APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1986, p. 451–454
0099-2240/86/020451-04$02.00/0

Vol. 51, No. 2

Stimulation of *Agrobacterium tumefaciens* Growth by *Azotobacter vinelandii* Ferrisiderophores

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Received 1 July 1985/Accepted 19 November 1985

Azotobacter vinelandii **stimulated the growth of** ***Agrobacterium tumefaciens*** **H2, H23, H24, H27, and ATCC 15955 on media containing insoluble iron sources. The** ***Azotobacter vinelandii*** **siderophores appeared to promote** ***Agrobacterium tumefaciens*** **growth by solubilizing mineral iron, and the ferrisiderophores so formed then acted as iron sources for** ***Agrobacterium tumefaciens*****. Agrobactin, the** ***Agrobacterium*** **siderophore, appeared to be inefficient in solubilizing mineral iron directly.**

The development of bacterial inoculants to enhance plant growth is becoming an attractive alternative to traditional chemical treatments. These inoculants may benefit the plant directly by supplying essential metabolites or plant growth hormones and indirectly by phytopathogen suppression (18). In the latter case, pseudomonads termed plant growth-promoting rhizobacteria (PGPR) have been found to rapidly colonize the roots of potato, sugar beet, and radish plants and cause statistically significant yield increases in field tests (7, 8, 18, 19). Evidence has been put forward by Kloepper and co-workers (7, 8) that PGPR exert their plant growth-promoting effect by depriving native microflora of iron by effectively complexing environmental iron with excreted natural chelators (siderophores). The ensuing colonization of the plant root by PGPR excludes these deleterious microbes from the rhizoplane and suppresses plant disease (16). Disease-suppressive soils often contain large populations of PGPR (16). These soils can be made conducive to plant disease by treating them with iron(III) EDTA, a form of iron that is readily available to most microbes. Conversely, conducive soils can be made suppressive by the addition of ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDA), which chelates iron into a form which is unavailable to those microbes that do not form high-affinity iron chelators.

Azotobacter vinelandii is a nitrogen-fixing soil organism which promotes plant growth by phytohormone production (2, 3). The siderophores produced by *Azotobacter vinelandii*, azotochelin and azotobactin, effectively release iron from insoluble ferriminerals commonly found in the soil (14) and transport the solubilized iron into the cell in a manner typical of siderophore systems (9). The yellow-green fluorescent siderophore, azotobactin, shares some structural similarities with pseudobactin, the *Pseudomonas* spp. siderophore involved in disease suppression (20). This study was initiated to determine whether *Azotobacter vinelandii* might also demonstrate phytopathogen suppression. In vitro screening indicated, however, that *Azotobacter vinelandii* enhanced the growth of strains of *Agrobacterium tumefaciens* and *Erwinia carotovora*. This stimulation was apparently siderophore mediated. Therefore, the nature of the growth stimulation of *Agrobacterium tumefaciens* by a potential PGPR was investigated further.

Agrobacterium tumefaciens H23 grew poorly on Burk medium (15), producing either very small colonies (diameter, 1 mm) after 4 days of incubation or a light lawn on spread plates after 48 h of incubation (Fig. 1). When *Azotobacter vinelandii* UW was applied to the center of a Burk medium plate preinoculated with a lawn of *Agrobacterium tumefaciens*, a zone of growth appeared around the *Azotobacter vinelandii* spot after 48 h of incubation (Fig. 1). This zone of growth contained *Agrobacterium tumefaciens* cells, not *Azotobacter vinelandii* cells. The diameter of the zone of *Agrobacterium tumefaciens* growth was dependent on incubation temperature, suggesting an increased rate of production or diffusion of growth-promoting substances from the *Azotobacter vinelandii* spot. This stimulation was not caused by an *Azotobacter* cell lysate, sterile iron-sufficient (18 μM $FeSO_4 \cdot 7H_2O$) culture supernatant fluid, *Azotobacter* capsular material, or concentrated surface protein (1) normally released by growing *Azotobacter vinelandii* (13). A similar zone of growth was caused by sterile *Azotobacter vinelandii* iron-limited (no added iron) culture supernatant fluid. In fact, the growth stimulation observed when *Azotobacter vinelandii* cells were present on the plate only occurred after the production of the fluorescent pigments (siderophores) characteristic of iron limitation in this species (9, 14). Growth stimulation was not observed, however, on iron-limited Burk medium plates despite the growth of and siderophore production by *Azotobacter vinelandii*. A similar application of sterile iron-limited *Azotobacter vinelandii* culture supernatant fluid to iron-limited Burk medium plates also did not enhance the growth of *Agrobacterium tumefaciens*. When 1 μg of $FeSO_4 \cdot 7H_2O$ was added with the iron-limited *Azotobacter* culture supernatant fluid, the growth of *Azotobacter tumefaciens* was clearly (radius, 5 to 7 mm) stimulated. Addition of more iron (10 μg) resulted in a larger zone (radius, 10 to 12 mm) of growth stimulation. Addition of iron without the siderophore solution, however, did not enhance *Agrobacterium* growth on Burk medium plates.

Azotobacter vinelandii UW caused similar stimulation of *Agrobacterium tumefaciens* H2, H23, H24, H27 (strongly tumorigenic on sunflower), and ATCC 15955 (active crown-gall former), although strain ATCC 15955 caused some inhibition of *Azotobacter vinelandii* growth and siderophore production. Similar stimulation of the *Agrobacterium tumefaciens* strains was caused by *Azotobacter vinelandii* ATCC 12837, ATCC 13705, and ATCC 12518. All of the *Azotobacter vinelandii* strains also caused stimulation of *Erwinia carotovora* subsp. *carotovora* ATCC 495 growth on Burk medium, although the zone of *Erwinia* growth had a 2- to 4-mm radius rather than the 15- to 20-mm radius observed

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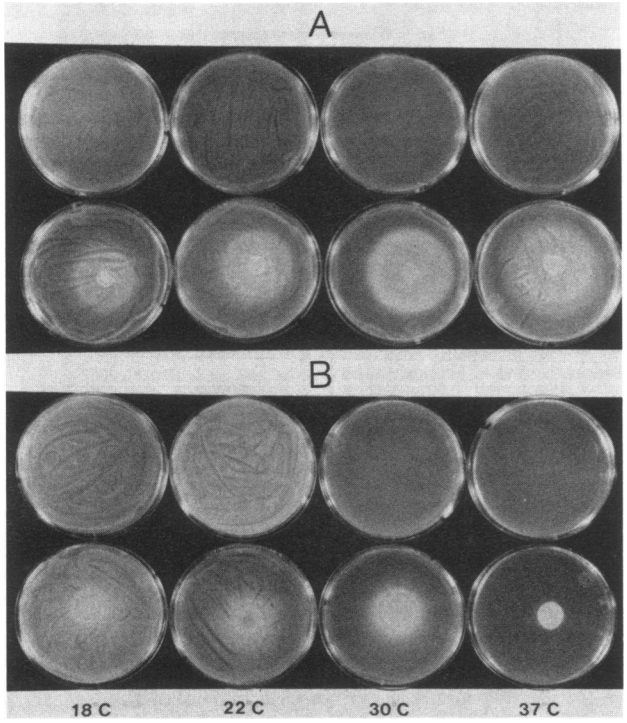

FIG. 1. Growth promotion by *Azotobacter vinelandii*. Plates of Burk medium were spread with *Agrobacterium tumefaciens* H23 (A) or *E. carotovora* subsp. *carotovora* ATCC 495 (B) alone or in combination with *Azotobacter vinelandii* UW (bottom row of each set). The plates were incubated at the temperatures indicated for 48 h.

with *Agrobacterium tumefaciens* strains. The upper limit of *Erwinia* growth and growth stimulation was 30°C (Fig. 1). Stimulation of *Agrobacterium tumefaciens* strains (radius, 5 to 12 mm at 30°C) also was caused by *Azotobacter paspali* strains PPD98, WR129, and ATCC 23833. *E. cartovora*, however, exhibited antibiosis against *Azotobacter paspali*. In all cases, the stimulation of the phytopathogen was not produced on iron-limited medium but appeared to be dependent on the *Azotobacter* species becoming iron limited on iron-containing medium.

Agrobacterium tumefaciens H23 did not appear to use the *Azotobacter* siderophores directly to promote iron uptake. Strain H23 produced the siderophore agrobactin in iron-limited culture (11, 12) and demonstrated the greatest rate of ^{55}Fe uptake in its own iron-limited culture supernatant fluid (Fig. 2). Iron-limited cells washed with 8 mM Tris hydrochloride (pH 7.8) also demonstrated good ^{55}Fe uptake when suspended in Burk medium containing 10 mM sodium citrate, which has been shown previously to promote iron transport in *Agrobacterium tumefaciens* (10). When washed cells were suspended in filter-sterilized *Azotobacter vinelandii* UW iron-limited culture supernatant fluid, there was relatively little ^{55}Fe uptake (Fig. 2). The only sample showing lower ^{55}Fe uptake was the control culture incubated on ice.

