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Citrate as a Siderophore in *Bradyrhizobium japonicum*†

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Under iron-limiting conditions, many bacteria secrete ferric iron-specific ligands, generically termed siderophores, to aid in the sequestering and transport of iron. One strain of the nitrogen-fixing soybean symbiont *Bradyrhizobium japonicum*, 61A152, was shown to produce a siderophore when 20 *B. japonicum* strains were screened with all six chemical assays commonly used to detect such production. Production by strain 61A152 was detected via the chrome azurol S assay, a general test for siderophores which is independent of siderophore structure. The iron-chelating compound was neither a catechol nor a hydroxamate and was ninhydrin negative. It was determined to be citric acid via a combination of thin-layer chromatography and high-voltage paper electrophoresis; this identification was verified by a specific enzymatic assay for citric acid. The inverse correlation which was observed between citric acid release and the iron content of the medium suggested that ferric citrate could serve as an iron source. This was confirmed via growth and transport assays. Exogenously added ferric citrate could be used to overcome iron starvation, and iron-deficient cells actively transported radiolabeled ferric citrate. These results, taken together, indicate a role for ferric citrate in the iron nutrition of this strain, which has been shown to be an efficient nitrogen-fixing strain on a variety of soybean cultivars.

Iron-containing proteins figure prominently in the nitrogen-fixing symbioses between bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium* and their respective plant hosts. Nitrogenase can constitute 10 to 12% of the total protein in a bacterial cell and leghemoglobin may represent as much as 25 to 30% of the total soluble protein in infected plant cells (47). For the synthesis of these and other iron-containing compounds, such as ferredoxin, hydrogenase, and cytochromes, plants and bacteria must have an adequate supply of iron.

This is well illustrated by the report that iron deficiency specifically limits nodule development in peanut plants inoculated with *Bradyrhizobium* sp. (29). Iron-stressed plants had fewer bacteroids present in the nodules, showed decreased amounts of leghemoglobin, and had lower specific nitrogenase activity on a fresh-weight basis (29). Additional evidence for the importance of iron in nitrogen-fixing symbioses is provided by a mutant of *Rhizobium leguminosarum* which forms white, ineffective nodules on peas and has an apparent defect in iron acquisition (24). These in planta results complement previous work on the effects of iron deficiency on free-living rhizobia (36). Cultures of iron-deficient cells had decreased cell yield, decreased cytochrome content, and diminished activity for the first two enzymes in the heme biosynthetic pathway (36). In addition to these reports, there is new evidence that iron plays a regulatory role in the nitrogen fixation process (11, 14), as well as playing an obviously central role as a component of compounds such as heme and nitrogenase. Thus, knowledge of how rhizobia acquire iron should help further our understanding of these economically important symbioses.

For rhizobia, acquisition of iron may be problematic both ex planta and in planta. The problem in the soil is not one of abundance, as iron ranks fourth among all elements on the surface of the earth, but rather one of availability in aerobic environments at biological pH. Under such conditions, iron tends to precipitate, forming oxyhydroxide polymers of the general composition FeOOH (28). In plants, iron availability may be restricted to prevent pathogens from establishing themselves (9, 28). To compete successfully in iron-limited environments, such as the soil or a plant or animal host, organisms have evolved specific, high-affinity mechanisms to acquire iron. In bacteria, these systems are composed of ferric iron-specific ligands, generically termed siderophores, and their cognate membrane receptors (27). Only limited information is available regarding siderophore systems in rhizobia (see M. L. Guerinot [*in* Y. Chen and Y. Hader, ed., *Iron Nutrition and Interaction in Plants*, in press] for a review). The nature of many of these reports makes it difficult to generalize about rhizobial siderophore production, but it appears that such production may be strain-specific and represents a wide range of siderophore structures (1, 5, 21, 25, 31, 33, 35, 39–43; Guerinot, in press).

In this report, we extend the list of siderophore-producing rhizobia to include the soybean symbiont *Bradyrhizobium japonicum*. Our observations also add bradyhizobia to the list of organisms which release acid under iron stress and provide the context for further molecular genetic studies of iron acquisition by this species.

MATERIALS AND METHODS

Materials. All of the chemicals used were reagent grade. Chrome azurol S, citric acid, malic acid, mannitol, and rhodoturulic acid were obtained from Sigma Chemical Co. (St. Louis, Mo.). The kit used to determine citric acid levels was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Radiolabeled ferric chloride ($^{55}FeCl_3$; specific activity, 2.1 mCi/mg) in 0.1 N HCl was purchased from Amersham Corp. (Arlington Heights, Ill.).

Bacterial strains. The strains used in this study are listed in Table 1.

Media and growth conditions. Cells were cultured in either yeast extract-mannitol medium (YEM) (48) or minimal me-

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TABLE 1. Strains of *B. japonicum* used in this study

Strain	Other designation	Source
61A50	USDA 50	Nitragin Co.[a]
61A63	USDA 29	Nitragin Co.
61A66	USDA 46	Nitragin Co.
61A69	USDA 66a	Nitragin Co.
61A76	USDA 324	Nitragin Co.
61A82	USDA 117	Nitragin Co.
61A101c		Nitragin Co.
61A118b	USDA 138	Nitragin Co.
61A152	532C	Nitragin Co.
61A182	8-0	Nitragin Co.
61A209		Nitragin Co.
61A212		Nitragin Co.
61A224		Nitragin Co.
61A227	587	Nitragin Co.
61A230	C33	Nitragin Co.
61A238		Nitragin Co.
61A259		Nitragin Co.
61A260		Nitragin Co.
61A262		Nitragin Co.
USDA 110	61A89	USDA[b]

[a] Milwaukee, Wis.
[b] U.S. Department of Agriculture, Beltsville, Md.

dium. The pH of both YEM and minimal medium was adjusted to 6.8 before autoclaving. The minimal medium had the following composition (grams per liter): $(NH_4)_2SO_4$, 0.33; MOPS (morpholinepropanesulfonic acid), 10.47; KH_2PO_4, 0.3; Na_2HPO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.12; $CaCl_2$, 0.05. Trace elements were prepared as a filter-sterilized stock and added to the minimal medium as follows (milligrams per milliliter): H_3BO_3, 10; $ZnSO_4 \cdot 7H_2O$, 1.0; $CuSO_4 \cdot 5H_2O$, 0.5; $MnCl_2 \cdot 4H_2O$, 0.5; $NaMoO_4 \cdot 4H_2O$, 0.1; $NiCl_2$, 1.19. After autoclaving, 10 ml of filter-sterilized 20% (wt/vol) mannitol was added per liter. When appropriate, the medium was supplemented with iron from a filter-sterilized stock of 70 mM ferric chloride in 0.1 N HCl. Sodium citrate or ferric citrate was supplied from filter-sterilized stocks when indicated. For uptake medium, minimal medium with no carbon source was prepared as described above, except that the pH was adjusted to 4.5. After autoclaving, the medium was kept for a week at room temperature to allow any iron salts to precipitate. The pH was then adjusted to 6.8 and the medium was filtered through an HA filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.). Adjustments to pH were made with a solution of 5 M NaOH–5 M KOH (3:1, vol/vol) as described by Langman et al. (18).

To determine the iron requirement of strain 61A152, cells were cultured initially in YEM. The cells were then diluted in minimal medium to which no iron was added. After one cycle of growth in minimal medium, the cells were once again diluted in minimal medium and various amounts of iron were added to each flask. Precautions were taken to minimize the iron contents of both the culture vessels and the medium. Glassware was washed with 6 N HCl and then rinsed with double-distilled water.

Unless otherwise noted, cells were incubated at 30°C with shaking.

Iron level determination. Iron levels were measured by flameless atomic absorption spectroscopy performed with a 503 spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) equipped with an HGA 2100 graphite furnace or by a colorimetric method using the chromogen ferrozine (45). The ferrozine method was used for media with high levels of organic material which interfered with the flameless atomic absorption spectroscopy. The sensitivity limit of the ferrozine method is approximately 0.3 μM. Less than 5 nM iron was found in the water used to prepare the medium. Minimal medium routinely contained less than 0.5 μM iron, and YEM medium contained approximately 5 μM iron.

Siderophore assays. All six chemical assays commonly in use to detect siderophore production were used on supernatants from cultures of *B. japonicum*. The chrome azurol S (CAS) assay, a general assay for siderophores, was performed as described by Schwyn and Neilands (38). Hydroxamate-type siderophores were measured by the Csaky method (8) and the method of Atkin et al. (4). The methods of Arnow (3), Rioux et al. (34), and Holzberg and Artis (15) were used to detect catechol-type siderophores. Siderophore assays were performed on supernatants directly and on supernatants which had been concentrated 10-fold by freeze-drying.

