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H_2-Dependent Mixotrophic Growth of N_2-Fixing *Azotobacter vinelandii*†

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Azotobacter vinelandii **can grow with a variety of organic carbon sources and fix N_2 without the need for added H_2. However, due to an active H_2-oxidizing system, H_2-dependent mixotrophic growth in an N-free medium was demonstrated when mannose was provided as the carbon source. There was no appreciable growth with either H_2 or mannose alone. Both the growth rate and the cell yield were dependent on the concentrations of both substrates, H_2 and mannose. Cultures growing mixotrophically with H_2 and mannose consumed approximately 4.8 mmol of O_2 and produced 4.6 mmol of CO_2 per mmol of mannose consumed. In the absence of H_2, less CO_2 was produced, less O_2 was consumed, and cell growth was negligible. The rate of acetylene reduction in mixotrophic cultures was comparable to the rate in cultures grown in N-free sucrose medium. The rate of [^{14}C]mannose uptake of cultures with H_2 was greater than with argon, whereas [^{14}C]sucrose uptake was unaffected by the addition of H_2; therefore, the role of H_2 in mixotrophic metabolism may be to provide energy for mannose uptake. *A. vinelandii* is not an autotroph, as attempts to grow the organism chemoautotrophically with H_2 or to detect ribulose bisphosphate carboxylase activity were unsuccessful.**

Azotobacter vinelandii is an obligate aerobic common soil bacterium "capable of fixing nitrogen in a nitrogen-free medium with an organic carbon source" (1). Due to its rapid growth and high N_2 fixation rate, *A. vinelandii* is commonly used to study the physiology and biochemistry of N_2 fixation. Even though *A. vinelandii* is capable of oxidizing a wide variety of organic substrates, growth of *A. vinelandii* in most natural environments is thought to be severely limited by organic carbon (8). In addition to the production of NH_4^+, nitrogenase evolves H_2. An enzyme that apparently aids in the efficiency of the N_2 fixation process is hydrogenase. When fixing N_2, *Azotobacter* hydrogenase oxidizes the nitrogenase-evolved H_2 at high rates, and the electrons from H_2 are carried through an electron transport chain to O_2, yielding H_2O as the final product. H_2 oxidation is thought to serve primarily two beneficial purposes for N_2-fixing *Azotobacter* species (21, 27). These are to provide ATP, which could then be used in the nitrogenase reaction, and to augment respiratory protection by removal of O_2, thereby preventing damage to the O_2-labile nitrogenase.

Due to an active H_2 oxidation system, some N_2-fixing bacteria, such as *Rhizobium japonicum* (11), *Azospirillum* spp. (22), and *Derxia gummosa* (16), are capable of growing chemoautotrophically with H_2 and CO_2. However, *Azotobacter* species are not autotrophic, as attempts to grow *A. vinelandii* and *Azotobacter chroococcum* this way have been unsuccessful (16; unpublished data). Nevertheless, we thought that H_2 might, in energy-limited conditions, supply sufficient energy for growth of this heterotrophic bacterium. In this report, we demonstrate growth and N_2 fixation of *A. vinelandii* that require both an inorganic energy source (H_2) and an organic carbon source. This type of growth is characteristic of mixotrophy (4, 19).

(A preliminary account of this work has been presented [Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K32, p. 177].)

MATERIALS AND METHODS

Screening carbon sources for ability to support H_2-dependent growth. *A. vinelandii* OP was grown in nitrogen-free Burk (7) liquid medium containing 2% sucrose. A drop of mid-log-phase cells (approximately 2×10^7 cells) was spread evenly onto a carbon-free Burk agar plate. A Taxo carbohydrate disk (BBL Microbiology Systems, Cockeysville, Md.) was placed onto the agar plate for the following carbon sources (see Table 1): adonitol, arabinose, glucose, dulcitol, galactose, inositol, inulin, lactose, levulose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. A sterile blank disk was also included as a control. Sterile saturated solutions of the other carbon sources used (see Table 1) were prepared. A sterile disk was saturated with the solution and placed onto the plate. The plates were then transferred to an anaerobic vented jar (product no. 60608; BBL). An amount equal to about 20% of the jar volume of either H_2 or Ar was injected into the container. The plates were incubated at 30°C for at least 3 days, and the plates were removed and checked for growth. For liquid growth, side-armed flasks (volume, 180 ml) containing 20 ml of N-free Burk medium with the indicated organic compound were autoclaved for 15 min. An aliquot (0.05 ml) of early-log-phase cells grown on Burk medium with 2% sucrose was then inoculated into each flask. The flasks were tightly stoppered with sterile rubber stoppers, and 50 ml of H_2 or Ar gas that had been filtered through a sterile membrane filter (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) was injected into the flasks. Excess pressure inside the flasks was then released by injecting a sterile needle through the stopper for 2 to 5 s. The flasks were then placed in a water bath shaker (30°C) at 250 rpm.

