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Carbon and Ammonia Metabolism of *Spirillum lipoferum*

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Intact cells and extracts from *Spirillum lipoferum* rapidly oxidized malate, succinate, lactate, and pyruvate. Glucose, galactose, fructose, acetate, and citrate did not increase the rate of O_2 uptake by cells above the endogenous rate. Cells grown on NH_4^+ oxidized the various substrates at about the same rate as did cells grown on N_2. Added oxidized nicotinamide adenine dinucleotide generally enhanced O_2 uptake by extracts supplied organic acids, whereas oxidized nicotinamide adenine dinucleotide phosphate had little effect. Nitrogenase synthesis repressed by growth of cells in the presence of NH_4^+ was derepressed by methionine sulfoximine or methionine sulfone. The total glutamine synthetase activity from N_2-grown cells was about eight times that from NH_4^+-grown *S. lipoferum*; the response of glutamate dehydrogenase was the opposite. The total glutamate synthetase activity from N_2-grown *S. lipoferum* was 1.4 to 2.6 times that from NH_4^+-grown cells. The levels of poly-β-hydroxybutyrate and β-hydroxybutyrate dehydrogenase were elevated in cells grown on N_2 as compared with those grown on NH_4^+. Cell-free extracts capable of reducing C_2H_2 have been prepared; both Mg^{2+} and Mn^{2+} are required for good activity.

Spirillum lipoferum can fix N_2 as a free-living organism or in association with the roots of several economically important grasses (9, 27). Döbereiner and Day (7, 9) characterized the factors affecting growth and N_2 fixation by *S. lipoferum*, and their observations have been verified (Albrecht and Okon, Plant Physiol. 56[Suppl. 2]:73, 1975). Organic acids, such as malate, lactate, pyruvate, and succinate, are the best carbon and energy sources for growth on N_2 (microaerophilic conditions) or NH_4^+ (aerobic conditions); glucose supported little or no growth. The addition of NH_4^+ to an enrichment medium completely repressed N_2 fixation (14).

The carbon and nitrogen metabolism of *S. lipoferum* was compared with other N_2-fixing organisms (15) on N_2 or on fixed nitrogen. The present paper is concerned with the metabolism of ammonia and carbon compounds by *S. lipoferum*.

MATERIALS AND METHODS

Organism and growth conditions. *S. lipoferum* ATCC 29145 was grown in 1-liter Roux bottles containing 150 ml of a modified Döbereiner and Day (9) medium with or without NH_4Cl (0.25%); the medium contained 0.005% yeast extract (Difco), 0.05% agar, and 0.5% sodium malate. The cultures were incubated at 30°C for 48 h (Roux bottles stagnant and lying flat), and the cells were collected and washed three times by centrifugation from 0.05 M phosphate buffer, pH 7.0.

Oxygen uptake by cell suspensions and cell-free extracts. The washed cells were resuspended in the pH 7.0 phosphate buffer, and rates of O_2 uptake were measured with a Rank O_2 electrode and recorder or with a Gilson volumometer at 30°C. The reaction mixture was composed of 2 ml of cell suspension, with an absorbance at 560 nm of 0.9 corresponding to 7×10^8 colony-forming units/ml by plate count, and 0.2 ml of substrate to give a final concentration of 0.04 M. The reaction was followed for 1 h with the volumometer or for 15 min with the O_2 electrode. Endogenous respiration was subtracted, and O_2 uptake rates were expressed as microliters of O_2 taken up per (hour × milligrams of N). Total nitrogen was determined by the Johnson method (reference 26, p. 259).

Crude, cell-free extracts of *S. lipoferum* were obtained by breaking the buffer-washed cells in a French press at 12,000 lb/in² at 4°C. The extract containing cell debris and agar was centrifuged at 17,000 × *g* for 30 min at 4°C, and the supernatant was used for measuring O_2 uptake and other enzyme activities. The extracts could be stored for at least 4 days at −18°C. The rates of O_2 uptake by cell-free extracts of *S. lipoferum* were estimated by a modification of the method of Stone and Wilson (22) with a Rank O_2 electrode with recorder for 15 min at 30°C. The reaction mixture contained 1 ml of 0.2 M potassium phosphate buffer, pH 7.0, 0.02 M final concentration of pH 7.0 $MgSO_4$, 1 ml of extract (1 to 2 mg of N per ml) in 0.1 M potassium phosphate buffer, pH 7.0, and 0.2 ml of substrate to give a final concentration of 0.04 M. The respiration without added substrate was subtracted, and rates of O_2 uptake were expressed as microliters of O_2 taken up per (hour × milligrams of N in the extract).