High-voltage paper electrophoresis. The ionophoretic mobilities of compounds were determined by using a flat-bed device in either pyridine-acetic acid-water (14:10:930), pH 5.2 or 4.0, or water-acetic acid-formic acid (451:12.5:36), pH 1.5. Samples were spotted on Whatman no. 1 paper, subjected to electrophoresis (ca. 40 V/cm, 60 min), and detected by one of the following indicator sprays: (i) 0.25% (wt/vol) ninhydrin in acetone, (ii) 3% ferric chloride in 95% ethanol, or (iii) CAS solution (38). Rhodotorulic acid, citric acid, and malic acid were used as reference standards.

TLC. Eight different solvent systems were used for thin-layer chromatography (TLC) on silica gel plates: (i) acetic acid-ethyl acetate-dibutyl ether, 1:10:10; (ii) acetic acid-ethyl acetate-dibutyl ether, 1:5:15; (iii) acetic acid-ethyl acetate-dibutyl ether, 1:15:5; (iv) ethanol-dibutyl ether, 1:1; (v) methanol-water, 9:1; (vi) 95% ethanol; (vii) 50% ethanol, and (viii) 47.5% ethanol. Spots were visualized by spraying the chromatograms with CAS solution (38), 0.25% ninhydrin in acetone, or 3% ferric chloride in 95% ethanol. Chromatograms were also viewed under UV light for detection of fluorescent compounds.

Citric acid determination. Citric acid was assayed enzymatically as recommended by the manufacturer, with a kit obtained from Boehringer Mannheim. Briefly, this assay measures the amount of NADH oxidized as citrate is converted to oxaloacetate and acetate in the presence of the enzymes citrate lyase, malate dehydrogenase, and lactate dehydrogenase (22). The amount of NADH oxidized is stoichiometric with the amount of citrate present in the sample. This assay can detect free acid, as well as salts of citric acid (e.g., ferric citrate). The minimum amount of citrate detectable is approximately 24 μM. Therefore, samples containing less than 24 μM must be concentrated via lyophilization before determination.

Uptake studies with cultured cells. Bacterial cells were grown in either iron-deficient or iron-sufficient minimal medium to an optical density of 0.6 at 420 nm. The cells were harvested by centrifugation at 7,719 × *g* for 15 min at 4°C, and the cell pellet was suspended in 5 ml of uptake medium. The cells were then preincubated at 30°C in a shaking water bath for 15 min before uptake studies were initiated by addition of 5 ml of radiolabeled ferric citrate. When indicated, cells were incubated for 10 min with 2,4-dinitrophenol (final concentration, 0.4 mM) or for 20 min on ice in a shaking water bath before addition to radiolabeled ferric citrate. ^{55}Fe complexes were prepared 30 min before the start of transport experiments by addition of 0.8 μCi of $^{55}FeCl_3$ to uptake medium which contained 10 μM $FeCl_3$ and

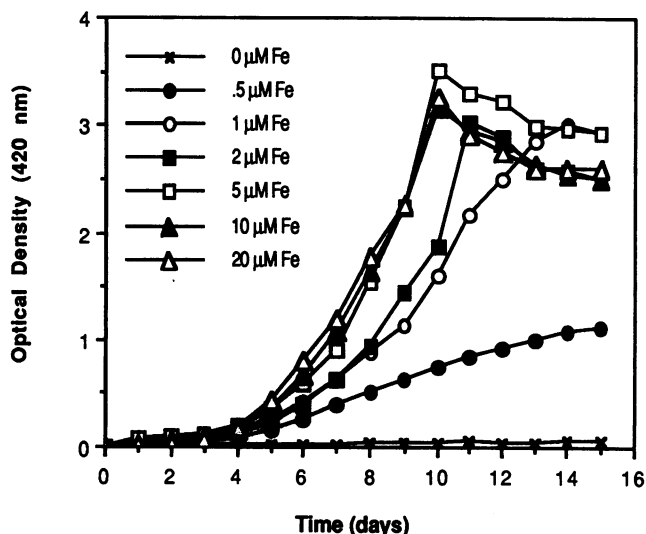

FIG. 1. Effect of iron concentration on the growth of *B. japonicum* 61A152 in minimal medium. Cells of strain 61A152 were grown without iron for one cycle in minimal medium and then inoculated into minimal medium with different initial iron concentrations. Iron was supplied as ferric chloride in 0.1 N HCl.

2 mM citrate (prepared as a 3:1 mixture of the sodium and potassium salts). Citrate was present at a 200-fold excess to ensure formation of ferric dicitrate complexes (20). Therefore, all of the iron available for uptake in these experiments should be in the form of ferric citrate. At timed intervals, 1.0-ml samples were removed in duplicate and filtered rapidly under vacuum through either a Millipore HA filter or an HVL membrane filter (0.45-μm pore size) which had been previously soaked for 30 min in uptake medium. The bacteria trapped on the filter were immediately washed three times with 3 ml of 0.5% (wt/vol) sodium thioglycolate. Thioglycolate had previously been shown to solubilize iron retained on the filter and to aid in removal of iron associated with the cell surface (6). The filters were dried and counted in Aquasol-2 (Du Pont Co., Wilmington, Del.) in an LS3800 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). All values were corrected for binding of the iron complexes to the filter.

Protein determination. Protein was determined by a modification of the Lowry method (37) with bovine serum albumin as the standard. Cells were precipitated with 10% trichloroacetic acid before protein determination.

RESULTS

Siderophore production. Siderophore production normally occurs when cells are limited for iron. Having established conditions for iron-limited growth of *B. japonicum* (Fig. 1 shows a representative set of growth curves for strain 61A152), we monitored cultures for siderophore production during growth in iron-limited minimal medium (medium containing less than 1 μM iron). The strains tested are listed in Table 1. No siderophores were detected, even when culture supernatants were lyophilized, concentrated 10-fold, and assayed (data not shown). However, when cells were cultured in YEM, we did detect an iron-chelating compound in the supernatant of stationary-phase cells of *B. japonicum* 61A152 by using the CAS assay. When siderophore production began, iron had reached undetectable levels in the culture medium, as determined by the ferrozine method.

TABLE 2. Levels of citrate and iron found in the culture supernatant of strain 61A152 during growth in minimal medium

No. of days postinoculation	Fe concn (μM)	Citrate concn (μM)
0	50	<0.2
8	1.2	81.8
11	<0.3	195

Identity of the siderophore. The CAS assay is based on the observation that when a strong chelator removes dye from a CAS dye-iron III-detergent complex, the dye turns from blue to orange (38). This assay is therefore independent of the structure of the siderophore and provided no clues as to the nature of the iron-binding compound released by strain 61A152. The identity of the compound responsible for the positive CAS signal was determined to be citric acid via a combination of TLC and high-voltage paper electrophoresis and confirmed by a specific enzymatic assay for citric acid. For TLC, the supernatant from YEM-grown cells was examined in eight different solvent systems; the compound migrated well in only two of the eight solvent systems. Preparations typically showed one spot after TLC on silica in 47.5% and 50% ethanol and had respective R_f values of 0.39 and 0.42 as visualized by CAS. In high-voltage paper electrophoresis, the iron-binding compound behaved identically to citric acid. When citric acid was cospotted with the unknown, only one spot was visualized. This spot was ninhydrin negative, indicating that the material in the spot probably did not contain a primary amino functional group. The identity of the siderophore was confirmed by using a specific enzymatic assay for citric acid (22).

Citrate production. Initially, we monitored siderophore production during growth of strain 61A152 by using the CAS assay. Once the identity of the siderophore was determined, the experiments were repeated by using a specific test for citric acid. The enzymatic assay for citric acid also allowed us to quantify citric acid release in minimal medium, which had previously not been possible with the CAS assay (Table 2). In the CAS assay, the sensitivity to citric acid is approximately 500 μM (its sensitivity for rhodotorulic acid, e.g., is 6 to 7 μM). In all cases, levels of citric acid production in minimal medium were found to be less than 500 μM. We also rescreened the remaining 19 strains for citric acid production by using the enzymatic assay for citric acid. No production was detected (the minimum amount detectable would be 2.4 μM).

For cells of strain 61A152 cultured in either YEM or minimal medium, production of citric acid was correlated with the amount of iron in the medium (Table 2). Cells grown with sufficient iron never produced citrate (data not shown); thus, citrate production did not correlate with entry into the stationary phase. Cells which are cultured in media with decreasing amounts of iron began producing citric acid at progressively earlier times (data not shown). Citric acid production was also turned off by addition of ferric chloride to the growth medium (data not shown), also indicating that citric acid production was correlated with the amount of iron available in the medium.

Citrate utilization. Once we had determined that 61A152 cells released citric acid apparently in response to iron stress, we wanted to determine whether exogenously supplied ferric citrate or sodium citrate would rescue cells from iron starvation. Addition of ferric citrate to iron-starved cells growing in minimal medium resulted in a rate of growth and

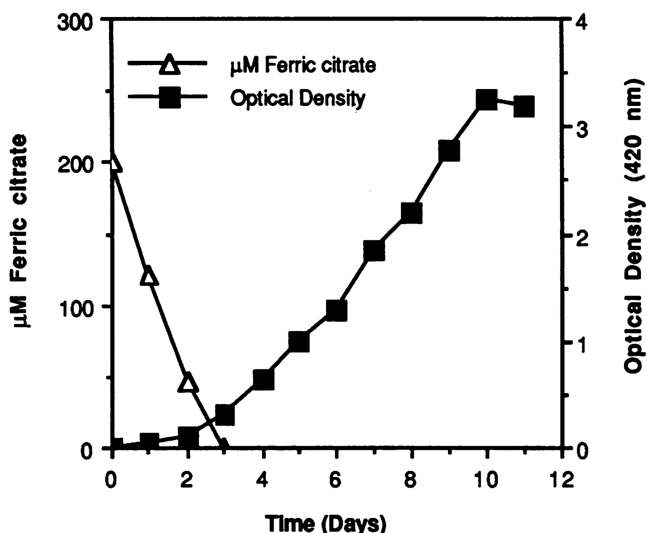

FIG. 2. Growth response of *B. japonicum* 61A152 to addition of ferric citrate. Cells of strain 61A152 were grown without iron for one cycle in iron-limited minimal medium and then inoculated into iron-limited minimal medium with 200 µM ferric citrate. Cells inoculated into iron-limited minimal medium with no additions showed no growth.

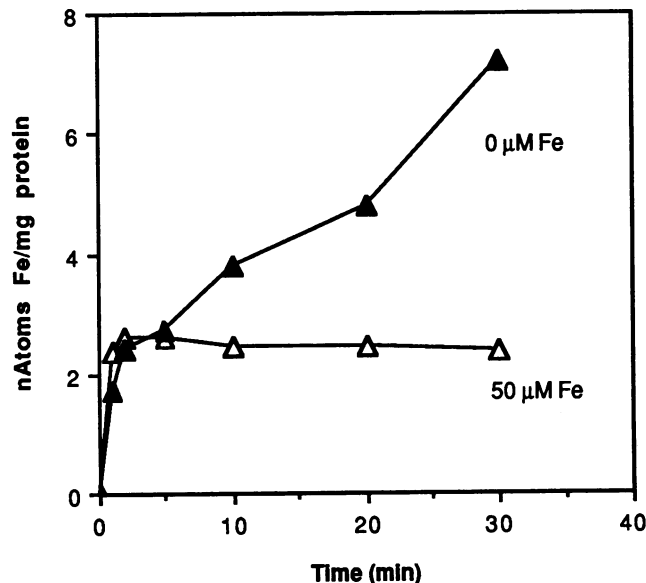

FIG. 3. Uptake of radiolabeled ferric citrate by cells of strain 61A152 grown in minimal medium in either 1 mM citrate–50 µM ferric chloride or 1 mM citrate only. The datum points are means of duplicate samples.

final cell density similar to those seen for cells grown with ferric chloride as an iron source (Fig. 2; compare with the growth curve for cells grown with various levels of iron in Fig. 1). Ferric citrate reached undetectable levels in the medium by day 3 (Fig. 2). Addition of citrate alone was also shown to increase the growth rate, and the cells were able to reach a final optical density of 1.4 after 11 days of growth (data not shown). Presumably, the citrate was able to mobilize previously unavailable iron associated with the culture flask and culture medium. When extreme care is taken to remove all traces of iron from culture flasks and media, citrate alone does not enhance growth. Addition of citrate or ferric citrate did not alter the pH of the medium, which was buffered with MOPS and adjusted to a pH of 6.8. Controls with citrate and no mannitol showed no growth, demonstrating that citrate was not used as a sole carbon source under these experimental conditions (data not shown).

Uptake of radiolabeled ferric citrate. To demonstrate that ferric citrate was used directly as an iron source, the uptake of radiolabeled ferric citrate by *B. japonicum* 61A152 was characterized. