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Growth of *Azotobacter vinelandii* on Soil Nutrients

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Azotobacter vinelandii **cells grew well in a medium made from soil and distilled water which contained little or no carbohydrate. They utilized *p*-hydroxybenzoic acid and other phenolic acids, soil nitrogen, and water-soluble mineral substances. Seventeen soils which supported excellent growth of *A. vinelandii* contained 11 to 18 different phenolic acids each, including *p*-hydroxybenzoic, *m*-hydroxybenzoic, vanillic, *p*-coumeric, syringic, *cis*- and *trans*-ferrulic, and other unidentified aromatic acids. Three white, chalky "caliche" soils which were taken from areas where no plants grew failed to support the growth of *A. vinelandii*, and these contained no, two, and three phenolic acids, respectively. *A. vinelandii* did not fix nitrogen when growing in dialysates of soils which contained numerous phenolic acids. Growth was ample and rapid in most of the soils tested, but cell morphology was different from that usually seen in chemically defined, nitrogen-free media which contain glucose.**

The ability of azotobacters to fix nitrogen was definitively established by Beijerinck in 1901 and has served as the distinguishing characteristic of these bacteria since that time. However, it has been shown that they fix nitrogen gas only in the absence of even minute quantities of nitrogenous compounds, such as urea, NH_4^+, and NO_3^-, and will utilize these preferentially when they are available (6, 9, 15). There are sufficient data to assume that this is true whether the organism uses the molybdenum-dependent or an alternative system (2) for nitrogen fixation. In the absence of nitrogenous compounds, when carbohydrates are available, several enzymes must be synthesized before fixation of N_2 can begin (7, 12, 13), and these must then be protected from oxygen since nitrogenase reductase is oxygen labile (3, 17, 18). It is also likely that other metabolic pathways must be altered to convert N_2 to the various nitrogen compounds essential for growth (8).

On the basis of this knowledge, nitrogen fixation must be viewed as a process used only under very unusual conditions since there are probably few places in nature which are free of nitrogenous compounds, especially NO_3^-. As a result of numerous laboratory studies performed over a period of some 80 years, we have come to think that the azotobacters contribute to soil fertility by fixing nitrogen in the soil and that they acquire the energy to do so by oxidizing carbohydrate substrates (8, 11). Although no definitive, unambiguous information regarding nitrogen fixation by azotobacters in nature is available in the literature (8), the assumptions made by Beijerinck in 1901 have gone unchallenged and are still part of every microbiology textbook.

It is well established in the literature (16) that azotobacters have the ability to oxidize many aromatic compounds not susceptible to attack by many other bacteria. The degradation residue of vegetation (humus) is rich in such substances, and these accumulate in the soil as a result of their resistance to microbial degradation. Humus is slowly but constantly degraded, releasing a variety of monomeric components including some of the phenolic acids identified in these studies (see Table 1). These energy-rich substances are available only to a few members of the autochthonous flora of the soil and the azotobacters are prominent among these. The azotobacters probably do not have to compete for carbohydrates in nature if an ample supply of utilizable substrates such as the phenolic acids is available. In like manner, if urea, NH_4^+, and NO_3^- are available in the soil, they will probably be utilized in preference to N_2.

In a previous report (5), we showed that *Azotobacter vinelandii* ATCC 12837 grew well in a dialyzed soil solution. Since this soil dialysate supported fairly large populations of azotobacter, and since it contained only soil and distilled water, it was evident that all of the nutrients required for the growth of *A. vinelandii* ATCC 12837 were found in soil.

Knowing which substances in the dialyzed soil medium supported azotobacter growth would, by extension, tell something of the substrates which *A. vinelandii* may utilize in its natural habitat, the soil, and whether or not it will fix nitrogen in the soil medium. A thorough search of the readily available literature showed that such studies have not been previously reported.

MATERIALS AND METHODS

Organism. *A. vinelandii* ATCC 12837 was used in all experiments. It was maintained on slants of a modified Burk agar (14) and periodically checked for purity and strain confirmation. All experiments were started with inocula from mid-logarithmic-phase cultures grown in Burk liquid medium at 26 to 28°C on a reciprocal shaker. Populations were estimated on plates of dialyzed soil agar by the method described by Milles and Misra (10).