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TABLE 1. Growth of *A. vinelandii* in the presence of hydrogen or argon[a]

Carbon source	Growth of culture under:	
	H_2	Ar
Acetate	+	+
Adonitol	−	−
D(+)Arabinose	+/−	+/−
Benzoate	+	+
D(+)Cellobiose	−	−
i-Erythritol	+	+
α-L(−)Fucose	−	−
D(+)Galactose	+	+
Methylglucoside	−	−
myo-Inositol	−	−
Itaconic acid	−	−
β-D(−)Levulose	+	+
D-Mannitol	+	+
Phenol	+	+
Propionate	+	+
D(+)Raffinose	+	+
2-Deoxyribose	−	−
Salicin	−	−
Sucrose	+	+
Xylose	−	−
D(+)Mannose	+	−
Aconitate	+	+
Adipic acid	−	−
Azelaic acid	−	−
n-Butyrate	+	+
Dulcitol	−	−
Ethanol	+	+
β-D(−)Fructose	+	+
D(+)Glucose	+	+
Glutamate	+	+
Inulin	+	+
α-Lactose	−	−
Maltose	+	+
α-D(+)Melibiose	+	+
Pimelate	−	−
Pyruvate	+	+
D(−)Ribose	−	−
α-L-Rhamnose	+	+
D-Sorbitol	+	+
Trehalose	+/−	+/−

[a] Procedures are described in the text. Symbols: +, growth; −, no growth; +/−, very little growth.

Optical density at 540 mm was monitored; 0.1 optical density unit was equivalent to approximately 2.2×10^7 viable cells per ml. All growth experiments were performed in this closed gas system; therefore, concentrations of gases such as H_2 and O_2 decreased during the course of these experiments. All carbon sources were obtained from Sigma Chemical Co., St. Louis, Mo., with the exception of glucose and acetate, which were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J.

Sugar uptake experiments. D-[U-^{14}C]mannose and D-[U-^{14}C]sucrose were purchased from Amersham Corp., Arlington Heights, Ill., and had specific activities of 300 and 552 mCi/mmol, respectively. *A. vinelandii* cells were grown in Burk medium with 2% mannose plus H_2 for 24 h. The cells were harvested and then washed three times with 50 mM phosphate buffer (pH 7.5). The cells were then suspended in carbon-free Burk medium to about 2×10^8 cells per ml. The culture was then incubated at 30°C for approximately 2 h to deplete the endogenous substrate. A 2-ml portion of the substrate-depleted sample was then transferred to a 15-ml bottle, which was then tightly stoppered with a sleeve-type rubber stopper. Three milliliters of either H_2 or Ar was then injected into the bottle. A 30-μl volume of [^{14}C]mannose solution (0.5 μCi) was injected along with cold mannose into the sample to give a final mannose concentration of 150 μM. At the times indicated (see Fig. 3), 0.1 ml of the sample was withdrawn from the bottle and then diluted into 4 ml of ice-cold phosphate buffer. A 2-ml portion of this diluted sample was filtered through a membrane filter (pore size, 0.22 μm; GSWP 024 00; Millipore), and the filter was washed twice with 10 ml of ice-cold phosphate buffer. The filters were then placed in 5 ml of Aquasol-2 (New England Nuclear Corp., Boston, Mass.), and radioactivity was counted in a Packard 3375 liquid scintillation spectrometer. Counting efficiency was 0.55. For [^{14}C]sucrose uptake experiments, the same procedure as described above for mannose was used, except that the cells were grown in 2% sucrose instead of mannose before harvest. Control experiments on boiled cells demonstrated that there was no mannose or sucrose uptake in nonviable cells. Radioactivity counts in these experiments were less than 80 cpm, whereas similar experiments with viable cells gave counts of over 1,000 cpm after 5 min.

Other assays. Whole-cell N_2 fixation by *A. vinelandii* was performed as described by Bishop et al. (2). Gas chromatography conditions for monitoring C_2H_4 production were as described previously (9). Carbon dioxide production was determined by measuring thermal conductivity with a Perkin Elmer Sigma 3B gas chromatograph. A column (2 m by 2 mm [inner diameter]) with Porapak N (80-100 mesh) was used. The carrier flow rate was 20 ml of helium per min, the detector and injector temperatures were 175°C, and the oven temperature was 50°C. For measuring the CO_2 produced during the 9-h incubation period (see Table 3), the culture was acidified with trichloroacetic acid, to ensure elimination of dissolved CO_2, before removal of the gas sample.

RESULTS

Forty carbon sources were tested for their ability to support growth of *A. vinelandii* on N-free agar plates in a closed gas system with either H_2 or Ar added at a partial pressure of 20%. The results are shown in Table 1. With the carbon sources that supported some growth, added H_2 did not facilitate growth. However, growth of *A. vinelandii* was observed on the mannose-containing plates in an atmosphere containing H_2 (Table 1), whereas no growth was observed when argon was substituted for H_2.

The effect of added H_2 on the growth rate of *A. vinelandii* in carbon-containing N-free media was subsequently tested.

TABLE 2. Doubling time of *A. vinelandii* grown with different carbon sources with either H_2 or Ar added[a]

Substrate	Doubling time (h) for culture grown under	
	H_2	Ar
Acetate	4.0	3.3
Glucose	2.8	3.0
Sucrose	3.0	3.0
Mannose	3.8	>14.0
Xylose	NG[b]	NG
Trehalose	NG	NG

[a] Procedures are described in the text.
[b] NG, No growth.

The cell doubling times on mannose and other carbon sources were compared (Table 2). The addition of H_2 did not stimulate the growth rate in any of the carbon substrates tested, with the exception of mannose. Therefore, H_2-dependent mixotrophic growth was dependent on the use of mannose as a carbon source. The doubling time of *A. vinelandii* in mannose decreased from more than 14 h (without H_2) to 3.8 h (with H_2). This difference in growth rate due to added H_2 was observed only when cells were fixing N_2. In the presence of NH_4^+ (0.05% NH_4Cl in the medium) doubling times were 4.8 h (with H_2) and 4.5 h (without H_2).

We studied the effects of the H_2 concentration on the growth rate in mannose-containing medium. Figure 1 shows the growth curves of N_2-fixing *A. vinelandii* in 2% mannose plus various amounts of H_2. Both the growth rate and the cell yield increased with increasing amounts of H_2. Maximum growth was achieved by the addition of 25% partial pressure H_2. Further addition of H_2 above 25% partial pressure did not increase growth rate or yield. Figure 2 shows the dependence of cell growth on the carbon source mannose, in the presence of saturating (25% partial pressure) H_2. In this experiment, H_2 was added after 24 h of incubation with mannose (Fig. 2, arrow) to show the dependence of growth on the added H_2; there was no appreciable growth before H_2 addition (Fig. 2). The results show a concentration dependence effect on mannose for both growth rate and yield. Maximum growth occurred at 2% mannose concentration;

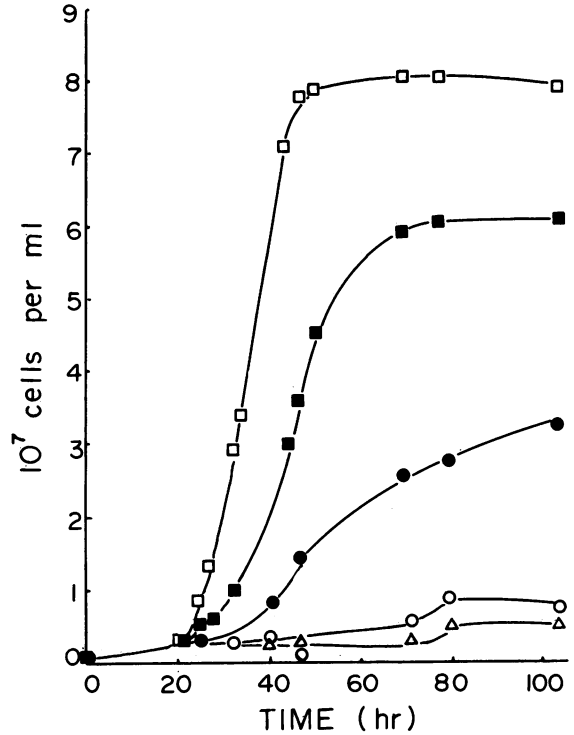

FIG. 1. Growth curves of N_2-fixing *A. vinelandii* in 2% mannose and various concentrations of H_2. H_2 was injected into the 150-ml stoppered flask 18 h after inoculation, and the cells were incubated and growth was monitored as described in the text. Cultures were grown under 50 ml of H_2 (□), 30 ml of H_2 plus 20 ml of Ar (■), 10 ml of H_2 plus 40 ml of Ar (●), 5 ml of H_2 plus 45 ml of Ar (○), or 50 ml of Ar (△).

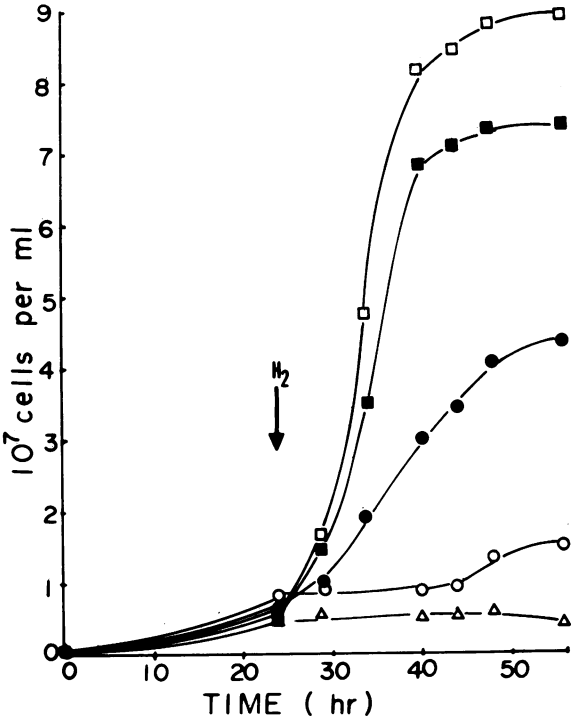

FIG. 2. Growth curves of N_2-fixing *A. vinelandii* with added H_2 plus 2% mannose (□), 1% mannose (■), 0.5% mannose (●), 0.125% mannose (○), or no mannose (△). Arrow shows the time at which H_2 (final 25% partial pressure) was added. Other conditions were as described in the legend to Fig. 1.

higher concentrations (3 and 6% [wt/vol]) were also tested (data not shown).

To confirm H_2- and mannose-dependent mixotrophic metabolism in *A. vinelandii*, we measured cell yield, CO_2 production, mannose consumption, and oxygen consumption in cultures with H_2 or argon added (Table 3). The cultures with added H_2 exhibited high rates of H_2 uptake

TABLE 3. Cell yield, CO_2 production, and mannose and O_2 consumption during mixotrophic growth of *A. vinelandii*[a]

Experiment no.	Addition	Total CO_2 production (mmol)	Mannose consumption (mmol)	Oxygen consumption (mmol)	Cell yield (viable cell number [10^7])
1	H_2	0.63	0.147	0.67	4.4
2	H_2	0.83	0.168	0.86	6.0
3	Ar	0.32	0.07	0.29	<0.5
4	Ar	0.40	0.05	0.35	<0.5

[a] The H_2 consumption rate for experiments 1 and 2 at the time of harvest was approximately 2.4 μmol of H_2 per h per 10^8 cells. All four cultures (10 ml of medium in a 180-ml flask) were initially inoculated with 2×10^6 cells and then incubated in a closed-flask system as described in the text. Gas samples (0.2 ml) were removed at the time of inoculation of each flask and again (9 h later) when cell yield was determined; the total amount of O_2 consumed or CO_2 produced was then calculated. Carbon dioxide was determined by gas chromatography as described in the text. Oxygen was determined polarographically as described previously (15). Mannose (the only organic carbon source in the medium) was also determined initially and at 9 h on 0.5-ml liquid samples after cells were removed by centrifugation. Mannose was quantitated by the phenol sulfuric acid method for hexose determination as described previously (12). Appropriate standards of CO_2, O_2, and mannose were made which included concentrations both above and below the concentrations of the experimental samples.

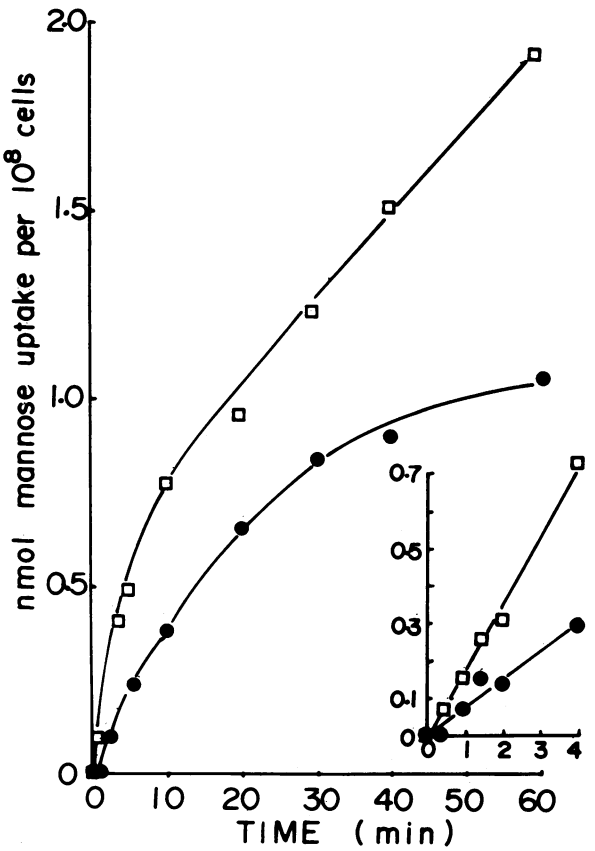

FIG. 3. [^{14}C]mannose uptake of *A. vinelandii* with added H_2 (□) or Ar (●). Cultures were incubated with [^{14}C]mannose for the indicated time with either H_2 or Ar, the cultures were rapidly filtered and washed on the filter, and radioactivity was determined as described in the text. The radioactivity count for the zero time culture (the sample of cells removed immediately after inoculation into the [^{14}C]mannose solution) was subtracted from all the other counts before the data were processed. Each point is the average of two independent determinations. (Inset) a similar experiment performed over a 4-min total time period.

activity (see the legend to Table 3) and grew from an inoculum of 2×10^6 cells to over 4×10^7 cells (Table 3, experiments 1 and 2). In contrast, the cultures without H_2 grew very little (to less than 5×10^6 cells). The cultures incubated with H_2 also consumed mannose, used an average of 4.8 mmol of O_2, and produced 4.6 mmol of CO_2 per mmol of mannose used for the two experiments with H_2 added. These are reasonable stoichimetries for O_2 production and CO_2 production via sugar metabolism by aerobic bacteria (10, 20).

The cultures incubated with Ar rather than H_2 still used mannose, produced some CO_2, and respired oxygen (Table 3, experiments 3 and 4). Therefore, these cultures are capable of some mannose-dependent metabolism, yet this must not be sufficient for growth. Even though no growth was observed in this condition, the fact that substrate was used and O_2 was consumed is still consistent with current views on maintenance metabolism requirements of bacteria (23). The lack of growth with mannose plus argon is not inconsistent with previous work on mannose metabolism in *A. vinelandii*; previous researchers have noticed that N_2-fixing *A. vinelandii* utilizes mannose for the production of acid, yet the cells are not capable of growth (24). Apparently, they did not test the effect of adding H_2.

To study the roles of mannose and H_2 in mixotrophic growth, we performed sugar uptake experiments. We compared the accumulated ^{14}C from mannose over a 60-min period in cells supplied with H_2 versus that in cells supplied with Ar (Fig. 3). The amount of ^{14}C accumulated in cells was greater in the presence of H_2 than without H_2. CO_2 measurements ruled out the possibility that mannose merely accumulated unchanged in the cells during transport studies. Little CO_2 production was observed until the substrate mannose was added, and CO_2 production was twofold greater in the presence of H_2 compared with that observed in the presence of argon; this indicates that the mannose transported is actually metabolized. Since sugars are rapidly metabolized after they enter cells, it is important to determine sugar uptake in the first few minutes. This gives a more accurate determination of the real uptake rate of the labeled sugar. The uptake rates of [^{14}C]mannose over a 4-min period in cultures grown with and without H_2 are also shown in Fig. 3 (inset). The uptake rate with H_2 provided was greater than that without H_2; after 4 min, the cells with H_2 accumulated more than two times as much ^{14}C-sugar as did cells without H_2. The results (Fig. 3) suggest that one role of H_2 may be to provide energy for mannose uptake. Also, the results demonstrate that the probable role of mannose is indeed as an intracellular carbon substrate for cells, rather than another role, such as providing a suitable osmotic environment for H_2-dependent growth. As a control, we also determined the effect of H_2 and Ar on [^{14}C]sucrose uptake. In this case, there was no difference in ^{14}C accumulation in cells with and without H_2 provided (data not shown).

Even though *A. vinelandii* took up mannose, it is important to rule out CO_2 as a major carbon source under H_2-dependent growth conditions. Attempts to detect appreciable whole-cell CO_2 fixation or ribulose bisphosphate carboxylase activity in permeabilized cells in H_2 plus mannose growth conditions were unsuccessful. Likewise, many attempts to grow *A. vinelandii* chemoautotrophically with H_2 were unsuccessful.

The rate of acetylene reduction by cells growing exponentially in mannose plus H_2 was 412 nmol of C_2H_4 produced per h per 10^8 cells. This value is comparable to the rates of N_2 fixation for this bacterium in N-free medium reported by others (3, 17). It is also comparable to the N_2 fixation rate we have observed in N-free sucrose medium (up to 550 nmol of C_2H_4 per h per 10^8 cells). Therefore, *A. vinelandii* growing mixotrophically presumably has no difficulty producing the energy and reductant needed for N_2 fixation.

DISCUSSION

Mixotrophy is the term used to describe the ability of an organism to use inorganic and organic compounds concomitantly as energy sources or carbon sources or both (4). We have demonstrated that the heterotrophic N_2-fixing bacterium *A. vinelandii* can grow mixotrophically. This type of growth in *A. vinelandii* was dependent on H_2 as the inorganic energy source and mannose as the organic carbon substrate. Most of the thiobacilli, hydrogen bacteria, nitrifying bacteria, and iron-oxidizing bacteria use the Calvin cycle to fix CO_2 and are chemolithoautotrophic. Mixotrophic growth is thought to be common among these chemolithoautotrophic bacteria (13, 18, 19). However, mixotrophy has been demonstrated in only a few heterotrophic bacteria. The anaerobe *Desulfovibrio* sp. can use either H_2 or organic compounds as the energy source and couples the electrons through cytochrome-dependent, energy-conserving reactions. Al-

though *Desulfovibrio* sp. can use H_2 as an energy source, it is not autotrophic, as it lacks the enzymes unique to autotrophy, namely phosphoribulokinase and ribulose diphosphate carboxylase. The mixotrophic mode of metabolism we describe for *A. vinelandii* is probably closest to the aerobic chemolithotrophic heterotroph metabolism of *Thiobacillus perometabolis* (14). This bacterium grows solely on organic compounds, but it can also oxidize sulfur compounds for energy generation. Also, some iron-oxidizing bacteria have been described that couple the oxidation of iron to organic substrate utilization (5, 6). Like *A. vinelandii*, these bacteria are heterotrophs; they do not fix CO_2 via the Calvin cycle.

Even though there are only these few reports of mixotrophy among heterotrophs, it has been considered that mixotrophy, even among nonautotrophic bacteria, may actually be widespread in natural environments (4, 13, 25). Since *A. vinelandii* is a common soil bacterium and H_2 is available in soil, mixotrophic growth of this heterotrophic bacterium may occur in nature. The metabolic flexibility of *A. vinelandii* may be of survival significance to the organism during competition in natural environments.

The basis for the requirement for H_2 to obtain growth on mannose is not known. However, since cells were able to grow on mannose plus NH_4^+ (non-N_2-fixing conditions), H_2 is required only when cells are fixing N_2. We suggest that the added energy demand for N_2 fixation requires the additional energy source, namely H_2. It may be important that under N_2-fixing conditions, the activity of hydrogenase markedly increases (compared with cells grown with NH_4^+), facilitating input of energy from H_2. Another possible role for H_2 is suggested by sugar transport studies. These studies showed that added H_2 can help support mannose uptake by *A. vinelandii*. Therefore, the role of H_2 probably includes not only production of ATP for N_2 fixation but also energy for mannose uptake.

A. vinelandii can grow in a variety of organic carbon sources and fix N_2 without the need for added H_2. However, H_2-dependent mixotrophic growth in N-free media by this organism was dependent on the use of mannose. Mannose must be a poor energy source for *A. vinelandii*; no growth was observed in mannose alone (without H_2). We have found a specific condition under which hydrogenase, with its associated electron transport pathway (26), can supply enough energy to permit N_2 fixation and growth in the presence of a poor carbon substrate. It is very important to rule out the possibility that *A. vinelandii* is an autotroph. Our attempts as well as attempts by others (16) to grow *A. vinelandii* autotrophically with H_2 have not been successful. Also, we have been unable to detect appreciable CO_2 fixation or the key CO_2-fixing enzyme, ribulose bisphosphate carboxylase, in cultures grown in H_2.

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