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Methods for Growing *Spirillum lipoferum* and for Counting It in Pure Culture and in Association with Plants

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Methods are described for growing *Spirillum lipoferum* in quantities sufficient to serve as inoculant in field trials of its associative N_2-fixing ability with higher plants and as a source of cells for the preparation of nitrogenase, cytochromes, respiratory enzymes, etc. A heavy inoculum of *S. lipoferum* grown on NH_4^+ was transferred to a medium of minimal nitrogen content, and initial rapid growth at the expense of residual combined nitrogen was replaced later by slower growth on N_2. Conversion to N_2 fixation was prompt upon exhaustion of fixed nitrogen; growth on N_2 was most rapid at a pO_2 of 0.005 to 0.007 atm. Numbers of *S. lipoferum* can be estimated by diluting soil, crushed roots, or other material, and inoculating diluted samples into a stagnant semisolid medium. Development of a characteristic subsurface layer of organisms and demonstration the these organisms can reduce C_2H_2 are presumptive evidence that they are *S. lipoferum*. With most-probable-number tables the observations can be converted to numbers of *S. lipoferum* in the samples. The most-probable-number method indicated that numbers of *S. lipoferum* may increase 100-fold or more in roots of maize removed from the plant and incubated for 24 h at 30°C at a pO_2 initially adjusted to 0.01 atm.

Reports that *Spirillum lipoferum* fixes N_2 in association with grass roots (3, 4, 6) have prompted numerous investigations of these associations both in the laboratory and the field. The physiology of *S. lipoferum* has been studied (1, 3), but little attention has been paid to the practical problems of producing inoculum for field trials and laboratory investigations or of counting the organisms as recovered from roots and soil. This paper describes a simple method for growing *S. lipoferum* in quantity and for evaluating the population of *S. lipoferum* by the most-probable-number (MPN) method.

MATERIALS AND METHODS

S. lipoferum ATCC 29145 was kept at room temperature on nutrient agar (Difco Laboratories) slants in screw-capped test tubes; these cultures remained viable for as long as 12 months. Before use, the culture was tested for purity by microscopic examination of wet mounts and by streaking it on a nutrient agar plate. Typical colonies are round to irregular, translucent, and hard, and they develop a pink pigment after about 1 week at 30°C. When *S. lipoferum* is grown on a semisolid nitrogen-free medium, it forms a typical thin layer of growth (pellicle) 1 to 4 mm below the surface (4).

The medium of Döbereiner and Day (4) was modified to provide increased buffering capacity, micronutrient elements, and a limited amount of NH_4Cl to aid in initiating aerobic growth. The medium contained the following (per liter of distilled water): K_2HPO_4 (6.0 g) and KH_2PO_4 (4.0 g) (mixed in 0.1 the final volume and autoclaved separately from the other medium constituents; later the phosphate solution was mixed with the cold medium), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.1 g), $CaCl_2$ (0.02 g), NH_4Cl (1.0 g), DL-malic acid (5.0 g), NaOH (3.0 g), Difco yeast extract (which shortened the lag in growth and aided vigorous growth) (0.1 g), $FeCl_3$ (10.0 mg), $NaMoO_4 \cdot 2H_2O$ (2.0 mg), $MnSO_4$ (2.1 mg), H_3BO_3 (2.8 mg), $Cu(NO_3)_2 \cdot 3H_2O$ (0.04 mg), and $ZnSO_4 \cdot 7H_2O$ (0.24 mg). The final pH was adjusted to 6.8.

For growth on N_2 under stagnant microaerophilic conditions, no NH_4Cl was added to the medium, but 0.5 g of agar per liter was added (dissolved by boiling before autoclaving). *S. lipoferum* will also form N_2-fixing colonies on solid, N-free medium (2% agar) of the above composition providing that the yeast extract is included to aid in the initiation of growth.

For optimal growth on N_2 in a liquid medium, the culture was maintained at a constant pO_2 of 0.005 to 0.007 atm by sparging with a mixture of N_2 and air, which was monitored by an O_2 electrode and controlled by an oxygenstat.

A 24- to 48-h slant culture suspended in 5 ml of 0.05 M sterile phosphate buffer (pH 7.0) was used as the initial inoculum. For subsequent cultures of higher volume, the inoculum was 10% (vol/vol) of a culture of *S. lipoferum* (pH below 7.8, late exponen-

tial phast) containing approximately 10^9 cells per ml. *S. lipoferum* was grown at 30°C in small Erlenmeyer flasks by shaking, in 3-, 10-, or 20-liter bottles with sparger and vigorous mixing, or in a 180-liter fermentor.

Acetylene reduction and bacterial counts. Maize roots from field or growth chamber experiments were collected, washed in distilled water, and incubated in 120-ml bottles under microaerophilic conditions by the method of Döbereiner and Day (4), and their acetylene reduction was measured (2). The roots were thoroughly washed with tap water to remove all loosely adhering soil particles. The washed roots (10 g [wet weight] each) were suspended in 90 ml of sterile 0.05 M phosphate buffer (pH 7.0) and were shaken for 1 h to release bacteria from the root surfaces. The roots were surface sterilized for 2.5 min in solution of sodium hypochlorite (approximately 1% consisting of 1 volume of commercial Clorox plus 5 volumes of water) and then were washed at least five times with sterile distilled water. The roots were crushed in the sterile phosphate buffer with a sterile mortar and pestle. Samples from these treatment, i.e., washed roots, surface-sterilized roots, and surface-sterilized and crushed roots, were immediately diluted serially in 10-fold steps in phosphate buffer. From each dilution, five 0.1-ml replicate samples were transferred to 9-ml cotton-plugged bottles containing 5 ml of nitrogen-free medium with 0.01% (wt/vol) yeast extract and 0.05% (wt/vol) agar added. Because the 9-ml bottles were filled with medium before sterilization in this experiment, the phosphate buffer was mixed together with the medium before sterilization. The inoculated cultures were incubated for 48 h at 30°C, and then each culture was examined for typical pellicle formation. The cotton plugs were exchanged for rubber serum stoppers in bottles showing pellicles (we never observed C_2H_4 formation in bottles without pellicles), and acetylene (12% of the total gas volume) was added; ethylene production was determined after 6 h of incubation. Positive identifications of *S. lipoferum* were recorded when there was typical pellicle formation and acetylene reduction activity. Negative identification was recorded for bottles without growth or bottles in which growth was positive but acetylene reduction was negative. MPN were calculated by using the probability tables of McGrady (5).

The cultures were also diluted in 0.05 M phosphate buffer for dilution plate counts in nutrient agar. Spectrophotometric measurements of the absorbance of samples at 560 nm in 1-cm light path cuvettes indicated the approximate concentrations of bacteria.

RESULTS AND DISCUSSION

The conditions required for growing *S. lipoferum* for specific applications are summarized in Table 1. Cells to be used as inoculum in the laboratory or in the field can be grown in NH_4Cl-supplemented medium under aerobic conditions. These convenient conditions give the fastest growth rate and the highest number of cells (10^9 cells per ml). Before the pH rises to 8.0, the cultures enter the stationary or declining growth phase. This growth method was useful for producing cells for enzyme extraction and for study of the physiology of the organism. Microaerophilic growth in semisolid agar under stagnant conditions was helpful for the isolation of the organism (4), since *S. lipoferum* grows in a typical pellicle 1 to 4 mm below the surface. This method was particularly useful for studying the substrates and growth conditons for N_2 fixation.

Studies of cell-free *S. lipoferum* nitrogenase were feasible when the organism was grown on N_2 at a constant, optimal pO_2 of 0.005 to 0.007 atm. If 10% inoculum grown with NH_4Cl under aerobic conditions is added to the nitrogen-free liquid medium, the organism grows rapidly (2-h generation time) until the combined nitrogen

TABLE 1. *Methods for growing S. lipoferum for specific uses*

Growth condition	Nitrogen source	Replication time	Late exponential phase				Suggested use
			pH	Absorbance at 560 nm	Approx no./ml	Yield of cells (g/liter)	
Aerobic (0.2 atm of O_2)	NH_4Cl	1–2 h	7.9	1.2	1×10^9	2.5–3.0	Inoculum for field experiments and cells for physiological studies and for preparation of enzymes and cytochromes
Microaerophilic (0.005 to 0.007 atm of O_2)	N_2	5.5–7 h	7.9	0.85	6.5×10^8	1.5–2.0	Cells for nitrogenase (may be adapted for continuous culture)
Microaerophilic (stagnant air with 0.05% agar)	N_2	20 h	7.9	0.80	6.0×10^8		Isolation, MPN counts, factors affecting growth and N_2 fixation
Aerobic (solid medium: 2% agar, 0.01% yeast extract)	N_2	Colonies formed in 48–72 h					Genetic studies, *nif*$^-$ mutants

in the medium is depleted, and then it switches to N_2 fixation with a 5.5- to 7.0-h generation time. In a period of 14 to 16 h, it is possible to obtain 1.5 to 2.0 g of cell paste per liter of culture; the cells have an active nitrogenase.

Bacterial counts of *S. lipoferum* by the pour plate method and by the MPN method agree reasonably well (Table 2). Attempts to count *S. lipoferum* in association with other bacteria on maize roots with nutrient agar pour plates were unsuccessful because many other types of bacteria overgrew *S. lipoferum* on the nitrogen-rich medium. Good results were obtained with the MPN technique by using typical pellicle formation and acetylene reduction as combined criteria for presumptive identification. The MPN method is not absolutely accurate (5), since the presence of many types of organisms on the root surface may affect its accuracy in counting *S. lipoferum*.

The data presented in Table 3 show how *S. lipoferum* was isolated and counted from roots from an inoculated field plot, whereas no *S. lipoferum* cells were detected even at the lowest dilution in a non-inoculated control plot giving a count of 8×10^6 bacteria per g of dry roots. Samples taken from the control field later in the growing season showed acetylene reduction activity, and *S. lipoferum* was then counted in cultures from the non-inoculated plot.

TABLE 2. *Numbers of S. lipoferum in pure culture as counted by the MPN and the pour plate method with nutrient agar*

Absorbance of 560 nm	No. of *S. lipoferum*[a]	
	Plate method	MPN
0.30	6.5×10^7	3.0×10^7
0.50	1.05×10^8	9.0×10^7
0.84	2.0×10^8	5.5×10^8

[a] Numbers are means of two replicates of each of two serial dilutions plated in duplicate or of five replicates for MPN. Standard deviations for numbers obtained by the pour plate method were within the ±10% range. Standard deviations for numbers obtained by MPN were in the ±20% range.

The total number of bacterial cells and *S. lipoferum* cells increased upon incubation of root samples under microaerophilic conditions at 30°C (Table 4). After incubation for 24 h, a 100-fold or greater increase often was observed in the number of *S. lipoferum* cells with a concomitant increase of acetylene reduction rates. High rates of N_2 fixation inferred from maize cultivars have been based on tests of roots for C_2H_2 reduction after their overnight incubation at low pO_2 (6). Proliferation of *S. lipoferum* during this preincubation may result in overestimation of the rates of N_2 (C_2H_2) reduction. Mass spectrometric analysis of the atmosphere in bottles containing roots being preincubated at low pO_2 (unpublished data) showed a decrease of the pO_2 and a rapid increase in the pCO_2. This suggested that an anaerobic metabolism had been established in the maize roots and that this probably produced organic acids (lactic acid, etc.) that supported vigorous growth of *S. lipoferum*. N_2 fixation rates based on measurement of C_2H_4 produced by preincubated roots probably are inaccurate because of the rapid proliferation of *S. lipoferum* cells during the period of preincubation at low pO_2.

Döbereiner and Day (4) reported localization of *S. lipoferum* within cortical root cells, and others have observed the organism inside root tissue. However, our measurements suggest that most of the *S. lipoferum* organisms before and after incubation are localized "outside" the roots and are easily separated by shaking the roots in buffer (Table 5). Surface "sterilization" sharply reduced the number of *S. lipoferum* and total bacteria, but the "inside" bacteria could be readily recovered after crushing the roots, although they were less numerous than the "outside" bacteria (Table 5). Many *S. lipoferum* cells could be counted after the roots were vigorously washed free of soil particles;

TABLE 3. *Effect of inoculation on the numbers of S. lipoferum and their acetylene reduction in roots of maize*

Field plot	Acetylene reduction[a] (nmol of ethylene/h per g [dry wt])	MPN method		
		No. of *S. lipoferum*/g (dry wt) of root[b]	Total no./g (dry wt) of root	
		Surface sterilized	Surface sterilized and crushed	
Inoculated	155 ± 20%[b]	1.1×10^3 ± 7%	6.5×10^6 ± 12%	7.0×10^6 ± 8%
Non-inoculated	0	0	0	8.0×10^6 ± 6%

[a] Roots were dried for 48 h at 80°C and weighed.

[b] Sweet corn roots (Wisconsin 900 hybrid variety) were collected from a field experiment in the Wisconsin River valley in August 1975. The roots were incubated overnight at room temperature (20 to 24°C) under microaerophilic conditions (started at a pO_2 of 0.01 atm); numbers represent means of two replicates from one maize root system divided into two equal parts.

TABLE 4. *Effect of incubation on maize roots under microaerophilic conditions on acetylene reduction and the number of S. lipoferum in plants from an inoculated maize field*[a]

Maize variety	Acetylene reduction (nmol of ethylene produced/min per g [dry wt])			MPN of *S. lipoferum*/g (dry wt)[b]		
	0 h	24 h	48 h	0 h	24 h	48 h
Wisconsin 900 hybrid						
Plant 1	0	425	2,424	1.0×10^4	2.0×10^6	2.8×10^7
Plant 2	0	275	1,008	1.5×10^3	3.2×10^6	1.1×10^7
Field maize						
Plant 1	0	8.8	72	1.3×10^4	3.2×10^5	7.0×10^5
Plant 2	0	14.0	75	5.7×10^3	1.9×10^5	2.9×10^6
Golden Cross Bantam hybrid						
Plant 1	0	12.0	240	1.5×10^3	1.4×10^6	1.1×10^7

[a] Maize roots from three plant varieties were collected from a field experiment in the Wisconsin River valley in September 1975. The roots were incubated at 30°C under microaerophilic conditions. The plant roots were divided into three equal parts to test acetylene reduction activity and to make bacterial counts at 0, 24, and 48 h.

[b] Roots were surface sterilized and then crushed. The total number of bacteria was two to three times higher than the number of *S. lipoferum*.

TABLE 5. *Localization of S. lipoferum in maize roots*[a]

Roots from:	Acetylene reduction (nmol of ethylene produced/h per mg [dry wt])		MPN of *S. lipoferum*/g (dry wt)[b]		Total no./g (dry wt) of root	
	0 h	24 h	0 h	24 h	0 h	24 h
Plant 1	0	678	(A) 3.6×10^5	1.35×10^7	3.6×10^5	7.5×10^8
			(B) 0	5.2×10^3	5.2×10^3	1.9×10^5
			(C) 3.3×10^3	5.0×10^3	7.5×10^5	4.2×10^7
Plant 2	0	866	(A) 6.6×10^4	9.0×10^7	3.6×10^6	1.6×10^8
			(B) 0	3.5×10^3	4.5×10^3	9.0×10^4
			(C) 4.0×10^3	1.6×10^6	4.0×10^6	2.5×10^6

[a] Roots of sweet corn (Golden Cross Bantam hybrid), inoculated with *S. lipoferum* and grown in a growth chamber, were divided into 2 equal fractions; one fraction was used to measure C_2H_2 reduction at 0 h and then was used for MPN counts of *S. lipoferum* and of total bacteria, whereas the second fraction of roots was incubated for 24 h and then was used in the same manner as the 0-h fraction. The experiment was repeated 2 more times with similar results.

[b] (A) Washed roots suspended in sterile buffer; (B) surface sterilized; (C) surface sterilized and crushed in sterile buffer.

this also indicates a very close association between *S. lipoferum* and roots.

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