Media. Dialyzed soil medium was prepared by pouring 10 g of finely powdered garden soil into washed dialysis tubing, which was then tied at both ends and placed in 50 ml of distilled water contained in 500-ml Erlenmeyer flasks. After 1 to 2 h, these were sterilized by autoclaving for 15 min at 121°C and then allowed to stand at room temperature for 24 h. The soil and dialysis tubes were then discarded, and bacterial inoculum was added to the clear dialysate. Dialyzed soil agar was prepared by adding 15 g of purified agar (Difco Laboratories, Detroit, Mich.) per 1,000 ml of soil dialysate and autoclaving again. The effect of autoclaving on this dialysate was estimated by comparing the growth of *A. vinelandii* to that obtained when the soil was dialyzed at 4°C

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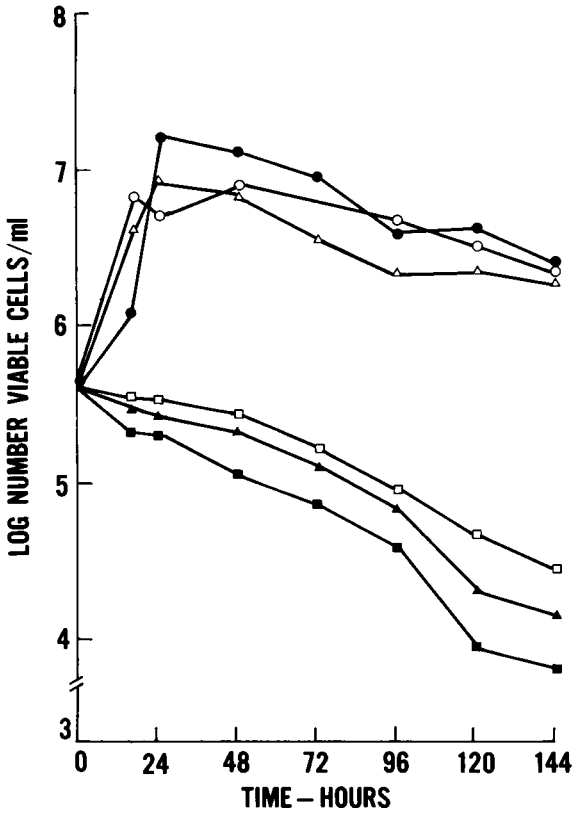

FIG. 1. Effect of various fractions of dialyzed soil medium on growth of *A. vinelandii*. The numbers of viable cells were determined on dialyzed soil agar. The following symbols indicate growth in different fractions: dialyzed soil medium (control), ○; aqueous fraction remaining after the ether- and pyridine-soluble substances had been removed from dialyzed soil medium, ●; ether-soluble substances fraction 1, □; pyridine-soluble substances, fraction 2, ■; phenolic acids, fraction 3, △; soil dialysate after removal of ether- and pyridine-soluble substances and phenolic acids, fraction 4, ▲. Fractions 1, 2, and 3 were dissolved in reconstituted ashed residue. All cultures were incubated at 26 to 28°C on a shaker.

and filter sterilized. The effect of dialysis was estimated by autoclaving the soil-water suspension, allowing the soil to settle, and decanting before centrifuging to obtain a particle-free supernatant. This was then autoclaved. No perceivable differences were noted in comparisons of the three methods. The other medium used was a modification of Burk nitrogen-free medium (14).

Ashing. The ash residue of dialyzed soil medium was prepared by evaporating a measured volume of medium to dryness on the steam table and then ashing at 1,000°C for 2 h in a muffle furnace. The ash was redissolved in a volume of distilled water equal to that of the original medium, and, if necessary, the pH was adjusted to its original value.

Fractionation. A 50-ml portion of dialyzed soil medium at pH 7 was mixed with 20 ml of reagent-grade diethyl ether, and the mixture was shaken intermittently for a period of 10 min. The ether phase was allowed to separate and was transferred to a 100-ml beaker, being careful to leave some ether behind rather than transferring ether-water emulsion at the interphase. This step was repeated three times and the ether extracts were pooled. This was then evaporated on a hot plate at 60°C under a stream of nitrogen. A 10-ml amount of water was added to the dry residue and this was evaporated as before. This step was repeated a second time to remove all traces of ether. The dried extract was labeled fraction 1 and stored at −20°C until needed. The aqueous phase was transferred to a rotary "flash evaporator" flask and evaporated to dryness at 60°C under vacuum (water aspirator). The dry medium residue was extracted with 10 ml of reagent-grade pyridine in the rotating flash evaporator flask at 60°C without vacuum. The pyridine was transferred to a second evaporator flask and the previous step was repeated two more times, pooling the three pyridine extracts. These were then evaporated to dryness at 60°C under vacuum, and residual pyridine was removed by adding 10 ml of water and evaporating three times. The dried pyridine extract was labeled fraction 2 and stored at −20°C until needed. The dry residue in the first evaporator flask was dissolved in 10 ml of water and this was evaporated to remove all traces of pyridine. It was then dissolved in 5.0 ml of water, and the pH was adjusted to the original value when necessary. This was treated as described below to extract the phenolic acids except that the aqueous phase was not saturated with NaCl. The dried ethyl acetate phase containing phenolic acids was labeled fraction 3 and stored at −20°C. The aqueous phase was evaporated to dryness under vacuum in the flash evaporator to remove all traces of ethyl acetate and redissolved in 50 ml of water or 50 ml of reconstituted ashed medium. This was labeled fraction 4.

Each fraction contained the selected organic material from 50 ml of soil dialysate and was dissolved in 50 of reconstituted ashed soil dialysate, the latter to serve as the source of minerals.

Extraction of phenolic acids. A 50-ml portion of dialyzed soil medium or fraction 3 (see above) was reduced to 5 ml at 50°C in a flash evaporator. This was then transferred to a glass-stoppered test tube, chilled in an ice bath, and acidified to pH 1 to 2 with ice-cold concentrated HCl. Sodium chloride was added to saturation and the medium was extracted three times with equal volumes of ethyl acetate. The combined extracts were placed in an ice bath and extracted three times with ⅓ volume of cold 10% (wt/vol) aqueous $NaHCO_3$. The $NaHCO_3$ washings were combined, chilled in an ice bath, and acidified to pH 1 to 2 with ice-cold concentrated HCl. This mixture was then brought to room temperature and saturated with NaCl before extracting three times with ⅓ volume of ethyl acetate. The ethyl acetate extracts were pooled and stored at −20°C until needed. These were evaporated to dryness with a stream of N_2, redissolved in 1 ml of ethyl acetate, reevaporated to dryness, and redissolved in 200 μl of ethyl acetate. Paper or gas chromatography was performed with 25, 50, or 100 μl as required.

Chromatography. The major soil phenolic acids were analyzed on two-dimensional chromatograms, using Whatman no. 1 filter paper. All chromatograms were examined under UV radiation before and after spraying with diazotized *p*-nitroaniline by the method of Armstrong et al. (1). Phenolic acids were identified by comparing (i) R_f values in three different solvent systems, (ii) color reaction with diazotized *p*-nitroaniline, (iii) UV fluorescence, and (iv) cochromatography with authentic substances.

The amount of *p*-hydroxybenzoic acid in the soil medium was estimated by comparison of spot size and color intensity with graded quantities of authentic *p*-hydroxybenzoic acid chromatographed with the organic phase of a mixture of benzene-methanol-acetic acid (45:8:4) and sprayed with diazotized *p*-nitroaniline.

TABLE 1. Phenolic acids found in 20 different soils obtained from various sites in central Texas, shown in order of elution from the gas chromatograph

Soil no.[a]	No. of acids	Phenolic acids[b]																	
		A	*p*-OH-benzoic	B	C	D	E	F	Vanillic	*cis-p*-Coumeric	G	Protocatechuic	H	Syringic	*cis*-Ferulic	*trans-p*-Coumeric	I	J	K	*trans*-Ferulic
1	18	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
2	12	−	+	−	+	+	+	−	+	+	+	+	−	+	−	+	−	+	−	+
3	12	+	+	−	+	+	−	−	+	+	+	+	+	+	−	+	−	−	−	+
4	16	+	+	−	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+
5	14	+	+	+	+	+	−	−	+	+	+	+	−	+	+	+	−	+	−	+
6	0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7	18	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
8	16	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
9	15	+	+	−	+	+	+	−	+	+	+	+	+	+	−	+	+	−	+	+
10	18	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
11	14	+	+	−	+	−	+	−	+	+	−	+	+	+	+	+	−	+	+	+
12	11	−	+	+	+	−	−	−	+	+	−	+	−	+	−	+	−	+	+	+
13	15	+	+	+	+	−	+	−	+	+	+	+	+	+	−	+	−	+	+	+
14	15	+	+	−	+	+	+	−	+	+	+	+	+	+	−	+	+	−	+	+
15	15	+	+	−	+	+	−	−	+	+	+	+	+	+	+	+	−	+	+	+
16	11	−	+	−	+	+	−	−	+	+	−	+	−	+	+	+	−	+	−	+
17	3	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−
18	2	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
19	12	+	+	+	+	+	−	−	+	+	−	+	−	+	+	+	−	+	−	−
20	12	+	+	−	+	+	−	−	+	+	−	+	+	+	−	+	−	−	+	+

[a] The soils were of various kinds ranging from black, rich soils such as no. 1, 7, 10, 14, and 15 to light, sandy loams such as no. 2, 3, 4, 5, 11, 12, and 13, to white, chalky caliche such as no. 6, 17, and 18. The phenolic acids shown were contained in 1 g of soil.

[b] Phenolic acids A through K were not identified. +, Presence of each phenolic acid; −, absence of phenolic acid or its presence in undetectable quantities.

The variety of phenolic acids in the soil was also determined by gas chromatography using the same extract as above. This was resolved with a 4-m glass column of 2-mm inside diameter packed with 3% OV17 in Chromosorb Q (Alltech Associates, Inc., Deerfield, Ill.) in a Hewlett-Packard chromatograph (model 5710A; Hewlett-Packard Corp., Avondale, Pa.) with flame ionization detector at 250°C. Nitrogen was used as the carrier gas at a flow rate of 35 ml/min. The injection port temperature was 250°C and the temperature program was started at 80°C for 8 min and then increased at a rate of 8°C/min up to 190°C.

Utilization of *p*-hydroxybenzoic acid. Utilization of *p*-hydroxybenzoic acid was shown by two different experiments. After 5 days of incubation, when growth ceased and *p*-hydroxybenzoic acid was not detectable in the culture, cells were removed by centrifugation and the supernatant was restored to its original volume and pH with distilled water and dilute NaOH or HCl, respectively. This was then sterilized by filtration (0.2 μm), and a sterile solution containing various quantities of authentic *p*-hydroxybenzoic acid was added. These supernatants were inoculated with *A. vinelandii* and incubated, and growth was measured (see Fig. 2). A second method was to add various quantities of *p*-hydroxybenzoic acid or the phenolic acid fraction of fresh soil dialysate to the ashed medium and reconstitute the original volume and pH before inoculation with *A. vinelandii*. These results were confirmed by showing that growth continued only when *p*-hydroxybenzoic acid was detectable in cultures and ceased when the quantity became undetectable by our methods.

Nitrogen fixation. Nitrogen fixation was measured by adding freshly made acetylene to 10 ml of culture in sealed bottles and measuring its conversion to ethylene after 10 min of incubation at 26 to 28°C on a reciprocal shaker. *A. vinelandii* grown in Burk medium alone and with 0.2% (wt/vol) NH_4Cl served as positive and negative controls, respectively. Acetylene and ethylene were measured by gas chromatography (Porapak T column at 80°C) in the Hewlett-Packard instrument.

RESULTS

Neither the ether-extractable (fraction 1) nor the pyridine-extractable (fraction 2) material of dialyzed soil medium (combined with ash residue) was capable of supporting growth or even survival of *A. vinelandii* (Fig. 1). The aqueous residue after removal of ether- and pyridine-soluble substances yielded the same amount of growth as did the whole soil medium (Fig. 1). When the phenolic acids (fraction 3) were extracted from the dialyzed soil medium, growth of *A. vinelandii* was not obtained in the remaining material (fraction 4). If the phenolic acids in fraction 3 were mixed with reconstituted ashed medium in proportions essentially identical to those of the original medium, ample growth was obtained, although neither alone supported growth. These data are representative of some 60 experiments and, in each case, the results were as those shown here.

