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CARLOS A. NEYRA AND PETER VAN BERKUM

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Nitrate reduction and nitrogenase activity in *Spirillum lipoferum*[1]

CARLOS A. NEYRA[2] AND PETER VAN BERKUM

EMBRAPA Km 47, Campo Grande 20.000, Rio de Janeiro, Brazil

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Nitrate and nitrite reduction under aerobic, microaerophillic, and anaerobic conditions was demonstrated in *Spirillum lipoferum* (ATCC 29145). Nitrite did not accumulate during assimilatory nitrate reduction in air. The nitrite produced during dissimilatory nitrate reduction accumulated in the medium but not in the cells. On exposure of the bacteria to nitrate and anaerobiosis, a low initial rate (lag) was followed by accelerated rates of nitrite accumulation. A 3-h anaerobic pretreatment, in the absence of nitrate, did not avoid the lag phase. No nitrate reductase activity (NRA) developed in the presence of chloramphenicol. The data suggest that induction of anaerobic NRA in *S. lipoferum* required nitrate and protein synthesis.

Anaerobic N_2ase activity by *S. lipoferum* was greatly stimulated in the presence of nitrate. The time course of nitrate reduction was coincidental with the pattern of nitrate-stimulated N_2ase activity indicating that a relationship exists between these two processes.

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On a démontré la réduction du nitrate et du nitrite en aérobiose, anaérobiose et dans des conditions microaérophiles chez *Spirillum lipoferum* (ATCC 29145). Le nitrite ne s'accumule pas durant la réduction assimilative en présence d'air. Le nitrite, produit durant la réduction dissimilative du nitrate, s'accumule dans le milieu mais non dans les cellules. En exposant les bactéries au nitrate et à l'anaérobiose, un taux initial faible (lag) d'accumulation du nitrite est suivi par un taux acceléré d'accumulation. Un prétraitement de 3 h en anaérobiose en l'absence de nitrate n'empêche pas cette phase de latence. En présence de chloramphénicol aucune activité de la nitrate réductase (NRA) ne se développe. Les données suggèrent que l'induction du NRA anaérobique chez *S. lipoferum* exige le nitrate et la synthèse des protéines.

L'activité anaérobie de N_2ase chez *S. lipoferum* est grandement stimulée en présence du nitrate. Le temps de la réduction du nitrate coincide avec le schème de stimulation de l'activité N_2ase par le nitrate, démontrant qu'il existe une corrélation entre ces deux processus.

[Traduit par le journal]

Introduction

The nitrogen-fixing bacterium *Spirillum lipoferum*, Beijerinck, has been isolated from roots of several field-grown tropical grain and forage grasses (5, 7, 9). The authors have indicated that *S. lipoferum* is responsible for most of the observed nitrogenase (N_2ase) activity in the roots of these plants. *Spirillum lipoferum* was identified following the original description by Beijerinck (2) and Bergey's Manual (3). Although *S. lipoferum* was omitted from the last edition of Bergey's Manual (1975), Krieg has included this organism in a recent review of Spirillaceae (13).

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[2]Author to whom correspondence should be sent.

Because of the potential economic importance most work with *S. lipoferum* has been related to nitrogen fixation. Several physiological aspects of nitrogen fixation and procedures for isolation and purification of *S. lipoferum* have been described (7). In contrast, nitrate metabolism in *S. lipoferum* has received little attention. The in vivo operation of nitrate and nitrite reductase in *S. lipoferum* has as yet not been shown. However, the processes of assimilatory and dissimilatory nitrate reduction in other bacteria are well known (4, 6, 11). Some microorganisms are able to use nitrate as a terminal electron acceptor for respiration under anaerobic conditions (4, 6), and Rigaud *et al.* (17) were able to couple anaerobic nitrate respiration to nitrogen fixation in soybean bacteroids.

A common procedure for determining in vivo nitrate reduction in bacteria involves the qualitative and (or) quantitative estimation of nitrite in the growth medium (18). The reduction of

nitrate to nitrite is known to be mediated through nitrate reductase (1, 4, 11). Similarly the disappearance of nitrite from the growth medium is evidence for nitrite reduction, a process mediated by nitrite reductase (4, 14).