Enzymes for nitrogen metabolism. Crude, cell-free extracts were prepared in 50 mM tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.0. The protein content in the extracts was determined with the microbiuret method (10). L-Glutamate:ammonia ligase (adenosine 5'-diphosphate-forming) (6.3.1.2) (glutamine synthetase [GS]) was estimated by measuring the absorbance of γ-glutamyl hydroxamate at 540 nm (16) or by the γ-glutamyltransferase assay in the presence of 1 mM $MnCl_2$ (19).

Relative adenylylation of GS in crude extracts was estimated by the oligonucleate 5'-nucleotidohydrolase (3.1.4.1) (snake venom phosphodiesterase [SVD]) method (3) and also from absorbancies obtained from the transferase assay conducted in the presence or absence of 60 mM $MgCl_2$ (3).

L-Glutamine:2-oxoglutarate aminotransferase (reduced nicotinamide adenine dinucleotide phosphate [NADPH]-oxidizing) (2.6.1.53) (glutamate synthase [GOGAT]) and L-glutamate:NAD^+ oxidoreductase (deaminating) (1.4.1.2) (glutamate dehydrogenase [GDH]) were estimated by the method of Meers et al. (13) by following NADPH or NADH oxidation at 340 nm. Specific activity was expressed as micromoles of NADPH or NADH oxidized per (minute × milligrams of protein). NADPH:(acceptor) oxidoreductase (1.6.99.1) (NADPH dehydrogenase) and NADH:(acceptor) oxidoreductase (1.6.99.3) (NADH dehydrogenase) were estimated by following the oxidation of NADPH or NADH at 340 nm. The reaction mixture contained 0.75 ml of 50 mM Tris-hydrochloride buffer, pH 7.6, 0.15 ml of 1.5 mM NADPH or NADH, and enzyme to 1 ml. D-3-Hydroxybutyrate:NAD^+ oxidoreductase (1.1.1.30) (β-hydroxybutyrate dehydrogenase) was estimated by following NAD^+ (or $NADP^+$) reduction at 340 nm in the presence of β-hydroxybutyrate. The reaction mixture consisted of 0.65 ml of 50 mM Tris-hydrochloride buffer (pH 7.6), 0.15 ml of 1.5 mM NAD^+ or $NADP^+$, 0.15 ml of 0.4 M sodium DL-β-hydroxybutyrate, and enzyme to 1 ml. Specific activity was defined as micromoles of NAD^+ or $NADP^+$ reduced per (minute × milligrams of protein). Background rates of NADH or NADPH oxidation were subtracted from the data recorded in the tables.

Nitrogenase. Suspensions of *S. lipoferum* grown in Roux bottles with N_2 as the nitrogen source were transferred to centrifuge tubes and incubated overnight at 30°C. The suspensions were then centrifuged at 5,000 × *g* for 10 min, and about 4 g of the cell paste recovered was resuspended under anaerobic conditions in 15 ml of 200 mM Tricine buffer, pH 8, plus 0.2 ml of 100 mM $Na_2S_2O_4$. This suspension was disrupted under anaerobic conditions in a French press and was centrifuged anaerobically at 39,000 × *g* for 30 min at 4°C; the supernatant was used as the crude enzyme preparation. Nitrogenase was estimated by the production of ethylene from acetylene after 5 or 10 min of shaking under anaerobic conditions (stoppered vessels evacuated and flushed three times with high-purity tank N_2 before C_2H_2 was added) at 30°C in a water bath (5). The reaction mixture is described in Table 6. Specific activity was expressed as nanomoles of ethylene produced per (minute × milligrams of protein).

Poly-β-hydroxybutyric acid. The method of Stockdale et al. (21) was used to determine the poly-β-hydroxybutyric acid content of *S. lipoferum* cell suspensions. A total of 8 ml of 5% commercial hypochlorite (Clorox) was added to 2 ml of a suspension of *S. lipoferum* cells, and the residue from hypochlorite digestion was washed twice with 10 ml of water, acetone and diethyl ether to remove soluble salts and non-poly-β-hydroxybutyrate lipids. The final pellet was dried and dissolved in 2 ml of concentrated H_2SO_4 to yield crotonic acid. The absorbance of crotonic acid at 235 nm was measured (21), and sodium DL-β-hydroxybutyrate was used as a standard.

