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Factors Affecting Growth and Nitrogen Fixation of *Spirillum lipoferum*

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Spirillum lipoferum grows vigorously on malate, succinate, lactate, or pyruvate, moderately on galactose or acetate, and poorly on glucose or citrate. It reduces $^{15}N_2$. Acetylene reduction rates decrease rapidly when the pH of the culture rises above 7.8. The organism is highly aerobic and had doubling times as low as 2 h when grown on NH_4^+. However, *S. lipoferum* reduces N_2 well only under microaerophilic conditions. The optimal pO_2 for acetylene reduction by stagnant cultures was 0.006 to 0.02 atm depending upon the cell density; aerated cultures grew well at a dissolved O_2 concentration corresponding to a pO_2 of about 0.008 atm. Shaking *S. lipoferum* with air temporarily inactivates its nitrogenase; reactivation is inhibited by chloramphenicol. The organism assimilated 20 to 24 mg of N/g of organic acid oxidized during growth. The strains studied can be placed in two groups based upon their morphology and physiological characteristics.

The recent isolation and characterization of *Spirillum lipoferum* from several tropical grasses in Brazil (6, 7, 12) has increased interest in the potential for N_2 fixation in microbial-grass associations and has prompted examination of the potential contributions of these associations in agricultural practice. Döbereiner's group in Brazil has observed *S. lipoferum* in the cortical cells of the roots of *Digitaria decumbens* (6) and *Zea mays* (12) and has reported that such roots can reduce acetylene. Albrecht and Okon (Plant Physiol. 56[Suppl.]: 73, no. 398, 1975) observed acetylene reduction in roots of maize and other grasses grown in association with *S. lipoferum* in field tests in Wisconsin; activities were less than reported for tropical conditions in Brazil.

S. lipoferum was first described by Beijerinck (2) and Schröder (11). *S. lipoferum* was neglected, and its description was withdrawn from the last edition of *Bergey's Manual* (4). Becking (1) and subsequently Döbereiner and Day (7) rediscovered and characterized it.

Growth and N_2 fixation in *S. lipoferum* are supported best by organic acids such as malate, lactate, and succinate as carbon and energy sources; it grows best on N_2 under microaerophilic conditions at a temperature from 32 to 38°C (6). *S. lipoferum* was described (7) as a highly motile, gram-negative, 1.5-turn spiral cell that has several lipid bodies filling most of the cell; colonies develop a pink pigment and a hardened and dry surface after a few days of incubation. Rates of acetylene reduction, specific activities, and efficiency of N_2 fixation have been reported (6); these were relatively high compared with other free-living N_2-fixing bacteria. We have studied the factors affecting growth and N_2 fixation in *S. lipoferum*.

MATERIALS AND METHODS

Organism and growth conditions. The organism used, *S. lipoferum* ATCC 29145, and organisms used for comparison were kindly supplied by J. Döbereiner and Day (7) enrichment medium containing the following in grams per liter of distilled water: K_2HPO_4 (6.0), KH_2PO_4 (4.0), $MgSO_4 \cdot 7H_2O$ (0.2), NaCl (0.1), $CaCl_2$ (0.02), $FeCl_3$ (0.01), $NaMoO_4 \cdot 2H_2O$ (0.1), $CaCl_2$ (0.02), $FeCl_3$ (0.01), $NaMoO_4 \cdot 2H_2O$ (0.002), and agar (Difco) 0.5 for semisolid medium or 20.0 for solid medium; it was adjusted to pH 6.8 with NaOH. Tenfold concentrated solutions of DL-malic acid, succinic acid, DL-lactic acid, pyruvic acid (pyruvic acid was sterilized by filtration), acetic acid or citric acid (neutralized to pH 6.8 with NaOH), and glucose or galactose, were sterilized separately and were added to the medium to give a final concentration of 0.5%. Yeast extract, peptone, Casamino Acids (all Difco) or KNO_3 were sterilized separately and were added aseptically to the medium to a final concentration of 0.005 to 0.01%. NH_4Cl (2.5 g/liter) was added to produce non-N_2-fixing cultures. Stock cultures of *S. lipoferum* were kept on slants of nutrient agar (Difco) at room temperature.