Azotobacter vinelandii siderophores, however, did reverse EDDA inhibition of strain H23 (Table 1). Therefore, the *Azotobacter* siderophores appeared to make iron available to *Agrobacterium tumefaciens* either from iron(III) EDDA or from the insoluble iron salts formed in Burk medium (9). The *Agrobacterium* siderophore, on the other

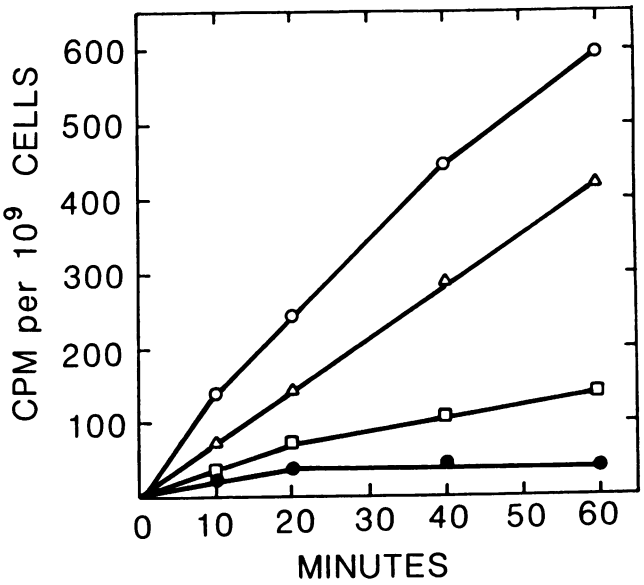

FIG. 2. ^{55}Fe uptake by *Agrobacterium tumefaciens*. Fe-limited *Agrobacterium tumefaciens* H23 cells were assayed for ^{55}Fe uptake with the corresponding iron-limited culture supernatant fluid (○), Burk medium containing 10 mM sodium citrate (pH 7.0) (△), *Azotobacter vinelandii* iron-limited culture supernatant fluid (□) incubated at 25°C, or *Agrobacterium tumefaciens* iron-limited culture supernatant fluid incubated on ice (●). The assay design and conditions were as previously described (9).

hand, was only able to release iron from iron(III) EDDA. A simple chelator like citrate also caused growth stimulation on Burk medium, presumably by solubilizing iron salts, but was unable to reverse EDDA inhibition.

The poor use of insoluble iron salts by *Agrobacterium tumefaciens* H23 was confirmed by adding ferriminerals as iron sources to iron-limited Burk medium and monitoring growth yield by protein assay (14). *Agrobacterium tumefaciens* was unable to use chemical FeS or any of the following minerals: marcasite (FeS_2), vivianite [$Fe_3(PO_4)_2 \cdot 8H_2O$], olivine [$(Mg,Fe)_2SiO_4$], hematite (Fe_3O_4), siderite ($FeCO_3$), goethite (FeOOH), pyrite (FeS_2), or illite [$2K_2O \cdot 3(Mg,Fe)O \cdot 8(Al,Fe)_2O_3 \cdot 24SiO_2 \cdot 12H_2O$]. In all cases, the protein content of the test culture was 75 to 100% of that of the iron-limited control culture. Viable numbers were at most twice as much as those of the iron-limited control culture after 20 h of incubation with minerals present

TABLE 1. Reversal of EDDA inhibition of *Agrobacterium tumefaciens* H23 by culture supernatant fluids containing siderophores

Test solution	Zone of growth stimulation (mm)	
	Burk medium	Burk medium plus EDDA[a]
Agrobacterium tumefaciens culture supernatant[b]	0	33
Azotobacter vinelandii culture supernatant[b]	30	45
Sodium citrate (10 mM; pH 7.0)	40	0

[a] Deferrated EDDA was added to make a final concentration of 1 mg/ml.
[b] Iron-limited culture supernatant fluid (100 μl) after 1 day of growth.

