Growth of *Spirillum lipoferum* at Constant Partial Pressures of Oxygen, and the Properties of its Nitrogenase in Cell-free Extracts

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SUMMARY

Spirillum lipoferum, an N_2 -fixing organism, was grown at constant concentrations of dissolved O_2 . When supplied with NH_4^+ aerobically, its doubling time was 1 h; when it fixed N_2 microaerophilically, its doubling time was 5.5 to 7 h and the optimal P_{O_2} for growth was 0.005 to 0.007 atm. At its optimal P_{O_2} for growth on N_2 , *S. lipoferum* assimilated 8 to 10 mg nitrogen/g carbon substrate used; its efficiency was less at higher P_{O_2} levels. Nitrogenase in cell-free extracts required Mg^{2+} and Mn^{2+} , and the Fe-protein was activated by *Rhodospirillum rubrum* activating factor. The nitrogenase had an optimal pH of 7.1 to 7.4 and an apparent K_m for acetylene of 0.0036 atm. Extracts of *S. lipoferum* lost their nitrogenase activity on storage at $-18^\circ C$, and activity was restored by adding purified Fe-protein from other N_2 -fixing bacteria.

INTRODUCTION

The cultural and physiological properties of *Spirillum lipoferum* ATCC29145 have been investigated recently (Day & Döbereiner, 1976). This is a N_2 -fixing bacterium that can associate with roots of several grasses (Döbereiner & Day, 1976; Von Bülow & Döbereiner, 1975; Albrecht & Okon, 1975). It will grow and fix N_2 under microaerophilic conditions with malate, succinate, lactate or pyruvate as carbon and energy sources.

This paper describes the growth of *S. lipoferum* at constant P_{O_2} and the properties of nitrogenase in cell-free extracts of this organism.

METHODS

Spirillum lipoferum ATCC29145, isolated from the roots of *Digitaria decumbens* (Döbereiner & Day, 1976), was grown in 3 or 20 l bottles containing 2.7 or 18 l, respectively, of the liquid medium of Döbereiner & Day (1976), modified to the following composition ($g\ l^{-1}$): K_2HPO_4 , 6; KH_2PO_4 , 4 (these were mixed to a tenth of the final volume and autoclaved separately to avoid precipitation; later the phosphate solution was mixed with the cold medium); $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; DL-malic acid, 5; NaOH, 3; Difco yeast extract, 0.1; and ($mg\ l^{-1}$) $FeCl_3$, 10; $NaMoO_4 \cdot 2H_2O$, 2; $MnSO_4$, 2.1; H_3BO_3 , 2.8; $Cu(NO_3)_2 \cdot 3H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.24; the final pH was adjusted to 6.8.

The inoculum was 10 % of the total volume of the culture and was grown aerobically at $30^\circ C$ in medium supplemented with 0.05 % (w/v) NH_4Cl . The culture had an initial E_{560}

of 0.09 to 0.11. The culture was sparged with a sterile mixture of N_2 and air, and was stirred with a magnetic bar; a thermistor probe in a well in the culture bottle controlled an external infrared lamp that maintained a temperature of 30 °C. A constant concentration of dissolved O_2 , in equilibrium with 0.002 to 0.018 atm O_2 , was maintained by using an O_2 -stat; an immersed sterilizable O_2 electrode (Borkowski & Johnson, 1967) was used to measure the O_2 concentration and to control the O_2 -stat.

Growth of the culture was followed by measuring E_{580} . After removing the organisms by centrifuging the culture at 5000 g for 10 min, total carbon in the supernatant was determined by the method described by Umbreit, Burris & Stauffer (1972*a*) using malic acid as a standard. The pellet was dried at 80 °C for 24 h and weighed, and total nitrogen in the organisms was measured (Umbreit *et al.*, 1972*b*).

Acetylene reduction in the growing culture was measured by transferring 2 ml samples with a syringe into 21 ml serum bottles, stoppered with rubber closures, that previously had been evacuated and filled with N_2 and O_2 to match the P_{O_2} of the culture. Acetylene, to a final pressure of 0.11 atm, was then added. The bottles were incubated at 30 °C and shaken at 150 cycles min^{-1} in a water bath, and the amount of ethylene formed by acetylene reduction was determined by gas chromatography using a flame ionization detector (Burris, 1974).

Spirillum lipoferum was harvested during the exponential growth phase under anaerobic conditions and centrifuged anaerobically at 8000 g for 10 min at 4 °C. The pellet (1.5 to 2.0 g wet wt/l medium) was stored in liquid N_2 without any apparent loss in nitrogenase activity.

Crude cell-free extracts of *S. lipoferum* were prepared under anaerobic conditions in 300 mM-Tris/HCl buffer, pH 8.7 (1 g wet wt of cell paste/4 ml buffer), containing dithiothreitol (0.1 mg ml^{-1}), DNAase and Na dithionite. The cells were broken in a French press at 80000 kPa. The final pH of the extract ranged from 7.5 to 8.1, depending on the batch of cells. When the buffer concentration was decreased or the buffer to cell ratio was lowered, the pH dropped below 5.0 and nitrogenase activity was lost completely. Osmotic shock treatment (Shah, Davis & Brill, 1972) caused only poor disruption of the organisms and yielded nitrogenase of low activity.

The extract was centrifuged anaerobically at 10000 g for 30 min at 4 °C, and this produced a turbid pinkish-brown supernatant (crude extract) and a white pellet. When this supernatant was centrifuged at 48000 g for 2 h at 4 °C, it gave a clear supernatant (soluble nitrogenase, brown colour with the pink of cytochromes) and a dark brown pellet (membrane fraction).

Nitrogenase activity was estimated by production of ethylene from acetylene in 21 ml bottles that had been shaken under anaerobic conditions (N_2 or H_2) at 30 °C in a water bath. For optimal activity, the reaction mixture (total vol. 1.35 ml) contained: 100 μ mol HEPES [2-(*N*-2-hydroxyethylpiperazine-*N'*-yl)ethanesulphonic acid] buffer, pH 7.3; 5 μ mol ATP, 40 μ mol creatine phosphate, 0.2 mg creatine kinase ml^{-1} ; 14.5 mM- $MgCl_2$; 0.44 mM- $MnCl_2$; 5 mM-Na dithionite; and 0.3 ml (approximately 2 mg protein) enzyme extract. Nitrogenase specific activity was expressed as nmol ethylene produced min^{-1} (mg protein) $^{-1}$. Protein concentration was determined by the microbiuret method (Goa, 1953).

The Fe-proteins of nitrogenases used in cross-testing for enzymic activity with the nitrogenase from *S. lipoferum* were purified by the following colleagues: R. Hageman, *Azotobacter vinelandii*; P. Ludden, *Rhodospirillum rubrum*; D. W. Emerich, *Bacillus polymyxa* and *Clostridium pasteurianum*. The purification methods will be published elsewhere.

Fig. 1. Typical growth curves of *S. lipoferum* grown at 0.2 atm O_2 in the presence of 0.1 % NH_4Cl (○), and on N_2 in a nitrogen-free medium at a P_{O_2} of 0.005 to 0.007 atm and a P_{N_2} of about 0.99 atm (□).

Table 1. Effect of P_{O_2} on doubling time, efficiency and acetylene reduction rates in cultures of *S. lipoferum* grown at constant P_{O_2}

Values of the doubling time are the ranges from at least two experiments. Efficiency of nitrogen assimilation was calculated by measuring the total nitrogen content of the culture and the carbon growth substrate consumed at four different times during the exponential phase of growth of the culture when fixing N_2 : efficiencies are expressed as mg nitrogen assimilated/g malate consumed. Acetylene reduction was measured when E_{560} was about 0.6, the cell density 4×10^8 cells ml^{-1} and the dry weight 0.5 mg ml^{-1} .

P_{O_2} (atm)	Doubling time (h)	Efficiency	Acetylene reduction (nmol h^{-1} ml^{-1})
0.002–0.003	9–10	11.9–12.5	250–280
0.005–0.007	5.5–7	8–10	280–300
0.009–0.011	7.5–8.5	2.5–4.0	80–100
0.014–0.018	8.5–9.5	1.2–2.1	50–75

RESULTS AND DISCUSSION

Spirillum lipoferum grew in an O_2 -stat at a constant P_{O_2} both on combined nitrogen and under N_2 -fixing conditions; as the O_2 demand of the culture increased, increasing amounts of O_2 at a constant P_{O_2} were supplied. Typically, the first phase of growth of *S. lipoferum* at 0.005 to 0.007 atm O_2 (bottom curve, Fig. 1; $\mu = 2$ h), depended on the combined nitrogen present in the yeast extract in the medium, and NH_4Cl carried over with the inoculum. Saturation of the NH_4Cl -containing medium with O_2 , at the level in air, at all stages of growth markedly increased the growth rate ($\mu = 1 \text{ h} \pm 10 \text{ min}$; top curve, Fig. 1). Thus, *S. lipoferum* is a fast-growing, fully aerobic organism when supplied with combined nitrogen.

