 18, 19). In VSY-4 medium with N_2O, although only a slight inhibitory effect on growth of *V. succinogenes* was observed at 10^{-3} atm (ca. 10^{-1} kPa) of C_2H_2 (Table 3), growth at the expense of N_2O reduction was completely inhibited at 5×10^{-2} atm of C_2H_2.

TABLE 1. *Growth of V. succinogenes with various combinations of electron donors and acceptors*

Substrate(s)[a]	E_{660} at 18 h
H_2	0.00
Formate	0.00
N_2O	0.01
$N_2O + H_2$	0.03[b]
N_2O + formate	0.29
Nitrate	0.01
Nitrate + H_2	0.15
Nitrate + formate	0.36

[a] Basal medium was similar to VSY-1, except for variations in electron donors and acceptors as indicated. The head space of experimental bottles contained 20 ml of N_2O or H_2 or both, equivalent to 82 mM on the basis of the volume of liquid medium (10 ml) in the bottle. On the same basis, formate and nitrate were 100 mM.

[b] After 40 h of incubation, the E_{660} increased to 0.14.

TABLE 2. *Effect of succinate on growth of V. succinogenes and reduction of N_2O to N_2 in the presence of formate*[a]

Succinate (mM)	E_{660}	N_2O consumed (ml)	N_2 formed (ml)
0	0.20	6.6	7.0
5	0.33	11.3	11.8
10	0.33	11.3	12.4
50	0.39	12.5	12.5

[a] *V. succinogenes* were grown with 20 ml of N_2O(82 mM) in VSY-1 medium containing 100 mM formate and various concentrations of succinate.

To clarify previous results regarding nitrite or ammonia formation from nitrate reduction (21), *V. succinogenes* was grown with 10 and 100 mM nitrate. If an enzyme system exists for production of trace N_2 from an intermediate of nitrate reduction, then N_2O, a precursor of N_2, should accumulate in cultures inhibited with C_2H_2 (see Table 3). This possibility was explored by incubating replicate samples with 5×10^{-2} atm of C_2H_2. When *V. succinogenes* was grown with 100 mM nitrate, the only end product was nitrite, which was recovered quantitatively (Table 4). However, the organism reduced 10 mM nitrate to ammonia quantitatively. Not even a trace of N_2O was detected in the presence of C_2H_2, indicating that gaseous nitrogen was not produced from nitrate reduction by *V. succinogenes*.

Examination of the data in Tables 1 and 4 reveals that cell growth, based on the cell density measurements (E_{660}), was directly correlated with the quantity of electrons transported to nitrate or N_2O. The E_{660} ratio of N_2O- and nitrate-grown cells was 0.81 (Table 1). Since almost all the added N_2O (+1) and nitrate (+5) were reduced to N_2 (0) and nitrite (+3), respectively, after the 18-h incubation, the ratio of electrons transported to 82 mM N_2O and to 100 mM nitrate was also $[(2 \times 82) \times 1]/(100 \times 2)$, or 0.82. Similarly, when 10 or 100 mM nitrate (+5) was reduced to ammonia (−3) or nitrite (+3) quantitatively, the ratio of electrons transported to 10 or 100 mM nitrate was $(8 \times 10)/(2 \times 100)$, or 0.40, which is identical with the E_{660} ratio (0.26/0.65) of these two systems (Table 4). These analyses suggest that the extent of nitrate reduction to nitrite or to ammonia by *V. succinogenes* may be regulated by the relative abun-

TABLE 3. *Inhibition of N_2O reduction by C_2H_2 in V. succinogenes*[a]

C_2H_2[b]	E_{660}	Inhibition (%)	N_2O consumed (ml)	Inhibition (%)
0	0.82	0	19.4	0
10^{-3} (10^{-1})	0.80	3	16.6	14
10^{-2} (1)	0.29	65	4.7	76
5×10^{-2} (5)	0.01	99	0	100
10^{-1} (10)	0.01	99	0	100

[a] *V. succinogenes* were grown with 20 ml of N_2O (82 mM) in VSY-4 medium.

[b] Each value represents atmospheres. Approximate metric equivalents (kiloPascals) are given in parentheses.

TABLE 4. *Ammonia or nitrite accumulation from nitrate reduction by V. succinogenes*[a]

Nitrate (mM)	C_2H_2 (5×10^{-2} atm)[b]	E_{660}	Final concn (mM)			
			Nitrite	Ammonia	N_2O	N_2
10	−	0.26	0	9.5[c]	0[d]	0
10	+	0.26	0	10.0[c]	0	0
100	−	0.65	94[c]	ND[e]	0	0

[a] *V. succinogenes* were grown in VSY-4 medium with either 10 or 100 mM potassium nitrate. To one group of bottles with 10 mM nitrate, 2.5 ml of C_2H_2 was added.