The objectives of this work were to show in *S. lipoferum*: (*a*) nitrate and nitrite reductase activities; (*b*) anaerobic nitrate reduction, and (*c*) coupling between nitrate reduction and nitrogenase activity under anaerobic conditions.

Materials and Methods

Bacteria and Procedure of Inoculation

The strain of *S. lipoferum* used in this work is an isolate from the roots of *Digitaria decumbens* cv. Transvala (American Type Culture Collection (ATCC) 29145). Stock cultures of this bacterium were kept on slopes of potato infusion agar (5), stored at room temperature (27°C). Two-millilitre lots of semisolid NFb media (10) in 7-ml serum bottles were inoculated from the stock cultures and grown at 36°C for 24 h before 0.5-ml inoculants were distributed to growth vessels by means of 1-ml sterile syringes or pipets.

N-free Growth Medium

Spirillum lipoferum was grown in a modified medium by Day and Döbereiner (7) (A) containing in g/litre of distilled H_2O: K_2HPO_4, 0.25; KH_2PO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; Fe(EDTA) (B.D.H. Chemicals Ltd., Poole, England), 0.06; $Na_2MoO_4{\cdot}2H_2O$, 0.02; $MnSO_4{\cdot}H_2O$, 0.01; KOH, 4.9; malic acid, 5.0; pH adjusted to 6.9. Batches of 30-ml liquid cultures, inoculated as described above, were grown at 30°C in 120-ml powder bottles with a sparging gas mixture of pO_2 0.01 atm in N_2 (7).

Glutamate or Nitrate Growth Medium

Medium A with the following modifications in g/litre of distilled H_2O was used (medium B): Fe(EDTA), 0.656; $Na_2MoO_4{\cdot}2H_2O$, 0.2; potassium glutamate, 10.0. A 2-litre batch culture provided with air sparging at 100 ml min^{-1} was grown at 32°C to supply samples of bacterial cells. To investigate nitrate reduction, 10-ml aliquots were dispensed into 27-ml vials fitted with a "Suba-seal" and provided with N_2 bubbling throughout to achieve anaerobic conditions. After the addition of 20 m*M* KNO_3, the time course of nitrite accumulation was followed. A similar 2-litre batch culture containing medium B, but with KNO_3 (2.0 g/litre) instead of glutamate was used to grow cells on nitrate as sole N source.

Anaerobic Nitrogenase Studies

Medium A provided with 0.5 g/litre agar was used to grow *S. lipoferum* in 200-ml medical flasks at 36°C for 48 h. After they were mixed, 4.0-ml aliquots were dispensed into 27-ml vials fitted with "Suba-seals." After the atmosphere was replaced in the vials with N_2 (for 30 min), the samples were left to equilibrate for 4 h at 27°C before KNO_3 (10 m*M*) and 12% C_2H_2 were added. Nitrite accumulation and C_2H_2 reduction were both followed in cultures which were shaken vigorously.

Assay Procedures

Nitrate was determined with a Corning Nitrate Electrode connected to an Orion Ionanalyzer specific ion meter model 470A. Nitrite concentrations were determined on aliquots (usually 0.1 or 0.2 ml) removed from the cultures, by adding to them 2.0 ml of a 1:1 (v/v) freshly mixed solution of 0.02% *N*-1-naphthyl-ethylenediamine dihydrochloride and 1% sulphanilamide in 1.5 *M* HCl. Distilled H_2O was added to complete 4.0 ml and colour permitted to develop for 15 min before reading at 540 nm. Acetylene reduction was determined by gas chromatography using a Poropak N column (2 m × 3 mm) at 110°C and H_2 flame-ionization detection.

Results

Nitrate and Nitrite Reduction

The ability of *S. lipoferum* to reduce nitrate and nitrite was investigated by following the time course of nitrite accumulation and disappearance in 48-h-old, N-free, liquid cultures sparged with 0.01 atm O_2 in N_2 (Fig. 1). On the addition of nitrate a steady accumulation of nitrite occurred, providing evidence for in vivo nitrate reduction. At the time of maximal nitrite accumulation, most or all of the nitrate added had been utilized. After this point the nitrite concentration of the medium declined steadily, providing evidence that *S. lipoferum* further reduced nitrite in vivo.

Assimilatory and Dissimilatory Nitrate Reduction

Spirillum lipoferum was grown in a 2-litre batch culture with KNO_3 as a nitrogen source.

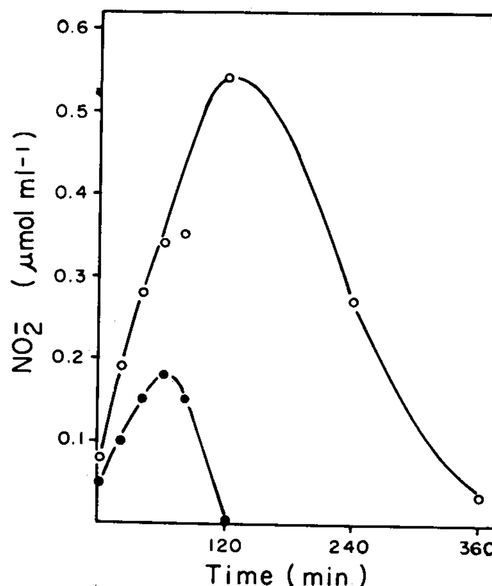

FIG. 1. Nitrite accumulation and disappearance from *S. lipoferum* cultures at pO_2 0.01 atm. KNO_3 ((○), 0.5 m*M*; (●), 0.1 m*M*) was added to 24-h-old cultures grown on medium A (see Materials and Methods) at 30°C.

A continuous sparging of sterile air was provided for the first 20 h of culturing, after which time the air was replaced by N_2 to achieve anaerobic conditions. Optical density (660 nm) and nitrate and nitrite concentrations were monitored from the time of inoculation (zero time). The nitrate concentration in the medium dropped from 20 to 12.5 m*M* and nitrite did not accumulate during the first 20 h of aerobic incubation (Fig. 2). The disappearance of nitrate was accompanied by an increase in optical density of the culture (insert Fig. 2). When changing from air to N_2 sparging nitrate reduction continued at a faster rate, nitrite accumulated, and the optical density failed to increase further (Fig. 2). These observations suggest that *S. lipoferum* assimilates nitrate under aerobic conditions for growth, and that under conditions of limiting O_2 nitrate is dissimilated.

Induction of Anaerobic Nitrate Reductase Activity

Spirillum lipoferum was grown aerobically in liquid glutamate medium for 24 h, then transferred to anaerobic conditions (N_2 bubbling). Nitrate (20 m*M*) was added either immediately or 3 h after transfer. Upon exposure to nitrate a low initial rate (lag phase) was followed by accelerated rates of nitrite accumulation (Fig. 3). Cultures pretreated for 3 h with N_2 bubbling showed a similar pattern of nitrite accumulation (insert Fig. 3). The addition of $(NH_4)_2SO_4$ (10 m*M*) decreased nitrite accumulation in the growth medium. In the presence of chloramphenicol (50 µg/ml) a lack of nitrite accumulation indicated that nitrate reductase activity did not develop.

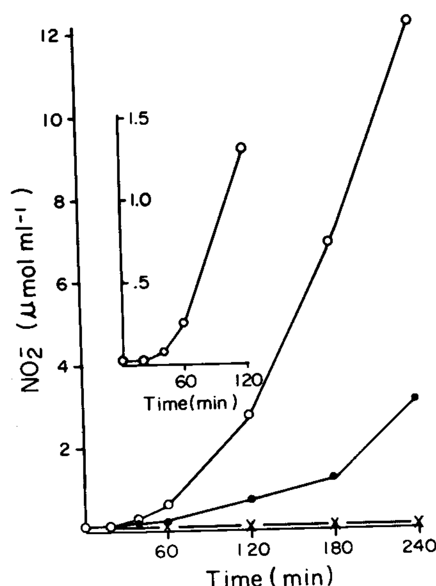

FIG. 3. Anaerobic nitrate reduction in aerobically grown *S. lipoferum*. The organism was grown in medium B at 32°C and KNO_3 (20 m*M*) added after transfer to anaerobic conditions (N_2 bubbling). Control (O); plus 10 m*M* $(NH_4)_2SO_4$ (●); plus chloramphenicol (50 µg/ml) (×). The insert shows a similar experiment, but cultures received a 3-h anaerobic pretreatment before addition of KNO_3 (20 m*M*).