RESULTS

Oxygen uptake by cell suspensions. Substrates that supported growth of *S. lipoferum* (malate, succinate, lactate, and pyruvate) supported vigorous uptake of O_2 by washed cell suspensions. There was a mutual enhancement of O_2 uptake between organisms grown and tested on metabolically related substrates; e.g., cells grown on malate oxidized succinate well, and cells grown on lactate oxidized pyruvate well and vice versa (Table 1).

Glucose, galactose, fructose, acetate, and citrate failed to enhance O_2 uptake over endogenous rates in cell suspensions of *S. lipoferum*. No significant differences in O_2 uptake rates on various substrates were observed between cells grown on malate with N_2 or NH_4^+ as the nitrogen source.

Oxygen uptake by crude cell-free extracts. Tricarboxylic acid cycle intermediates, as well as lactate and pyruvate, supported O_2 uptake by cell-free extracts from *S. lipoferum*; activities were compatible with an operative trichloroacetic acid cycle (Table 2).

Succinate supported the highest rates of O_2 uptake, but other tricarboxylic acid cycle intermediates also were oxidized actively. The

TABLE 1. *Uptake of O_2 by intact cells of S. lipoferum*[a]

Grown on 0.04 M:	Oxygen uptake [QO_2(N)] tested on 0.04 M:			
	Malate	Succinate	Lactate	Pyruvate
Malate[b]	870 ± 31	840 ± 35	458 ± 37	342 ± 16
Succinate	725 ± 15	875 ± 65	520 ± 33	385 ± 35
Lactate	285 ± 68	250 ± 51	663 ± 22	730 ± 66
Pyruvate	242 ± 31	276 ± 44	519 ± 45	630 ± 39

[a] Data are given as QO_2(N), which equals microliters of O_2 uptake per (hour × milligrams of N in cells). Data given are the means of three replicates in each of three experiments with different batches of cells; both a Gilson volumometer and an O_2 electrode were used. Endogenous respiration was subtracted from total O_2 uptake. Rates are followed by the standard deviations of the pooled data.

[b] There were no appreciable differences in O_2 uptake when cells were grown with malate on either NH_4^+ or N_2 as the nitrogen source.

addition of NAD^+ enhanced O_2 uptake on malate, citrate, isocitrate, fumarate, oxaloacetate, α-ketoglutarate, lactate, or pyruvate. $NADP^+$ by itself increased O_2 uptake somewhat, but had little effect on the oxidation of the substrates tested. The addition of flavine adenine dinucleotide (FAD) to succinate or α-ketoglutarate reaction mixtures caused only a slight increase in the rate of O_2 uptake (Table 2).

There were no significant differences in O_2 uptake between extracts from cells grown on N_2 or NH_4^+. Glucose, fructose, galactose, glucose 6-phosphate, gluconate 6-phosphate, fructose 6-phosphate, and acetate failed to enhance O_2 uptake appreciably above the rate without added substrate, whereas a slight increase was observed with added fructose 1,6-diphosphate, glyceraldehyde 3-phosphate, and phosphoenol pyruvate (Table 2). The addition of coenzyme A to an acetate-containing extract did not affect O_2 uptake, and acetate did not enhance the rate of O_2 uptake when added together with malate or fumarate (22).

Ammonia metabolism. The addition of methionine sulfoximine or methionine sulfone (20 mg/ml) to cultures growing in the presence of NH_4^+ derepressed nitrogenase synthesis (Table 3).

GS. Activity was determined in extracts from cells grown on NH_4^+ or N_2. The biosynthetic assay indicated specific activities eight times higher for N_2-grown cells as compared with NH_4^+-grown cells (Table 4). The transferase assay and the SVD assay (3), applied to extracts from N_2-grown cells harvested at the log and stationary growth phases, showed high activities in the presence of Mg^{2+} plus Mn^{2+} as compared with Mn^{2+} alone (relative adenylylation $Mn^{2+}/[Mn^{2+} + Mg^{2+}] = 0.78$). There was no effect of preincubation with SVD (relative SVD adenylylation [SVD Mn^{2+} + Mg^{2+}]/[Mn^{2+} + Mg^{2+}] = 1), whereas an extract from log-phase NH_4^+-grown cells gave a positive response (Table 4) (relative adenylylation of 3.05 and SVD relative adenylylation of 2.28). A cell-free extract from NH_4^+-grown cells harvested in the stationary phase (72 h) showed a deadenylylated GS (Table 4).