[b] Approximately 5 kPa.

[c] Ammonia concentrations were corrected by subtracting the concentration in the uninoculated medium. Nitrite in the uninoculated medium was negligible, as was nitrite in the inoculated control without nitrate.

[d] Minimum detectability of N_2O by gas chromatography was 8×10^{-3} mM (2 μl of N_2O in a 50-ml bottle).

[e] ND, Not determined.

dance of the electron donor.

Nitric oxide (NO) is a precursor of N_2O during denitrification (13). Since *V. succinogenes* reduces nitrate to ammonia and reduces N_2O to N_2, we investigated whether NO serves as a precursor of N_2O and not of ammonia. *V. succinogenes* did not grow with 4 to 40 mM NO substituted for N_2O in VSY-4 medium. No further study was carried out to attempt to find conditions for growth of the organisms with NO.

DISCUSSION

Nitrate can serve (i) as sole source of nitrogen for the synthesis of cellular materials of many microorganisms (assimilatory nitrate reduction) and also as (ii) an electron acceptor (nitrate respiration or dissimilatory nitrate reduction) for some facultative and obligate anaerobes (15). Dissimilatory nitrate reduction by these microorganisms can be classified into several types. Some organisms such as *Escherichia coli* reduce nitrate only to nitrite (4, 20), and some organisms such as *Clostridium perfringens* and *Klebsiella aerogenes* reduce nitrate to ammonia but not to N_2 (9, 22, 7). The classical denitrifying bacteria reduce nitrate, nitrite, and N_2O to N_2. Some strains of *Pseudomonas fluorescens* and *P. chlororaphis* (6), and *Corynebacterium nephridii* (8), reduce nitrate only to N_2O, but cannot produce N_2. *V. succinogenes* has a unique array of pathways for dissimilatory reduction of the oxides of nitrogen. It produces ammonia but not N_2O or N_2 from nitrate. However, it retains a similarity to the classical denitrifiers with its ability to reduce N_2O to N_2.

It was demonstrated that the reduction of nitrate to nitrite by *K. aerogenes* was the only step in the reduction of nitrate to ammonia that was coupled with an energy-yielding reaction (7). Also, the results of Hasan and Hall (9) suggest that only a partial step of nitrate reduction by *C. perfringens* was coupled with an adenosine triphosphate-yielding reaction. Although two different pathways for reduction of nitrogen oxides are operative in *V. succinogenes*, cell growth from the reduction of nitrate to nitrite, nitrate to ammonia, and N_2O to N_2 were in a constant ratio to the quantity of electrons transported to these nitrogen oxides. This suggests that the growth efficiency of *V. succinogenes* is primarily controlled by the coupling of electron transport from formate to these nitrogen oxides and that not only the reduction of nitrate to nitrite but also further reduction to ammonia are coupled with energy-yielding reactions. Since this would be the first observation that an organism can use eight electrons transported to a nitrate molecule for energy-yielding reactions, it will be interesting to investigate the growth yields from the reductions of nitrate to nitrite and nitrite to ammonia by *V. succinogenes*. Koike and Hattori (11) found almost identical growth yields of *P. denitrificans*, when expressed on an electron basis, in continuous culture with nitrate, nitrite, or N_2O. They concluded that oxidative phosphorylation occurs to a similar extent in the electron-transport chains associated with the reduction of nitrate to nitrite, nitrite to N_2O, and N_2O to N_2.

Our findings may provide a new perspective on the nitrogen cycle. Since N_2O is a natural agent for the destruction of ozone in the stratosphere, many studies on the sources and sinks of N_2O, as well as measurements of atmospheric N_2O concentrations, have been reported in recent years. Although the removal of N_2O from the environment has been attributed to the activity of denitrifiers, the activity of non-denitrifiers such as *V. succinogenes* that reduce N_2O to N_2 may also have a potential significance as

an N_2O sink in the environment.

It is obvious that the accumulation of ammonia in the environment by assimilatory nitrate reduction is far less significant than by dissimilatory nitrate reduction. Recent findings indicate that ammonia is produced more often than N_2 during nitrate reduction in coastal marine sediments (12, 18). Therefore, it would be useful to find organisms in various ecosystems that are capable of reducing both nitrate to ammonia and N_2O to N_2 and evaluate the significance of their nitrogen transformations. A curved-rod bacterium similar to *V. succinogenes* in its ability to reduce fumarate, aspartate, nitrate, and N_2O in the presence of H_2 or formate was isolated recently from a sewage sludge sample in this laboratory. Characterization of this organism by various biochemical tests and a study of conditions for its optimal growth are in progress.