Each time a sample was removed from these cultures, an aliquot was tested for nitrogen fixation. In all, more than 70 cultures were checked, but none indicated that the cells therein were capable of fixing nitrogen. All control cultures showed that the cells were capable of nitrogen fixation when grown in Burk nitrogen-free medium (see Table 2).

Analyses by paper chromatography revealed six phenolic acids which could be easily and reproducibly detected: vanillic, ferulic, *p*-hydroxycinnamic, *m*-hydroxybenzoic, dihydroferulic, and *p*-hydroxybenzoic acids. Several others appeared only as faint traces on chromatograms and could not be identified. Comparison of R_f values in three solvent systems, color of UV fluorescence, color after spraying with diazotized *p*-nitroaniline, and cochromatography with authentic compounds in three solvent systems confirmed the

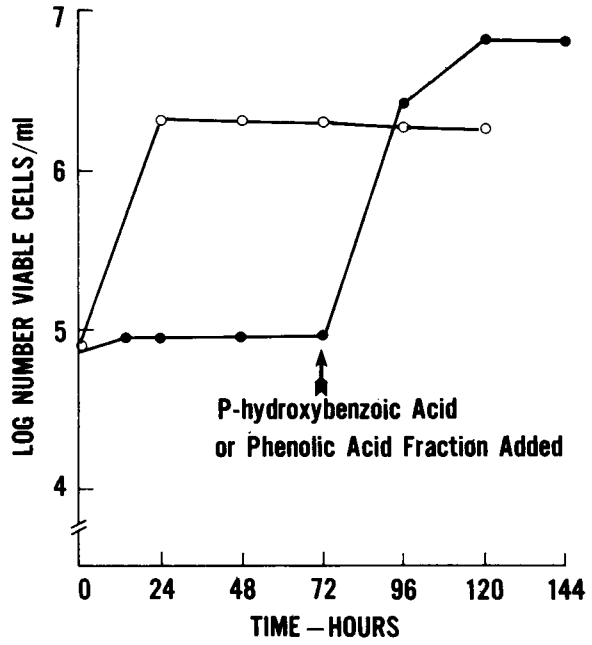

FIG. 2. Effect of *p*-hydroxybenzoic acid added to spent medium on growth of *A. vinelandii*. The following symbols indicate the growth of *A. vinelandii* in: ○, dialyzed soil medium; ●, "spent" culture filtrate with added *p*-hydroxybenzoic acid (1 mg/ml) or the phenolic acid fraction from another aliquot of soil medium added at ↑. All cultures were inoculated with washed, mid-log-phase cells grown in Burk medium.

identification of each phenolic acid observed on the paper chromatograms. Analysis by gas chromatography showed that the dialyzed soil medium contained many more phenolic acids than those detectable by paper chromatography (Table 1). In addition, no differences in the phenolic acid "profiles" were observed when the analyses were carried out before or after lipid and carbohydrate extraction. Retention time and cochromatography were used to identify the phenolic acids observed by gas chromatography.

To determine which acids were used as oxidizable substrate by *A. vinelandii*, dialyzed soil medium cultures were incubated for 5 days and the cells were then removed by centrifugation. Uninoculated media were incubated concomitantly and served as references. Assays performed by paper chromatography showed that only *p*-hydroxybenzoic acid of the six detected by this method was utilized by *A. vinelandii*, while other acids were not used even after 30 days of incubation. Assay by gas chromatography showed that *A. vinelandii* utilized *p*-hydroxybenzoic and protocatechuic acids and those acids designated C, G, and H in Table 1.

The variety of phenolic acids found in different soils is shown in Table 1. All dark, porous soils obtained from areas with plant growth (no. 1 to 5, 7 to 16, and 19 and 20) contained 11 to 18 phenolic acids each. Three white, chalky ("caliche") soils in areas with no plant growth contained no, two, and three phenolic acids. Azotobacter growth was ample in media made with the soils containing 11 to 18 phenolic acids, but no growth was observed in the white, chalky soils.

The data in Fig. 2 show that *p*-hydroxybenzoic acid could support the growth of *A. vinelandii*. In every experiment performed, growth of *A. vinelandii* was immediate and abundant, showing that this organism would grow well on the *p*-hydroxybenzoic acid normally found in the soil. Chromatographic analyses showed that growth ceased when *p*-hydroxybenzoic acid was exhausted, but this may have been due to exhaustion of other phenolic acids as well. The quantitative relationship between authentic, reagent-grade *p*-hydroxybenzoic acid and azotobacter growth is shown in Table 2. Since the soil dialysate contains many aromatic acids, all in unknown quantities, it was not possible to establish equivalence between these and *p*-hydroxybenzoic acid. An extensive series of tests also showed that cells growing in soil dialysate failed to fix atmospheric nitrogen (Table 2). It was plainly evident that they utilized inorganic nitrogenous compounds from the soil and that these were available in ample amounts. Measurement of total nitrogen (Kjeldahl) in the soil dialysate, before and after growth, showed that the amount of nitrogen removed when the cells were harvested ranged from 10 to 20% of total soil nitrogen in the various soils used. The average value for total (Kjeldahl) soil nitrogen in 20 samples of soil dialysate (made from dark, rich soils) used for these experiments was 11.6 ± 7.0 mg of N per liter.

On the other hand, analysis of 20 different soil dialysates showed that the amount of *p*-hydroxybenzoic acid ranged from 2.8 to 4.4 mg/liter of medium. The quantities of the other phenolic acids utilized were not measured but many experiments such as that described Fig. 2 showed clearly that the limiting substrate was the carbon source. When pure *p*-hydroxybenzoic acid was added to reconstituted ashed medium, all dialyzed soil medium constituents were in the same concentrations as those of the original soil dialysate, but *p*-hydroxybenzoic acid had to be added in fairly large quantities. The resultant growth did not correspond stoichiometrically to the amount of *p*-hydroxybenzoic acid contained in the soil dialysate. However, since other acids were also used, it was obvious that the differences observed were a measure of the utilization of acids other than *p*-hydroxybenzoic acid.

The cells in dialyzed soil medium were very similar in appearance, size, morphology, and growth rate to those

TABLE 2. Effect of various quantities of authentic *p*-hydroxybenzoic acid on growth and nitrogen fixation of *A. vinelandii* incubated at 26 to 28°C on a reciprocal shaker for 5 days

p-Hydroxybenzoic acid (mg/ml)[a]	Viable cells per ml	Nitrogen fixation as acetylene reduction[b]
0.4	1×10^3	–
0.8	1.1×10^3	–
1.2	1.6×10^5	–
1.6	2.0×10^7	–
2.0	1.6×10^7	–
3.0	1.0×10^7	–
4.0	6×10^4	–
6.0	1.0×10^3	–
Dialyzed soil, medium control	8.1×10^7	–
Burk medium, positive control	1.0×10^7	+
Burk plus NH_4Cl, negative control	6.4×10^6	–

[a] Authentic *p*-hydroxybenzoic acid and 50 ml of distilled water were added to the ashed residue from 50 ml of dialyzed soil medium, and the pH was adjusted to 7.0. All flasks were inoculated with growth from mid-log-phase cells (10^3 per ml) grown on Burk medium and washed three times with distilled water. Values given are representative of those obtained in separate experiments.

[b] –, No acetylene reduced; +, acetylene reduction.