TABLE 2. *Uptake of O_2 by cell-free extracts of S. lipoferum*[a]

Expt and substrate[b]	μl of O_2/(h × mg of N)
1. 0.04 M succinate	100–200[c]
2. 0.04 M succinate + 0.2 mM FAD	150–225
3. One of the following: 0.04 M malate, citrate, α-ketoglutarate, oxaloacetate, fumarate, isocitrate, lactate, or pyruvate	10–30
4. As in expt 3 + 0.2 mM NAD^+	30–60
5. As in expt 3 + 0.2 mM $NADP^+$	10–30
6. One of the following: 0.04 M fructose 1,6-diphosphate, glyceraldehyde 3-phosphate, or phosphoenol pyruvate	1–5
7. One of the following: 0.04 M glucose, galactose, fructose, glucose 6-phosphate, gluconate 6-phosphate, or fructose 6-phosphate	0–0.5

[a] Rates have been corrected for respiration without added substrate.

[b] Final concentrations are recorded; measurements were made in the O_2 electrode vessel and were linear for 15 min.

[c] Values recorded represent the range obtained with three different batches of cell extracts with three replicate measurements for each batch. Cells were grown on either NH_4^+ or N_2 as the nitrogen source.

TABLE 3. *Effect of various derepressing substances on acetylene reduction by NH_4^+-grown S. lipoferum* [a]

Addition (per ml)	Rate of acetylene reduction as a function of time of incubation (h)			
	2	6	8	16
0.8 mg of NH_4Cl	0	0	0	0
NH_4Cl + 20 mg of methionine sulfoximine	0	1.5	3	15
NH_4Cl + 20 mg of methionine sulfone	0	0	0	20

[a] Data are given as nanomoles of C_2H_4 produced per (hour × milliliters of culture). Substances were added to 2 ml of 24-h Roux bottle cultures in semisolid medium that had been transferred to 9-ml bottles; the cultures at transfer had a pH of 7.5 and an absorbance at 560 nm of 0.5. After the indicated times of incubation, C_2H_2 was added and the C_2H_4 produced in 30 min was measured.

Other enzymes. *S. lipoferum* GOGAT was found to be $NADP^+$ dependent; the specific activities in extracts from N_2-grown cells were 1.4 times those from NH_4^+-grown cells at 48 h and 2.6 times at 72 h. GDH showed an NAD^+ dependency and had 1.5 times as much activity in extracts from NH_4^+-grown as from N_2-grown cells (Table 5). There were no significant differences in the activity between NADH dehydrogenase or NADPH dehydrogenase in extracts from NH_4^+-grown as compared with N_2-grown cells.

β-Hydroxybutyric acid metabolism. The specific activities of β-hydroxybutyric acid dehydrogenase were much higher in cell-free extracts from N_2-grown as compared with NH_4^+-grown cells harvested during the log phase (Table 5). Some increase was observed in β-hydroxybutyric acid dehydrogenase in extracts from NH_4^+-grown cells collected during the stationary phase.

TABLE 4. *GS in S. lipoferum*

Growth medium	Time of harvest (h)	Biosynthetic assay	Transferase assay			Relative adenylylation	
			Mn^{2+}	$Mn^{2+} + Mg^{2+}$	SVD $Mn^{2+} + Mg^{2+}$	$Mn^{2+}/(Mn^{2+} + Mg^{2+})$[a]	(SVD $Mn^{2+} + Mg^{2+}$)/($Mn^{2+} + Mg^{2+}$)[b]
N_2	48	0.025[c]	0.44	0.57	0.57	0.78	1.0
	72		0.32	0.39	0.39	0.82	1.0
NH_4^+	48	0.0032	0.43	0.14	0.32	3.05	2.28
	72		0.22	0.26	0.31	0.85	1.19

[a] Column 4/column 5.

[b] Column 6/column 5.

[c] Specific activities are recorded as micromoles of γ-glutamyl hydroxamate formed per (minute × milligrams of protein) at 30°C. Experiments were repeated three times with each of three different extracts of cells.