The cultures were grown at 30°C under stagnant conditions in 9-, 21-, or 120-ml vaccine bottles, or 1-liter Roux bottles, containing 5,10, 30, or 150 ml of medium, respectively. Cultures also were grown in

125-ml Erlenmeyer flasks containing 25 ml of medium; these were shaken in a 30°C water bath (125 strokes/min). *S. lipoferum* inoculum was prepared by adding 10 ml of sterile pH 6.8 phosphate buffer (0.05 M) to a 48-h-old nutrient agar slant. Cultures generally were inoculated with 0.1 ml of such a fresh bacterial suspension. Microaerophilic conditions for the cultures were obtained by adding 0.05% agar (occasionally 0.17% agar) to the medium to restrict mixing of the stagnant medium in cotton-stoppered bottles or by exchanging the atmosphere in the rubber-stoppered bottles five times with argon and then refilling the bottles with argon and O_2 to the desired pO_2.

Growth in larger batch cultures. A 10-liter bottle containing 9 liters of the liquid medium described with 0.5% malate as the carbon and energy source was inoculated with 100 ml of a suspension of *S. lipoferum* previously grown for 24 h on the same liquid medium containing 0.1% NH_4Cl. The culture was sparged with a sterile gas mixture of N_2 and air and stirred with a magnetic bar; a thermistor probe controlled an external heat lamp to maintain a temperature of 30°C. A constant dissolved O_2 concentration (in equilibrium with 0.008 atm of O_2) was maintained by monitoring the dissolved O_2 concentration with a sterilizable O_2 electrode (3) immersed in the culture. Samples of the growing culture were siphoned directly into serum bottles for assaying acetylene reduction and total nitrogen content. Spectrophotometric measurements of absorbance at 560 nm (A_{560}) of culture samples in 1-cm light-path cuvettes were used to calculate growth rates and approximate concentrations of bacteria. Bacteria also were counted by dilution plate counts; cultures were diluted in 0.05 M phosphate buffer for plating in nutrient agar. Total nitrogen and total carbon in the culture were determined by the methods of Johnson (8, 9).

Acetylene reduction. The amount of ethylene formed from acetylene reduction was determined by gas chromatography with a flame ionization detector. The column, 2 mm inside diameter and 150 cm long, was packed with Porapak R and was operated at 50°C (5). Final partial pressure of acetylene in the test vials was 0.11 atm. Activity was expressed as nanomoles of ethylene produced/(hour × milliliters of suspension).

Fixation of $^{15}N_2$. The method described in Quispel's book (5) was used. A 20-ml sample of *S. lipoferum* culture was transferred from a Roux bottle to a 60-ml serum bottle. The atmosphere was exchanged five times with argon, and then the bottle was filled with pA, 0.69, pO_2, 0.01, and $p^{15}N_2$, 0.30 atm. The bottle was shaken for 30 min at 30°C, and the reaction was stopped by adding 5 ml of 7.5 N H_2SO_4. The gas phase was analyzed mass spectrometrically for ^{15}N concentration, the cells were digested by the Kjeldahl procedure, and the NH_3 was distilled into acid. The NH_3 was oxidized to N_2 with alkaline hypobromite, and the ^{15}N concentration of the N_2 was determined with a Consolidated-Nier isotope-ratio mass spectrometer. N_2 fixation was expressed as nanomoles of N_2 fixed/(hours × milliliters of suspension). All experiments were repeated at least three times with three or more replicates in the experiments.

RESULTS

Growth and acetylene reduction in a nitrogen-free medium. Figure 1 shows growth rates, acetylene reduction, and pH changes in the medium in cultures of *S. lipoferum*. Malate, succinate, lactate, or pyruvate supported faster

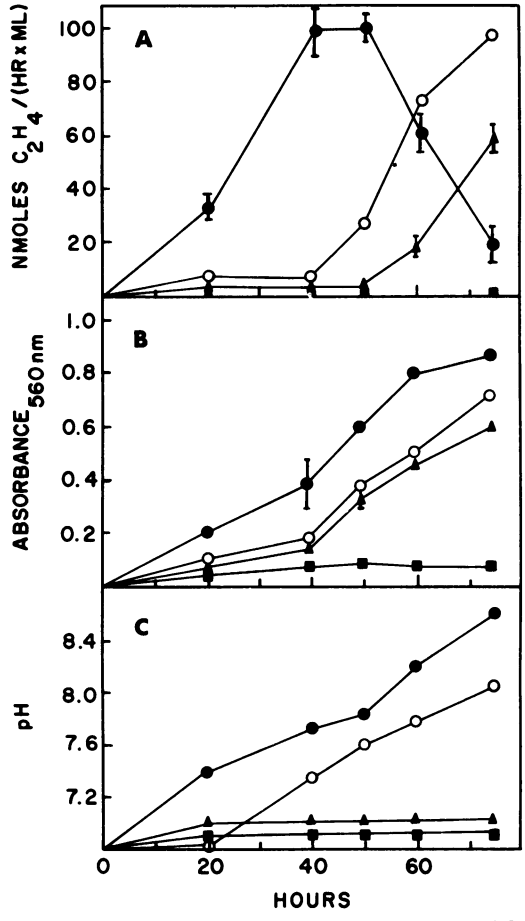

FIG. 1. *Growth curves and C_2H_2 reduction of S. lipoferum in "nitrogen-free" medium. Symbols: (●) Malate, succinate, lactate, or pyruvate 0.5% + yeast extract, KNO_3 or niacin, 0.005%; (○) malate, succinate, lactate, or pyruvate, no yeast extract, KNO_3 or niacin; (▲) 0.5% galactose or acetate + 0.005% yeast extract; (■) 0.5% glucose or citrate + 0.005 or 0.01% yeast extract. Bacteria were incubated in 9-ml bottles with 5 ml of enrichment medium; each measurement (abosrbance, C_2H_2 reduction, pH) was carried out three times on three replicates. A typical experiment is shown, and when the standard deviation among the grouped substrates was greater than the diameter of the symbol for the point on the line, an error bar to indicate the standard deviation is shown.*

growth rates (generation time, 20 h) and higher acetylene reduction specific activities than did galactose or acetate. With glucose or citrate as a carbon and energy source, *S. lipoferum* cultures reached absorbance levels of 0.1 to 0.12, and then growth stopped. Addition of small amounts of combined nitrogen (starter levels of 0.01% or 0.005% N) enhanced the growth rate and shortened the lag phase of the cultures (Fig. 1); KNO_3 or yeast extract was most effective, and peptone or Casamino Acids were less effective. In some cases acetylene reduction could be detected at the stage before growth ceased in the glucose cultures, but no further development or increase in acetylene reduction was observed. If the data for the top curve of Fig. 1B are plotted with log absorbance versus hours, the growth on malate, succinate, lactate, or pyruvate is approximately exponential between 20 and 60 h.

When the sodium salts of the organic acids were metabolized, the pH rose rapidly. The capacity for C_2H_2 reduction also rose rapidly until the pH approached 7.8; after the pH reached 7.9, the rate of acetylene reduction decreased rapidly. An increase in phosphate buffer concentration up to 10 g/liter prolonged growth and nitrogenase activities, and the cultures reached absorbance levels above 0.8 at 560 nm. Concentrations of phosphate higher than 10 g/liter inhibited growth and acetylene reduction by *S. lipoferum* cultures.

Oxygen requirements. *S. lipoferum* did not grow or fix N_2 under totally anaerobic conditions with the substrates tested. In a malate-containing enrichment medium without agar, *S. lipoferum* grew near the bottom of the 9-ml bottles and supported acetylene reduction. With 0.05 to 0.17% agar in the medium, the organism grew well and typically formed a pellicle about 2 mm below the surface; apparently diffusion of O_2 balanced O_2 use at this depth to produce an optimal concentration of dissolved O_2 for growth of *S. lipoferum* on N_2. An agar concentration of 0.05% in the medium supported good growth and acetylene reduction rates and permitted easy manipulation of bacterial suspensions and direct measurement of their turbidity to estimate growth. A curve of absorbance of 0.05% agar medium plotted versus the number of colony-forming units counted by the poured plate method was linear (A_{560} of 0.9 was equivalent to 7×10^8 colony-forming units/ml). Disturbance of suspensions of *S. lipoferum* by shaking gently or by transferring cultures from one vessel to another produced sharp decreases of acetylene reduction rates; apparently the organism's nitrogenase is poorly protected against O_2.

To determine the optimal pO_2 for acetylene reduction, *S. lipoferum* was grown in 1-liter Roux bottles containing 150 ml of malate medium with 0.05% agar. Ten-milliliter portions of culture were transferred to 120-ml bottles and were incubated stagnant for 4 h in air. After stoppering the vials with rubber vaccine stoppers, the atmospheres were exchanged five times with argon, and then acetylene, argon, and O_2 at different concentrations were added; these manipulations were performed with minimal agitation of the cultures. The bottles then were shaken vigorously in a 30°C water bath. The acetylene reduction rates measured are shown in Fig. 2. Cultures preincubated for 4 h after transfer to the test bottles gave good replication, but replication was poor when acetylene reduction by the cultures was estimated immediately after they were transferred from a Roux bottle. Generally, the specific activities for acetylene reduction were five to six times higher in shaken as compared with stagnant control cultures.

The optimal pO_2 (as estimated from the pO_2 in the atmosphere above the cells rather than from dissolved O_2) for acetylene reduction was lower with light suspensions (pO_2 = 0.006 atm at A_{560} of 0.18) and higher with heavy cultures (pO_2 = 0.02 atm at A_{560} of 0.5). No acetylene reduction activity was observed in the system without O_2 or above a pO_2 of 0.04 atm. The optimal pO_2 for $N_2(C_2H_2)$ reduction covers a very narrow range.

Batch liquid cultures of *S. lipoferum* were grown in a 10-liter bottle by controlling N_2 and O_2 flow to maintain a suitable dissolved O_2

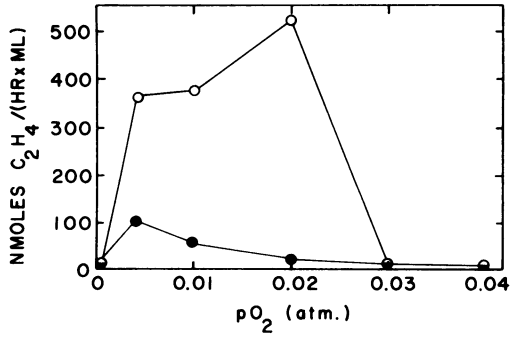

FIG. 2. *Effect of the pO_2 on C_2H_2 reduction by S. lipoferum. Symbols: (●) A_{560} = 0.18; (○) A_{560} = 0.5. Bacteria were incubated in 120-ml bottles, 10 ml of suspension of S. lipoferum was grown on enrichment medium with 0.05% agar. The atmosphere was exchanged five times with argon and then filled with argon, O_2, and acetylene. Cultures were shaken vigorously at 30°C for 30 min. Production of ethylene was linear during the 30-min test.*

concentration corresponding to a pO_2 of 0.008 atm O_2 and was monitored with a sterilizable O_2 electrode (3). The growing culture consumed increasing amounts of O_2, and although it was difficult to maintain constant dissolved O_2 manually, we observed an approximately linear increase in total N over a 28-h period and obtained a yield of 2.1 g (wet weight) of cells per liter at A_{560} of 1.1. The increase in absorbance was irregular; a plot of log absorbance against time indicated a generation time of about 8 h before the 10-h point and about 16 h for the culture between 10 and 28 h.

Samples (5 ml when A_{560} = 1.01) were taken from the batch culture and were incubated in bottles under stagnant conditions at 30°C at different pO_2 levels. Rates of acetylene reduction were measured periodically, and the results are presented in Fig. 3. In this liquid culture, a pO_2 near 0.004 atm was optimal for acetylene reduction. Immediately after transferring the suspensions from the batch culture, acetylene reduction activity was lost completely, but good activity was restored after 1.5 h. Addition of 50 μg of chloramphenicol per ml to cultures of *S. lipoferum* after they had been shaken for 1 min in air completely inhibited the return of acetylene reduction activity as compared with cultures without added chloramphenicol (Table 1); this suggests a need for protein synthesis to restore nitrogenase activity. Addition of the same concentration of chloramphenicol to a preincubated stagnant culture inhibited acetylene reduction only slightly during the test period.

Fixation of $^{15}N_2$. Fixation of $^{15}N_2$ by *S. lipoferum* cultures was measured in 60-ml bottles at pO_2, 0.01, $p^{15}N_2$, 0.3 and pA, 0.69 atm. An enrichment of 0.50 atm% ^{15}N excess was found in the culture after 30 min of incubation corresponding to 15.0 nmol of N_2 fixed/(h × ml of culture).

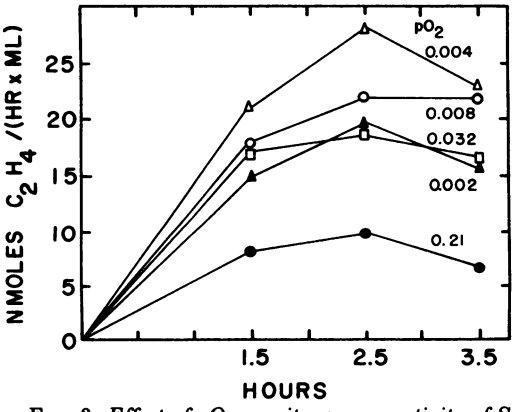

FIG. 3. *Effect of pO_2 on nitrogenase activity of S. lipoferum in liquid culture. The pO_2 in the atmosphere above the culture is indicated on the figure.*

TABLE 1. *Effect of chloramphenicol on C_2H_2 reduction by stagnant cultures of S. lipoferum after they have been shaken aerobically*

Treatment[a]	nmol of ethylene produced/(h × ml of cell suspension)	
	30 min	5 h
Stagnant	50	70
Shaken	0	60
Stagnant + 50 μg of chloramphenicol per ml	50	40
Shaken + 50 μg of chloramphenicol per ml	0	0

[a] Five-milliliter cultures of *S. lipoferum* in 9-ml bottles were stoppered and shaken in air in the presence of 50 μg of chloramphenicol per ml for 1 min. Then acetylene was added at zero time, and the ethylene produced was measured after 30 min. After 5 h of incubation (stagnant) the culture again was stoppered, and acetylene again was added; ethylene produced in the ensuing 30 min was analyzed.

Effect of NH_4^+. Addition of 0.25% NH_4Cl to a liquid or semisolid medium enhanced bacterial growth but completely inhibited acetylene reduction. Very fast growth rates were observed in shaken cultures, in contrast to the slower growth rates obtained under stagnant conditions (Fig. 4). *S. lipoferum* should be considered as a highly aerobic organism; the restrictions imposed by the sensitivity of nitrogenase to O_2 limit its growth rate under conditions that require it to fix N_2 rather than to use externally supplied NH_4^+. Pyruvate, succinate, lactate, or malate supported the best growth with NH_4^+, whereas little or no growth was observed with glucose, galactose, citrate, or acetate as the carbon and energy source (Fig. 4). Addition of yeast extract to the NH_4^+-containing medium shortened the lag phase but did not affect the growth rate (Fig. 4).

Efficiency. The efficiency of growth of *S. lipoferum* was expressed as milligrams of N assimilated per gram of carbon substrate consumed. Measurements were made in stagnant cultures by determining the initial and final total nitrogen in the cells and the carbon substrate content in the medium. Efficiencies measured after 72 h when 0.05% substrate was supplied appeared higher than when 0.5% substrate was supplied (Table 2). However, no marked differences in efficiency were observed when cultures supplied with 0.5% substrate for 36 h were compared with cultures supplied with 0.05% substrate for 72 h. There were no particular differences in efficiency among the substrates tested.

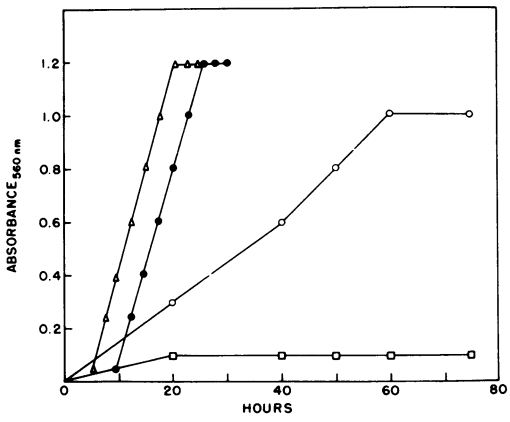

FIG. 4. *Growth curves of S. lipoferum with 0.25% NH_4Cl. Symbols: (△) 0.5% malate, succinate, lactate, or pyruvate + 0.005% yeast extract; (●) 0.5% malate, succinate, lactate, or pyruvate, no yeast extract; (□) 0.5% glucose, galactose, acetate, or citrate + 0.005 or 0.01% yeast extract. Above, 25 ml of liquid medium in a 125-ml Erlenmeyer flask shaken at 30°C, 125 strokes/min. (○) 0.5% malate, succinate, lactate, or pyruvate + 0.005% yeast extract and 0.05% agar; 5 ml in 9-ml bottles incubated under stagnant conditions.*

TABLE 2. *Efficiency of conversion of substrates to cells as judged by total N assimilated by S. lipoferum*[a]

Substrates	mg of N assimilated/g of substrate	
	36 h[b]	72 h
Malate (0.5%)	24.0	8.0
Lactate (0.5%)	21.0	7.9
Succinate (0.5%)	22.0	7.7
Malate (0.05%)		18.5
Lactate (0.05%)		20.0
Succinate (0.05%)		21.0

[a] *S. lipoferum* was grown at 30°C on 5 ml, 0.05% agar medium in 9-ml bottles.

[b] Total nitrogen was measured at time zero and after 36 or 72 h of incubation. Total C in the supernatant was measured at time 0, 36, and 72 h after centrifuging the culture at 12,000 × *g* for 10 min.

Morphology and growth of various isolates of *S. lipoferum*. Similarities and differences among several isolates of *S. lipoferum* are summarized in Table 3. The isolates can be separated into two main groups. Group 1 cultures are not capable of growing or fixing N_2 with glucose as a sole carbon and energy source; colonies developed a pink pigment and dry surfaces after 1 week of incubation on nutrient agar in petri dishes. Group 2 cultures are capable of growing and fixing N_2 on glucose; colonies have a wetter appearance and dry more slowly, and a pink pigment is observed only after 4 weeks of incubation. Differences were not consistently correlated with plant source. Group 1 organisms all were isolated at the Kilometer 47 station in Brazil (6), whereas two isolates out of three of group 2 were isolated from geographical regions other than Kilometer 47.

DISCUSSION

Our results corroborate in most details the observations by Döbereiner and Day (7) and Day and Döbereiner (6) on the factors affecting growth and N_2 fixation by *S. lipoferum*. The $^{15}N_2$ fixation assay has verified the ability of *S. lipoferum* to fix N_2. Malate, succinate, lactate, pyruvate, and galactose supported growth and acetylene reduction in a medium containing 0.05% agar. The agar increased viscosity sufficiently so that growth could be established under microaerophilic conditions about 2 mm below the surface, but it did not interfere appreciably with bacterial counts or measurements of absorbance. Döbereiner and Day (7) used 0.17% agar, which is suitable for isolating *S. lipoferum* but interferes with absorbance measurements. Increasing the phosphate salts in the medium to 10 g/liter prolonged growth and acetylene reduction activity and increased the yield of cells in batch cultures. As observed by Day and Döbereiner (6), specific activities for acetylene reduction decreased sharply after a pH of 7.8 to 7.9 in the medium was reached.

Acetylene reduction by *S. lipoferum* with glucose, in combination with a small amount of carbon starter compound such as malate or yeast extract, was reported by Day and Döbereiner (6); they did not report measurements of growth. We observed only minimal growth on glucose plus yeast extract (ATCC 29145), although in some cultures there was measurable acetylene reduction and a slight increase in absorbance after 24 h of incubation. Growth and activity probably stopped after the carbon starter compound was depleted, and no further increase in absorbance was observed during 2 weeks of incubation. Schröder (11) reported good growth on glucose. For 3 of 12 isolates of *S. lipoferum* that we examined, there was good growth and acetylene reduction with glucose as a sole carbon source.

Addition of NH_4^+ to the medium completely repressed nitrogenase but enhanced growth rates of *S. lipoferum* under aerobic conditions; evidently *S. lipoferum* is a fully aerobic organism when supplied available fixed nitrogen. It was surprising that the rapid growth on NH_4^+ (Fig. 4) was linear rather than exponential; the

TABLE 3. *Characteristics of various isolates of S. lipoferum*

Isolate	Growth on enrichment medium, 0.05% agar	Growth on solid enrichment medium, 2.0% agar	Growth on nutrient agar
Group 1 *Spirillum lipoferum* 4, 7, and 13 from *Digitaria decumbens* (Kilometer 47, Brazil) 81, 82, 80, and 75 from maize (Kilometer 47, Brazil) 60, 5le from wheat (Kilometer 47, Brazil)	Typical pellicle below surface Good growth and acetylene reduction on malate, succinate, lactate and pyruvate; very similar growth curves; see Fig. 1 Slower growth on galactose No growth or acetylene reduction on glucose No need for yeast extract as starter	Round or irregular wet, hard, opaque colonies; a pink pigment develops after 2 weeks as well as a dark spot in the center of the colony 5le has very strong pink pigment	Round or irregular, translucent colonies are dry and develop a pink pigment after 1 week of incubation
Group 2 59, from wheat (Kilometer 47, Brazil) RG from wheat (Rio Grande do Sul, Brazil) USA_8 from grass (Pullman, Wash.)	(1) As group 1 (2) As group 1 (3) As group 1 (4) Growth and acetylene reduction on glucose (5) Need for yeast extract as starter or heavy inoculum	Growth only when yeast extract is present in the medium	Dry and develop a pink pigment only after 5 weeks of incubation

early doubling time was about 2 h (7- to 9-h period) and then increased to about 5 h (9- to 14-h). The requirement of *S. lipoferum* for microaerophilic conditions when fixing N_2 is well established. Good acetylene reduction activities obtained in roots of grasses infected with *S. lipoferum* have been associated with the low pO_2 inside the root (7). The optimal pO_2 range for N_2 fixation is very narrow. In liquid medium we found a pO_2 of 0.004 atm optimal, whereas Day and Döbereiner (6) reported a pO_2 of 0.01 to 0.02 atm. Relatively dense cultures (A_{560} = 0.5) in 0.05% agar medium were assayed for their C_2H_2 reduction in 30 min; they exhibited an optimal pO_2 of 0.02 atm, whereas relatively light culture had an optimal pO_2 of 0.006 atm. The data are in terms of the pO_2 in the gas phase supplied rather than in terms of dissolved O_2. When 9-liter batch cultures were grown in a liquid medium, it proved difficult to maintain the dissolved O_2 manually, because there was an increasing demand for O_2 by the growing culture. We are developing apparatus to maintain a constant level of dissolved O_2 in liquid cultures to aid in defining the optimal pO_2 more accurately.

Disturbing or shaking a culture under aerobic conditions produced an immediate loss of nitrogenase activity and indicated that nitrogenase in *S. lipoferum* is poorly protected against O_2. On the other hand, shaking a culture under microaerophilic conditions at its optimal pO_2 in a stoppered bottle increased its rate of acetylene reduction five to six times over stagnant cultures. Addition of chloramphenicol to cultures that had been shaken for 1 min in air inhibited return of acetylene reduction activity; this indicated a need for protein synthesis to restore nitrogenase activity. This result differed from that observed with the azotobacter, as switching nitrogenase on and off with a change in pO_2 did not involve de novo protein synthesis (10). Day and Döbereiner (6) reported high efficiency in the utilization of substrate carbon to support the assimilation of cellular nitrogen of *S. lipoferum* grown at a low pO_2; tests were made with a semisolid agar medium and with a low substrate concentration. We obtained about half their reported efficiencies in cultures incubated in air, without shaking, in a medium containing only 0.05% agar. After a short incubation period, efficiency appeared to be higher (0.5% substrate) than when cultures were incubated for 72 h. Perhaps most growth was complete in 36 h, and subsequently the organisms supplied with 0.5% substrate oxidized substrates with minimal coupling of the oxidation to growth. We observed no marked differences in efficiency among the substrates tested, whereas Döbereiner and Day (7) reported higher efficiency with lactate than malate (lactate is the more reduced compound).

Several *S. lipoferum* isolates were placed in two main groups. Different isolates from different plants (Table 3) showed almost identical growth curves on malate and galactose in group 1 and on malate, galactose, and glucose in group 2. It was not possible to relate group differences or similarities to plant species from which the organisms were isolated. Two isolates out of three in group 2 were from geographical regions different from the region of origin of

group 1 isolates, and this may account partially for their differences in morphology and requirements for growth. More detailed work is needed to establish firmly the taxonomy of *S. lipoferum* and to determine whether the two groups represent a single bacterial species.

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LITERATURE CITED

1. **Becking, J. H.** 1963. Fixation of molecular nitrogen by an aerobic *Vibrio* or *Spirillum*. Antonie van Leeuwenhoek 29:326.
2. **Beijerinck, M. W.** 1925. Über ein *Spirillum* welches freien Stickstoff binden kann? Zentbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 63:353–359.
3. **Borkowski, J. D., and M. J. Johnson.** 1967. Long-lived steam-sterilizable membrane probes for dissolved oxygen measurement. Biotechnol. Bioeng. 9:635–639.
4. **Buchanan, R. E., and N. E. Gibbons (ed.).** 1974. Bergey's manual of determinative bacteriology, 8th ed., The Williams & Wilkins Co., Baltimore.
5. **Burris, R. H.** 1974. Methodology, p. 9–33. *In* A. Quispell (ed.), The biology of nitrogen fixation. North-Holland Publishing Co., Amsterdam.
6. **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.
7. **Döbereiner, J., and J. M. Day.** 1975. Associative symbiosis in tropical grasses: characterization of microorganisms and dinitrogen fixing sites. International symposium on N_2 fixation-interdisciplinary discussions, 3–7, June 1974, Washington State University. Washington State University Press, Pullman.
8. **Johnson, M. J.** 1941. Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem. 137:575–586.
9. **Johnson, M. J.** 1949. A rapid micromethod for estimation of non-volatile organic matter. J. Biol. Chem. 181:707–711.
10. **Postgate, J.** 1974. Prerequisites for biological nitrogen fixation in a free-living heterotrophic bacterium, p. 663–686. *In* A. Quispel (ed.), The biology of nitrogen fixation. North-Holland Publishing Co., Amsterdam.
11. **Schröder, M.** 1932. Die Assimilation des Lufstickstoff durch einige Bakterien. Zentbl. Bacteriol. Parasitenkd. Infektionskr. Abt. 2 85:177–212.
12. **Von Bülow, J. F. W., and J. Döbereiner.** 1975. Potential for nitrogen fixation in maize genotypes in Brazil. Proc. Natl. Acad. Sci. U.S.A. 72:2389–2393.