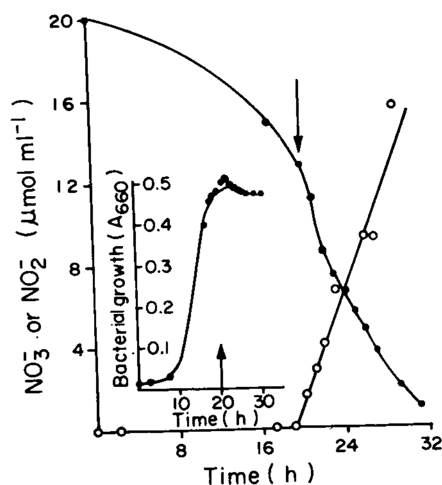

FIG. 2. Assimilatory and dissimilatory nitrate reduction in *S. lipoferum*. The bacteria were grown in a 2-litre batch culture with KNO_3 as N source. The arrows indicate the time of switching from aerobic to anaerobic conditions. Nitrate (●), nitrite (O), and optical density at 660 nm (insert) were monitored from the time of inoculation.

Subsequent experiments revealed that nitrite reduction can also be induced by a KNO_3 (0.1 m*M*) anaerobic pretreatment (Fig. 4). *Spirillum lipoferum* cultures grown in liquid N-free malate medium with 0.01 atm O_2 in the gas phase were preincubated for 3 h with or without 0.1 m*M* KNO_3 under anaerobic conditions (N_2 bubbling). After the period of pretreatment $NaNO_2$ (0.5 m*M*) was added to the cultures and the disappearance of nitrite from the medium followed. Faster and linear rates of nitrite utilization were observed for cells pretreated with nitrate. The cultures not previously exposed to nitrate showed an initial lag in nitrite reduction (Fig. 4).

Anaerobic Nitrate Reduction and Nitrogenase Activity

Because *S. lipoferum* is able to reduce nitrate under anaerobic conditions and because of the

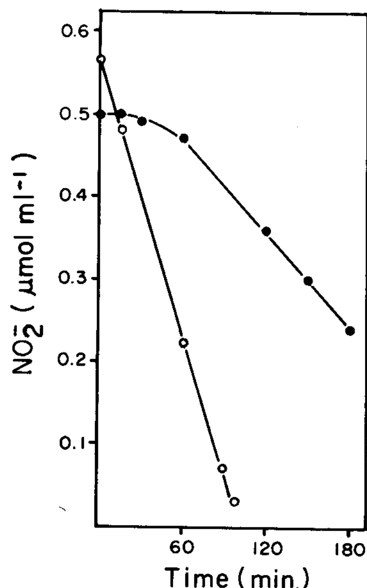

FIG. 4. Anaerobic nitrite reduction in *S. lipoferum* cultures. The organism was grown in medium A at pO_2 0.01 atm for 24 h before transfer to anaerobic conditions (N_2 bubbling) and $NaNO_2$ (0.5 m*M*) addition. (●), Control; (○), KNO_3 (0.1 m*M* for 3 h) pretreatment.

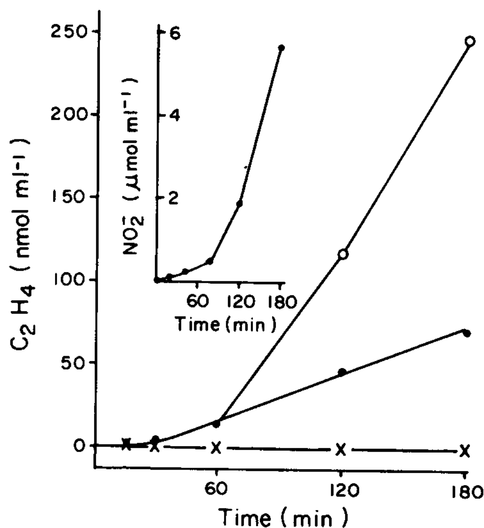

FIG. 5. Nitrate reduction and anaerobic nitrogenase activity in *S. lipoferum*. Control (●); plus 10 m*M* KNO_3 (○), plus 5 m*M* $(NH_4)_2SO_4$ (×). The insert shows the time course of nitrate reduction. For experimental details see Materials and Methods.

work of Rigaud *et al.* in soybean bacteroids (17), we decided to investigate the relationship between nitrate reduction and anaerobic N_2ase activity in *S. lipoferum*. The time course of C_2H_2 reduction by *S. lipoferum* with and without NO_3^- or NH_4^+ (10 m*M* N) under anaerobic conditions is presented in Fig. 5. Addition of NO_3^- stimulated, but NH_4^+ completely inhibited N_2ase activity. The time course of nitrate reduction (insert Fig. 5) was coincidental with the pattern of NO_3^--stimulated anaerobic N_2ase activity. Stimulation of N_2ase activity occurred with the development of the accelerated rates of nitrate reduction. No stimulation of N_2ase was evident during the first 45 to 60 min of exposure to NO_3^- because of the lag phase in nitrate reduction.

Relatively high levels of nitrite accumulated in the cultures and several reports had indicated that nitrite inhibits N_2ase activity (12, 17). To provide an explanation, cultures were fractionated by low-speed centrifugation and each fraction subjected to nitrite analysis. Almost all the nitrite formed was recovered in the supernatant, suggesting that *S. lipoferum* is somehow able to exclude nitrite.

Discussion

Nitrate and nitrite reduction under aerobic, microaerophillic, and anaerobic conditions were demonstrated in *S. lipoferum*. This microorganism was able to grow aerobically with nitrate as sole N source, demonstrating assimilatory nitrate reduction. Nitrite did not accumulate in the growth medium. Nitrate reduction continued after switching the culture from aerobic to anaerobic conditions (N_2 bubbling), and nitrite accumulated in the growth medium. The nitrite produced by dissimilatory nitrate reduction was apparently excluded by the bacteria and accumulated in the growth medium, thereby preventing toxic levels of nitrite inside the cells. However, at least part of the nitrite so produced is expected to undergo further reduction because *S. lipoferum* was observed to reduce nitrite anaerobically. Denitrification by several strains of *S. lipoferum* has also recently been demonstrated (15). All these observations indicate that dissimilatory nitrate reduction occurs at the membrane level and is consistent with reports that respiratory nitrate reductase is membrane-bound in several microorganisms (4).

The induction of nitrate reductase activity by nitrate in microorganisms (4) and higher plants (1) has been reported. The rate of nitrate reduction by *S. lipoferum* under anaerobic

conditions appears to increase with time of exposure to nitrate suggesting the inducible nature of NRA. The lag phase in NRA was not avoided by the anaerobic pretreatment, and did not develop at all in the presence of chloramphenicol. These observations suggest that induction of anaerobic NRA in *S. lipoferum* requires nitrate and protein synthesis.

Nitrogenase activity in *S. lipoferum* is optimal at pO_2 0.01 to 0.02 atm, and decreasing pO_2 reduces N_2ase activity (7) probably because of adenosine 5′-triphosphate (ATP) limitation. All nitrogenases studied require ATP to reduce N_2 or any other substrate (8). The ATP requirement for N_2ase activity in cell-free extracts of *S. lipoferum* has also recently been confirmed (16). Nitrate reduction by *S. lipoferum* under anaerobic conditions may therefore yield sufficient energy (ATP) to meet the requirement for N_2ase activity. This probably explains the observed relationship between nitrate reduction and N_2ase activity under anaerobic conditions. Similar conclusions were reached by Rigaud *et al.* (17) working with soybean bacteroids. However, they reported that anaerobic N_2ase activity was absolutely dependent on nitrate, while in *S. lipoferum* some C_2H_2 reduction was observed in the controls without nitrate. The possible entry of some O_2 into the vials with the addition of solutions and subsequent gas samplings cannot be excluded and may have prevented fully anaerobic conditions. Nevertheless, the O_2 concentration was clearly suboptimal for C_2H_2 reduction when comparing to cultures provided with 0.01 atm O_2 in the gas phase.

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1. BEEVERS, L., and R. H. HAGEMAN. 1969. Nitrate reduction in higher plants. Annu. Rev. Plant Physiol. **20**: 495–522.
2. BEIJERINCK, M. W. 1925. Uber ein *Spirillum*, welches freien Stickstoff binden kann? Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2, **63**: 353–359.
3. BREED, R. S., E. G. D. MURRAY, and N. R. SMITHS. 1957. Bergey's Manual of determinative bacteriology. 7th ed. Williams and Wilkins, Baltimore, Md., U.S.A.
4. BROWN, C. M., D. S. MACDONALD-BROWN, and J. L. MEERS. 1974. Physiological aspects of microbial inorganic nitrogen metabolism. Adv. Microb. Physiol. **11**: 1–52.
5. BÜLOW, J. F. W. VON, and J. DÖBEREINER. 1975. Potential for nitrogen fixation in maize genotypes in Brazil. Proc. Natl. Acad. Sci. U.S.A. **72**: 2389–2393.
6. BURNS, R. C., and R. W. F. HARDY. 1975. Nitrogen fixation in bacteria and higher plants. Springer-Verlag, New York-Heidelberg-Berlin.
7. DAY, J. M., and J. DÖBEREINER. 1976. Physiological aspects of N_2-fixation by a *Spirillum* from *Digitaria* roots. Soil Biol. Biochem. **8**: 45–50.
8. DILWORTH, M. J. 1974. Dinitrogen fixation. Annu. Rev. Plant Physiol. **25**: 81–114.
9. DÖBEREINER, J., and J. M. DAY. 1974. Associative symbioses in tropical grasses: characterization of micro-organisms and dinitrogen fixing sites. Proceedings of the International Symposium on N_2-fixation Interdisciplinary Discussions. Washington State University, Washington State University Press.
10. DÖBEREINER, J., I. E. MARRIEL, and M. NERY. 1976. Ecological distribution of *Spirillum lipoferum* Beijerinck. Can. J. Microbiol. **22**: 1464–1473.
11. HEWITT, E. J. 1975. Assimilatory nitrate–nitrite reduction. Annu. Rev. Plant Physiol. **26**: 73–100.
12. KENNEDY, I. R., J. RIGAUD, and J. C. TRINCHANT. 1975. Nitrate reductase from bacteroids of *Rhizobium japonicum*: enzyme characteristics and possible interaction with nitrogen fixation. Biochim. Biophys. Acta, **397**: 24–35.
13. KRIEG, N. R. 1976. Biology of the chemoheterotrophic spirilla. Bacteriol. Rev. **40**: 55–115.
14. NEYRA, C. A., and R. H. HAGEMAN. 1974. Dependence of nitrite reduction on electron transport in chloroplasts. Plant Physiol. **54**: 480–483.
15. NEYRA, C. A., J. DÖBEREINER, R. LALANDE, and R. KNOWLES. 1977. Denitrification by N_2-fixing *Spirillum lipoferum*. Can. J. Microbiol. **23**: 300–305.
16. OKON, Y., S. L. ALBRECHT, and R. H. BURRIS. 1976. Fixation of nitrogen by *Spirillum lipoferum* alone and with plants. Plant Physiol. **57**: S-369.
17. RIGAUD, J., F. J. BERGERSEN, G. L. TURNER, and R. M. DANIEL. 1973. Nitrate dependent anaerobic acetylene-reduction and nitrogen-fixation by soybean bacteroids. J. Gen. Microbiol. **77**: 137–144.
18. SKERMAN, V. B. D. 1959. A guide to the identification of the genera of bacteria. Williams and Wilkins, Baltimore, Md., U.S.A.