TABLE 2. Growth of *Agrobacterium tumefaciens* alone or in coculture with *Azotobacter vinelandii*

Iron source[a]	Relative viable no. per ml[b]			Coculture supernatant fluid relative absorbance[d]	
	Agrobacterium tumefaciens		*Azotobacter vinelandii* coculture		
	Alone	Coculture		A_{310}[d]	A_{380}[e]
None	1.0	1.6	0.12	1.0	1.5
Marcasite	1.0	8.7	0.22	2.3	NP[f]
Olivine	1.5	9.1	0.03	1.3	2.7
Pyrite	1.7	4.7	0.36	0.5	1.5
Illite	2.0	7.4	0.14	0.8	1.5

[a] Iron sources (50 mg) were added to 100 ml of iron-limited Burk medium and incubated as previously described (14).

[b] Viable number per milliliter relative to independent *Agrobacterium tumefaciens* H23 or *Azotobacter vinelandii* UW culture without added iron.

[c] Absorbance of coculture supernatant fluid relative to absorbance of corresponding independent *Azotobacter vinelandii* culture supernatant fluid. Both culture fluids were acidified to pH 1.8 before absorbance determination.

[d] A_{310} due to the catechols azotochelin [*N,N'*-bis(2,3-dihydroxybenzoyl)-L-lyzine] and 2,3-dihydroxybenzoic acid formed by *Azotobacter vinelandii* (14). Agrobactin did not contribute significantly to the A_{310} value.

[e] A_{380} due to azotobactin (yellow-green fluorescent peptide siderophore) formed by *Azotobacter vinelandii*. (14).

[f] NP, None produced (14).

(Table 2). When *Agrobacterium tumefaciens* cells were cocultured with *Azotobacter vinelandii*, which was able to solubilize iron from these sources (14), the *Agrobacterium* viable count increased considerably (representative data are shown in Table 2). The *Azotobacter* viability in the coculture was much less than that found when the cells were cultured independently in iron-limited culture. This inhibition was probably the result of unsuccessful competition with *Agrobacterium tumefaciens* for iron solubilized by the *Azotobacter* siderophores. This iron limitation of the *Azotobacter* population was demonstrated by the characteristically enhanced production of *Azotobacter* siderophores (14) in the coculture (Table 2).

In summary, these results show that the growth stimulation seen on Burk medium plates was the result of iron solubilization by *Azotobacter* siderophores followed by the scavenging of iron from these ferrisiderophores by *Agrobacterium tumefaciens*. The *Agrobacterium tumefaciens* siderophore alone did not solubilize iron from insoluble iron sources or from the insoluble iron complexes formed in Burk medium. It was probably only the high affinity of this siderophore for iron (4) that allowed the minimal growth of *Agrobacterium tumefaciens* on these media.

Catechols have been shown to be very efficient in the solubilization of ferriminerals (14), and the failure of agrobactin, a tricatechol (11, 12), to also do so is somewhat surprising. The solubilization of ferriminerals by simple catechols may be a chemical reaction that is more affected by the concentration of the reactants than by the chelation ability of the siderophores. *Azotobacter vinelandii* produces 250 nmol of total catechol per ml in iron-limited medium after 24 h of incubation (14), whereas *Agrobacterium tumefaciens* produces only 24 nmol of agrobactin catechol per ml in the same time (calculated from reference 12), which must limit the amount of ferrimineral solubilized by *Agrobacterium tumefaciens*. Agrobactin is also cell bound, which may further restrict access to insoluble iron sources (12).

Iron molecules bound to ferrisiderophores or in organic complexes are the most likely sources of iron for agrobactin and perhaps for other high-affinity chelators that are produced in small amounts. There may be a functional hierarchy of siderophores in soil with abundant simple catechols, reductants including soil humus and organic acids (6, 17) being involved primarily in iron mobilization from soil particles and the more efficient chelators involved in scavenging this organically complexed iron. The producers of even small amounts of high-affinity siderophores, therefore, would likely be highly successful in competition for iron with other bacteria or plants but might be relatively unsuccessful in environments in which iron is not already in an organic form.

Currently, there is considerable interest in developing *Azotobacter vinelandii* as an inoculant to enhance plant growth (2, 3), and attempts to genetically modify *Azotobacter vinelandii* have begun (4, 5). It remains to be shown that this scenario derived from in vitro data would be operational in the soil, but the potential creation of a soil conducive to plant disease by inoculation should be investigated further.

We thank B. Terzaghi and J. Dobereiner for strains of *Azotobacter paspali* used in this study. Preliminary investigations were made with help of Marianne Huyer, Robert Carmichael assisted with photography, and Wade Bingle provided purified *Azotobacter vinelandii* surface protein.

This study was funded through grants from the Natural Sciences and Engineering Research Council of Canada and the Agricultural Research Council of Alberta Farming for the Future program.

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