TABLE 5. *Enzymatic activities of extracts from S. lipoferum cells grown on N_2 or NH_4^+ as a nitrogen source*

Nitrogen source	Time of harvest (h)	Enzymatic activity							
		GOGAT		GDH		NADPH dehydrogenase	NADH dehydrogenase	β-Hydroxybutyrate dehydrogenase	
		NADPH	NADH	NADPH	NADH			NAD^+	$NADP^+$
N_2	48	0.074[a]	0.0	0.0	0.017	0.006	0.015	0.040[b]	0.0
	72	0.067	0.0	0.0	0.033	0.006	0.014	0.036	0.0
NH_4^+	48	0.052	0.0	0.0	0.027	0.006	0.016	0.001	0.0
	72	0.025	0.0	0.0	0.042	0.002	0.017	0.012	0.0

[a] Specific activities are recorded as micromoles of NADH or NADPH oxidized per (minute × milligrams of protein).

[b] β-Hydroxybutyrate dehydrogenase specific activities are given as micromoles of NAD^+ or $NADP^+$ reduced per (minute × milligrams of protein). Assays were repeated two times with each of three different extracts.

The poly-β-hydroxybutyrate (PHB) content of *S. lipoferum* cells grown on N_2 was constant at different stages of growth (25 to 30% of the dry weight of the cells); PHB constituted 0.5 to 1.0% of the dry weight of NH_4^+-grown cells harvested during exponential growth. Absorption curves for PHB from *S. lipoferum* or for authentic β-hydroxybutyrate in concentrated H_2SO_4 (225- to 340-nm range) were nearly identical. The peak absorbance from the crotonic acid formed was at 235 nm in each case.

Nitrogenase in cell-free extracts. Crude cell-free extracts from *S. lipoferum* showed nitrogenase activity (Table 6) that was dependent on both $MgCl_2$ (14 mM) and $MnCl_2$ (0.45 mM).

TABLE 6. *Nitrogenase in cell-free extracts from S. lipoferum*

Determination	nmol of C_2H_4 produced/(min × mg of protein)
Complete[a]	3.5
− Mn^{2+}	0.020
− Mg^{2+}	0.025
− $Na_2S_2O_4$	0.000
− ATP-generating system	0.000

[a] Final concentrations in 2-ml total reaction volume were: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2.5 mM ATP, 20 mM creatine phosphate, 14.5 mM $MgCl_2$, 0.45 mM $MnCl_2$, and 0.1 mg of creatine kinase. A 1.1-mg amount of $Na_2S_2O_4$ (about 3 mM final concentration) in 0.1 ml of 0.02 M Tris at pH 7.4 was added, and the enzyme brought the volume to 2 ml.

DISCUSSION

Substances that supported growth and N_2 fixation by *S. lipoferum*, such as malate, succinate, lactate, and pyruvate, supported rapid O_2 uptake by cell suspensions, whereas no enhancement above endogenous rates was obtained with substances that supported poor growth and poor N_2 fixation. There was a mutual response between organisms grown and then tested on closely related substrates; e.g., cells grown on malate used succinate well. A similar adaptation to carbon sources has been observed with the rhizobia and azotobacter (6); the $QO_2(N)$ values for *S. lipoferum* are about 10 to 20% those for the azotobacter and similar to

those for the rhizobia. Tricarboxylic acid cycle intermediates as well as lactate and pyruvate supported active O_2 uptake in cell-free extracts from *S. lipoferum*; this was similar to the observations by Stone and Wilson (22) for the azotobacter and suggested that *S. lipoferum* has an operative tricarboxylic acid cycle. *Axotobacter vinelandii* has the most vigorous respiration reported for any organism. The respiratory rates of *S. lipoferum* in contrast are comparable to those of most aerobic bacteria, and the fact that the oxidation of tricarboxylic acid cycle intermediates by *S. lipoferum* is less rapid than by *A. vinelandii* should not be interpreted as evidence against a tricarboxylic acid cycle in *S. lipoferum*. Although citrate was not oxidized by suspensions of *S. lipoferum* cells, it did support O_2 uptake by cell-free extracts of *S. lipoferum* or the azotobacter (22). Electrons from tricarboxylic acid cycle intermediates, including isocitrate, were transferred to NAD^+ but apparently not to $NADP^+$. Benemann and Valentine (1) and Senior and Dawes (18) suggested that a $NADP^+$-linked isocitrate dehydrogenase may provide the major portion of the reducing equivalents required for N_2 fixation in azotobacter. Added acetate did not increase the rate of oxidation of malate or fumarate by extracts from *S. lipoferum*, although Stone and Wilson (22) observed such an increase with *A. vinelandii*. The utilization of acetate by *S. lipoferum* may require adaptation.