Growth rates under N_2 -fixing conditions during the second phase of growth were slower and depended on P_{O_2} being maintained within a narrow range; μ was 5.5 to 7 h at a P_{O_2} of 0.005 to 0.007 atm (Fig. 1, Table 1). Though several different P_{O_2} values in the range 0.004 to 0.04 atm have been reported as optimal for growth and N_2 fixation of *S. lipoferum* (Day & Döbereiner, 1976), none of these values was measured at a constant concentration of dissolved O_2 .

By using a 10 % inoculum grown on a medium containing NH_4Cl , *S. lipoferum* could be grown and harvested with an active nitrogenase in 14 to 16 h; the initial growth for a generation or so after transfer to the nitrogen-free medium was presumed to be on residual NH_4^+

Fig. 2. Effect of (a) MgCl₂, (b) MnCl₂ (optimal MgCl₂ present), (c) dithionite, and (d) pH on nitrogenase activity of cell-free extracts of *S. lipoferum*.

from the inoculum and after this the culture depended on N₂ fixation to support growth.

The O₂ concentration in the medium affected the nitrogenase activity, the total nitrogen in the culture, and the utilization and efficiency of use of carbon substrates (Table 1). At optimal P_{O_2} , the efficiency was 8 to 10 mg nitrogen fixed/g carbon substrate used, whereas efficiencies were lower at O₂ concentrations above the optimal (Table 1).

Very high efficiencies of substrate conversion (40 to 50 mg cell nitrogen produced/g carbon substrate utilized) have been reported in O₂-limited continuous cultures of *Azotobacter chroococcum* (Postgate, 1971), and by Day & Döbereiner (1976) in stagnant cultures of *S. lipoferum* on a semi-solid medium. With the same organism under similar conditions, values ranging from 10 to 25 have been obtained by Y. Okon *et al.* (unpublished observations). However, efficiency values of only 3 to 6 were reported by Fedorov & Kalininskaya (1961) for a so-called *Mycobacterium flavum*, a microaerophilic N₂-fixing bacterium.

The values for the efficiency of N₂-fixation presented here were derived from measurements

Fig. 3. Time course of acetylene reduction by nitrogenase in cell-free extracts from *S. lipoferum*. The reaction mixture contained 14.8 mM-MgCl₂ and 0.85 mg dithionite per assay: □, plus 0.44 mM-MnCl₂ and an activating protein from *R. rubrum*; △, plus 0.44 mM-MnCl₂; ○, minus MnCl₂ plus activating protein (coincident with curve with neither MnCl₂ nor activating protein). Gas samples (0.5 ml) were removed sequentially from reaction vessels at the times shown; control tests in which individual bottles were sampled at each time gave the same response within experimental error.

of the increase in total nitrogen of the organism and the consumption of malate as carbon substrate in a known period during the exponential phase of growth of *S. lipoferum*. As the culture was growing on N₂ at a constant P_{O_2} , we consider the data more meaningful than earlier data obtained with cultures growing under a variable P_{O_2} ; the efficiencies we observed are in the range reported for other aerobic N₂-fixing bacteria (Mulder & Brotonegoro, 1974).

The percentage of nitrogen in dry cells that had been grown under N₂-fixing conditions was only 4 to 5 %, whereas NH₄Cl-grown organisms contained 9 to 10 % nitrogen. This low percentage of nitrogen in N₂-grown cells reflects their high poly-β-hydroxybutyrate content (Y. Okon *et al.*, unpublished observations).

Measurements of acetylene reduction at different stages of growth gave only relative values, because transfer of samples to stoppered bottles altered the rates of acetylene reduction by changing the cell environment. However, maximum acetylene reduction rates were observed for samples taken from the cultures grown at the optimal P_{O_2} of 0.005 to 0.007 atm (Table 1).

Nitrogenase activity measured in cell-free extracts of *S. lipoferum* was affected by Mg²⁺ and Mn²⁺ in the reaction mixture (Fig. 2*a, b*). No nitrogenase activity was observed with less than 6.0 mM-MgCl₂; above this concentration nitrogenase activities increased sharply until an optimal level of 13.5 to 15.5 mM-MgCl₂ was reached. Concentrations above 16.0 mM-MgCl₂ were inhibitory. Addition of MnCl₂ to the reaction mixture (with MgCl₂ present) enhanced nitrogenase activity, the optimal concentration being 0.44 mM-MnCl₂.

Concentrations of dithionite above 1 mg/1.35 ml reaction mixture (about 4.3 mM) inhibited nitrogenase (Fig. 2*c*). Dithionite samples from various commercial sources were used to prepare anaerobic solutions, and all produced the same effect. The pH and volume of the reaction mixtures were constant for these assays.

The optimal pH for nitrogenase activity was from 7.1 to 7.4; preparations were inactive below pH 6.5 and above pH 8.2 (Fig. 2*d*). Nitrogenase in cell-free extracts from *S. lipoferum* was saturated at about 0.04 atm of acetylene and an apparent K_m of 0.0036 ± 0.0004 atm

Table 2. *Nitrogenase activity in different fractions of an extract from S. lipoferum, and cross-reactions with Fe-protein from several N₂-fixing bacteria*

Specific activities [expressed as nmol ethylene produced min⁻¹ (mg protein)⁻¹] were derived from the amount of product formed between 20 and 30 min of the assay and the protein content of crude extract of *S. lipoferum*. Concentrations of Fe-protein from supplementing bacteria were not determined, but these proteins were free of any background activity. The Fe-proteins used were tested for activity with their homologous MoFe-proteins at the time of the experiment. The 'inactive' Fe-protein of *R. rubrum* was activated by the activating protein remaining in the extract from *S. lipoferum* after storage at -18 °C; activated Fe-protein of *R. rubrum* supplemented the MoFe-protein of *S. lipoferum* to generate nitrogenase.

	Specific activity
Crude extract; supernatant after centrifuging at 10000 g for 20 min	8.0
Soluble nitrogenase; supernatant after centrifuging the crude extract at 48000 g for 2 h	10.0
Membranes; resuspended pellet after centrifuging the crude extract at 48000 g for 2 h	0.0
Crude extract stored 5 days at -18 °C	0.8
Addition to crude extract of <i>S. lipoferum</i> after 5 days storage:	
<i>A. vinelandii</i> Fe-protein	45.0
<i>R. rubrum</i> activated Fe-protein	9.0
<i>R. rubrum</i> 'inactive' Fe-protein	3.7
<i>B. polymyxa</i> Fe-protein	1.7
<i>C. pasteurianum</i> Fe-protein	0.8

(1.3×10^{-4} M) was obtained from a reciprocal plot of activity against acetylene concentration. Higher K_m values for acetylene have been reported for a partially-purified nitrogenase of *A. vinelandii*: 0.012 atm (Rivera-Ortiz & Burris, 1975), and 0.002 to 0.009 atm (Hardy, Burns & Parshall, 1971). As the K_m for acetylene depends on the MoFe:Fe-protein ratio (Shah, Davis & Brill, 1975), the relatively low apparent K_m observed for *S. lipoferum* nitrogenase may indicate a high MoFe:Fe-protein ratio.

We observed a non-linear time course of the activity of *S. lipoferum* nitrogenase with acetylene as a substrate in all our cell-free extracts (Fig. 3). Nitrogenase activity was enhanced by MnCl₂ and by a purified protein from *R. rubrum* (Ludden & Burris, 1976) capable of activating the Fe-protein of nitrogenase (Fig. 3).

Specific activities for nitrogenase of 10 nmol ethylene produced min⁻¹ (mg protein)⁻¹ were obtained when the enzyme was assayed under optimal conditions during the 20 to 30 min interval of the reaction after nitrogenase was fully activated. Nitrogenase was soluble; no activity was observed in the pellet obtained by centrifuging the crude extract at 48000 g (the membrane fraction, Table 2).

Extracts of *S. lipoferum* lost their nitrogenase activity rapidly on storage at -18 °C. Addition of purified Fe-protein of nitrogenase from *A. vinelandii*, *R. rubrum* (previously treated with activating protein) or *B. polymyxa* restored nitrogenase activity to the inactive extracts (Table 2), whereas *C. pasteurianum* Fe-protein failed to do so.

For activity, *S. lipoferum* nitrogenase required Mg²⁺, ATP furnished by an ATP-generating system, a pH near neutrality, a reductant and anaerobic conditions, similar to other nitrogenase systems (Ljones, 1974). Like *R. rubrum*, *S. lipoferum* has a nitrogenase that is apparently activated during the assay, and the activation requires Mn²⁺ in the reaction mixture (Fig. 3). A purified activating protein from *R. rubrum* enhanced *S. lipoferum* nitrogenase activity, and a crude extract from *S. lipoferum* was capable of activating purified inactive

Fe-protein from *R. rubrum* (Table 2); this emphasizes the similarity of the nitrogenase systems in the two organisms.