ACKNOWLEDGMENTS

I thank M. J. Wolin for his advice and encouragement throughout this investigation and A. Ballentine and J. Percent for technical assistance.

This work was supported in part by Public Health Service Biomedical Research Support grant RDSR 5S07 RR05649-12, National Institutes of Health.

LITERATURE CITED

1. **American Public Health Association.** 1975. Standard methods for the examination of water and wastewater. American Public Health Association Inc., New York.
2. **Balderston, W. L., B. Sherr, and W. J. Payne.** 1976. Blockage by acetylene of nitrous oxide reduction in *Pseudomonas perfectomarinus*. Appl. Environ. Microbiol. **31**:504-508.
3. **Chan, Y.-K., and R. Knowles.** 1979. Measurement of denitrification in two freshwater sediments by an in situ acetylene inhibition method. Appl. Environ. Microbiol. **37**:1067-1072.
4. **Cole, J. A., and J. W. T. Wimpenny.** 1968. Metabolic pathways for nitrate reduction in *Escherichia coli*. Biochim. Biophys. Acta **162**:39-48.
5. **Fedorova, R. I., E. I. Milekhina, and N. I. Il'Yukhina.** 1973. Possibility of using the "gas-exchange" method to detect extraterrestrial life: identification of nitrogen-fixing organisms. Akad. Nauk. SSSR. Izvestiia Ser. Biol. **6**:797-806.
6. **Greenberg, E. P., and G. E. Becker.** 1977. Nitrous oxide as end product of denitrification by strains of fluorescent pseudomonads. Can. J. Microbiol. **23**:903-907.
7. **Hadjipetrou, L. P., and A. H. Stouthamer.** 1965. Energy production during nitrate respiration by *Aerobacter aerogenes*. J. Gen. Microbiol. **38**:29-34.
8. **Hart, L. T., A. D. Larson, and C. S. McCleskey.** 1965. Denitrification by *Corynebacterium nephridii*. J. Bacteriol. **89**:1104-1108.
9. **Hasan, S. M., and J. B. Hall.** 1975. The physiological function of nitrate reduction in *Clostridium perfringens*. J. Gen. Microbiol. **87**:120-128.
10. **Kafkewitz, D.** 1975. Improved growth media for *Vibrio succinogenes*. Appl. Microbiol. **29**:121-122.
11. **Koike, I., and A. Hattori.** 1975. Energy yield of denitrification: an estimate from growth yield in continuous cultures of *Pseudomonas denitrificans* under nitrate-, nitrite- and nitrous oxide-limited conditions. J. Gen. Microbiol. **88**:11-19.
12. **Koike, I., and A. Hattori.** 1978. Denitrification and ammonia formation in anaerobic coastal sediments. Appl. Environ. Microbiol. **35**:278-282.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
14. **Niederman, R. A., and M. J. Wolin.** 1972. Requirement of succinate for the growth of *Vibrio succinogenes*. J. Bacteriol. **109**:546-549.
15. **Payne, W. J.** 1973. Reduction of nitrogenous oxides by microorganisms. Bacteriol. Rev. **37**:409-452.
16. **Ryden, J. C., L. J. Lund, and D. D. Focht.** 1979. Direct measurement of denitrification loss from soils: I. Laboratory evaluation of acetylene inhibition of nitrous oxide reduction. Soil Sci. Soc. Am. J. **43**:104-110.
17. **Ryden, J. C., L. J. Lund, J. Letey, and D. D. Focht.** 1979. Direct measurement of denitrification loss from soils: II. Development and application of field methods. Soil Sci. Soc. Am. J. **43**:110-118.
18. **Sørensen, J.** 1978. Capacity for denitrification and reduction of nitrate to ammonia in a coastal marine sediment. Appl. Environ. Microbiol. **35**:301-305.
19. **Sørensen, J.** 1978. Denitrification rates in a marine sediment as measured by the acetylene inhibition technique. Appl. Environ. Microbiol. **36**:139-143.
20. **Taniguchi, S., and E. Itagaki.** 1960. Nitrate reductase of nitrate respiration type from *E. coli*. I. Solubilization and purification from the particulate system with molecular characterization as a metalloprotein. Biochim. Biophys. Acta **44**:263-279.
21. **Wolin, M. J., E. A. Wolin, and N. J. Jacobs.** 1961. Cytochrome-producing anaerobic vibrio, *Vibrio succinogenes*, sp. n. J. Bacteriol. **81**:911-917.
22. **Woods, D. D.** 1938. The reduction of nitrate to ammonia by *Clostridium welchii*. Biochem. J. **32**:2000-2012.
23. **Yoshinari, T., R. Hynes, and R. Knowles.** 1977. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biol. Biochem. **9**:177-183.
24. **Yoshinari, T., and R. Knowles.** 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. **69**:705-710.