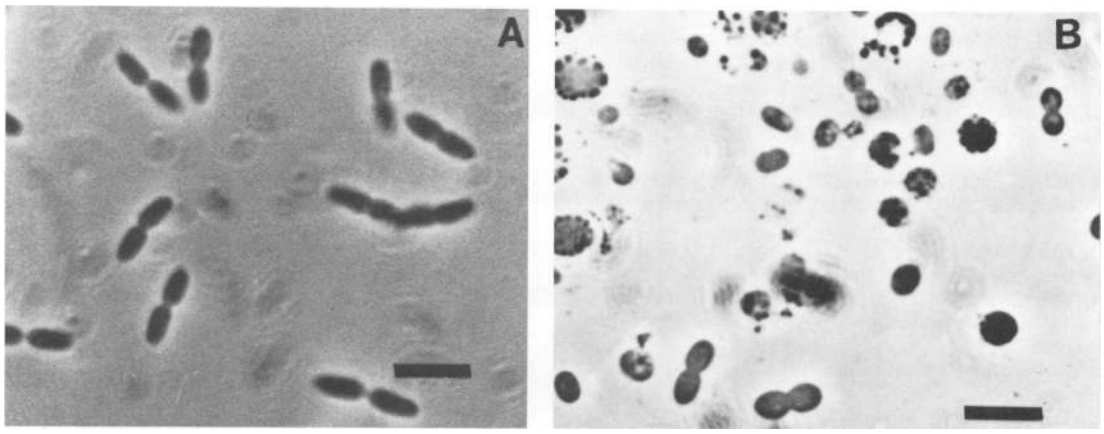

FIG. 3. Cells of *A. vinelandii* grown in Burk medium (A) and dialyzed soil medium (B). Pure cultures in the soil dialysate (B) show many forms including those with poly-β-hydroxybutyric acid inclusions and particles released from cells on lysis. All cultures were inoculated with mid-log-phase cells grown in Burk medium and incubated aerobically at 26 to 28°C on a shaker. Bar, 5 μm (for both A and B).

grown in the medium composed of ashed residue and reagent-grade *p*-hydroxybenzoic acid. In these media, *A. vinelandii* cells are seen to contain multiple intracellular particles which escape into the medium when the cells autolyze. Figure 3 shows the difference between cells grown in Burk medium and those grown either in the soil dialysate or on soil minerals plus *p*-hydroxybenzoic acid. These cultures contained large cells with peripheral spherical bodies, cells with small inclusions, free particles suspended in the medium, and cells which were quite similar to those seen in Burk medium. These forms of azotobacter were similar to those reported in the literature of the 1930s and also to those described in more recent reports (4, 5).

DISCUSSION

While it is generally impossible to perform in vitro studies that duplicate or even closely mimic the natural environment, it is possible to approach the conditions extant in nature by judicious design of laboratory experiments. To learn something of the role of *A. vinelandii* in nature, it was assumed that it would be more profitable to culture this bacterium in a medium made only from soil and distilled water rather than in the chemically defined, nitrogen-free media commonly used, because the natural habitat of azotobacter during its growth phase is much more likely to be an aqueous solution of soil chemicals than one devoid of nitrogen and containing large amounts of carbohydrate. While it is recognized that axenic cultures reflect nature only obliquely, this is a shortcoming shared with the vast majority of laboratory experiments, including those which described the growth of azotobacter in nitrogen-free media, but one (usually) overcome by judicious interpretation of results.

Using dialyzed soil cultures, we found that *A. vinelandii* cells did not require carbohydrates for growth since they grew as well on the phenolic acids normally found in all soils (Fig. 2, Table 2) as they did on glucose. In like manner, since *A. vinelandii* utilizes NO_3^- and other simple forms of nitrogen in preference to N_2 (6, 9, 15), it follows that it would not fix nitrogen as long as even traces of these forms of nitrogen were available. Our data show that this was the case in the dialyzed soil cultures. The data in Table 2 describing nitrogen fixation are part of those obtained from more than 70 determinations, and all showed that atmospheric nitrogen was not fixed by cells growing in soil dialysates. It was also found that as many as three cell crops could be harvested from the same batch of soil dialysate if *p*-hydroxybenzoic acid was added (as in Fig. 2) and that cells in the third crop still did not fix nitrogen. Two cell crops were obtained from more than 20 other cultures, and nitrogen fixation was not detected in multiple samples (two to five from each culture) from any of these. We interpreted these findings as indicative of the fact that there was ample nitrogen for growth in the soil dialysate and that nitrogen fixation was not necessary for growth in the soil medium.

The morphology of *A. vinelandii* in these soil cultures was quite different from that observed in the chemically defined, nitrogen-free media generally used in laboratory studies. While we do not claim that soil dialysate provides the same environment as the natural habitat of azotobacter does, we do assume that it is more like the soil environment than Burk medium is, and on that basis we suggest that the morphology depicted in Fig. 3 may be more like that of azotobacter in nature than that seen in the laboratory media commonly used.

In summary, the data presented show that *A. vinelandii* cells grew well on a medium made only of soil and distilled water and that they obtained carbon and energy from the oxidation of phenolic acids, undoubtedly derived from soil humus. Only *p*-hydroxybenzoic acid was studied in detail, but other acids (Table 1) were also utilized. *A. vinelandii* consumed nitrogen found in the soil and, under these conditions, failed to fix atmospheric nitrogen.

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