Adding Fe-protein from *A. vinelandii* to an extract of *S. lipoferum* inactivated by storage at -18°C , resulted in a specific activity five times higher than that of the active crude extract from *S. lipoferum*; this suggested that some of the Fe-protein had been lost or inactivated during the preparation of the extract from *S. lipoferum* cells. Nitrogenase of *S. lipoferum* is poorly protected from O_2 (Day & Döbereiner, 1976), and the rapid loss of nitrogenase on aeration of N_2 -fixing cells is apparently caused by inactivation of the O_2 -labile Fe-protein.

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REFERENCES

- ALBRECHT, S. L. & OKON, Y. (1975). Nitrogen fixation by *Spirillum lipoferum*. *Plant Physiology Supplement* **56**, 73.
- BORKOWSKI, J. D. & JOHNSON, M. J. (1967). Long-lived steam-sterilizable membrane probes for dissolved oxygen measurement. *Biotechnology and Bioengineering* **9**, 635-639.
- BURRIS, R. H. (1974). Methodology. In *The Biology of Nitrogen Fixation*, pp. 9-33. Edited by A. Quispel. Amsterdam, New York: North Holland Publishing Co.
- DAY, J. M. & DÖBEREINER, J. (1976). Physiological aspects of N_2 -fixation by a *Spirillum* from *Digitaria* roots. *Soil Biology and Biochemistry* **8**, 45-50.
- DÖBEREINER, J. & DAY, J. M. (1976). Associative symbioses in tropical grasses: characterization of micro-organisms and dinitrogen fixing sites. In *Proceedings of the 1st International Symposium on Nitrogen Fixation*, pp. 518-538. Edited by W. E. Newton and C. J. Nyman. Pullman, Washington, U.S.A.: Washington State University Press.
- FEDOROV, M. Y. & KALININSKAYA, T. A. (1961). The relation of the nitrogen fixing *Mycobacterium* (*Mycobacterium* species 301) to various carbon sources and to additional factors. *Mikrobiologiya* **30**, 7-11.
- GOA, J. (1953). A micro biuret method for protein determination. Determination of total protein in cerebrospinal fluid. *Scandinavian Journal of Clinical Laboratory Investigation* **5**, 218-222.
- HARDY, R. W. F., BURNS, R. C. & PARSHALL, G. W. (1971). The biochemistry of N_2 fixation. In *Bioinorganic Chemistry*, pp. 219-247. Edited by R. F. Gould. Washington, D.C.: American Chemical Society.
- LJONES, T. (1974). The enzyme system. In *The Biology of Nitrogen Fixation*, pp. 617-638. Edited by A. Quispel. Amsterdam, New York: North Holland Publishing Co.
- LUDDEN, P. & BURRIS, R. H. (1976). *Science* (in the Press).
- MULDER, E. G. & BROTONEGORO, S. (1974). Free-living heterotrophic nitrogen-fixing bacteria. In *The Biology of Nitrogen Fixation*, pp. 37-85. Edited by A. Quispel. Amsterdam, New York: North Holland Publishing Co.
- POSTGATE, J. R. (1971). Fixation by free-living microbes: physiology. In *The Chemistry and Biochemistry of Nitrogen Fixation*, pp. 161-190. Edited by J. R. Postgate. London, New York: Plenum Press.
- RIVERA-ORTIZ, J. M. & BURRIS, R. H. (1975). Interactions among substrates and inhibitors of nitrogenase. *Journal of Bacteriology* **123**, 537-545.
- SHAH, V. K., DAVIS, L. C. & BRILL, W. J. (1972). Nitrogenase. I. Repression and derepression of the iron-molybdenum and iron proteins of nitrogenase in *Azotobacter vinelandii*. *Biochimica et biophysica acta* **256**, 498-511.
- SHAH, V. K., DAVIS, L. C. & BRILL, W. J. (1975). Nitrogenase. VI. Acetylene reduction assay: dependence of nitrogen fixation estimates on component ratio and acetylene concentration. *Biochimica et biophysica acta* **384**, 353-359.
- UMBREIT, W. W., BÜRRIS, R. H. & STAUFFER, J. F. (1972). *Manometric and Biochemical Techniques*, (a) p. 268, (b) p. 259. Minneapolis, U.S.A.: Burgess Publishing Co.
- VON BÜLOW, J. F. W. & DÖBEREINER, J. (1975). Potential for nitrogen fixation in maize genotypes in Brazil. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 2389-2393.