Sugars and sugar phosphates, such as glucose, fructose, galactose, glucose 6-phosphate, gluconate 6-phosphate, and fructose 6-phosphate, failed to enhance O_2 uptake above rates without added substrates in cell-free extracts from *S. lipoferum*, whereas fructose 1,6-diphosphate, glyceraldehyde 3-phosphate, and phosphoenol pyruvate were oxidized slowly.

The results suggest that the glycolytic and pentose phosphate pathways of metabolism are only weakly functional in *S. lipoferum*. Glucose is oxidized slowly by cell-free extracts from *A. vinelandii* (22). The optimal pO_2 for *S. lipoferum* is very low when it is fixing N_2, but good aeration enhances growth on NH_4^+. However, there is no significant difference in uptake of O_2 by N_2- or NH_4^+-grown cells or their extracts; likewise, there is no significant difference in the specific activities of their NADH or NADPH dehydrogenases.

The possible involvement of GS in the regulation of nitrogenase has been reported recently (2, 4, 23, 25). In the presence of excess NH_4^+, methionine sulfoximine and methionine sulfone, which inhibit GS, derepress nitrogenase in *A. vinelandii* and *Klebsiella pneumoniae* (11) and in *Anabaena cylindrica* (20); the agents also suppress adenylylation in *K. pneumoniae* GS (3; Gordon and Brill, unpublished data). Similar results have been obtained with *S. lipoferum*. Adenylylation of GS in *Escherichia coli* and several other bacteria has been compared by Tronick et al. (24), and they showed that the azotobacter, *K. pneumoniae*, and *Rhizobium japonicum* GS cross-reacted with *E. coli* GS antibodies. With the SVD method, Tronick et al. (24) demonstrated that variation in the relative adenylylation was affected by the amount of combined nitrogen in the medium.

With the methods and criteria of Tronick et al. (24) for adenylylation, Bishop et al. (2, 3) concluded that NH_4^+ causes an apparent repression of GS as well as an increase in adenylylation in free-living cells of *K. pneumoniae* and *R. japonicum*. Similar results were observed with *S. lipoferum* GS, as it apparently was adenylylated in extracts of NH_4^+-grown cells and deadenylylated in extracts from N_2-grown cells; this was not observed with *R. japonicum* bacteroids (3).

Activities of GOGAT in N_2-fixing bacteria generally are higher when the organisms are growing on N_2 rather than on NH_4^+ or other sources of combined nitrogen; the opposite is true for GDH (8). We have observed the same response with *S. lipoferum*. The coenzyme dependency, NAD^+ or $NADP^+$ for GOGAT or GDH, may vary among organisms (8). We observed that *S. lipoferum* has an $NADP^+$ GOGAT and an NAD^+ GDH.

The role of PHB and of β-hydroxybutyrate dehydrogenase in N_2 fixation is not well defined. Senior and associates (17, 18) suggested that the accumulated PHB in *Azotobacter beijerinckii* may favor N_2 fixation by serving as a sink to reduce potentially inhibitory levels of acetyl coenzyme A and NADPH formed under O_2-limited conditions. They also suggested (18) a possible role for PHB as an energy source for nitrogenase when the carbon source in the medium was depleted. Jones and Redfearn (12) demonstrated an NAD^+-linked β-hydroxybutyrate dehydrogenase in extracts from *A. vinelandii*; in combination with a cytochrome system it oxidized β-hydroxybutyrate rapidly, and it was suggested that PHB may aid in excluding O_2 from nitrogenase as well as acting as an electron donor. *S. lipoferum* cells grown on N_2 had a more highly active NAD^+-dependent β-hydroxybutyrate dehydrogenase than did cells grown on NH_4^+, and this indicates a possible relationship of PHB content and dehydrogenase activity in N_2 fixation.

In preliminary studies, we have obtained a cell-free extract from *S. lipoferum* capable of reducing acetylene. The activities have been rather low compared with preparations from other N_2-fixing bacteria. The extracts exhibit the normal requirements for adenosine 5'-triphosphate (ATP) and a low-potential electron donor. Both Mg^{2+} and Mn^{2+} are required; a similar requirement for Mn^{2+} has been observed with extracts from *Rhodospirillum rubrum* (Ludden and Burris, Science, in press). Purification of the MoFe and Fe proteins from *S. lipoferum* will permit a more detailed examination to establish whether its nitrogenase is comparable to other nitrogenases or whether its proteins have unique properties.

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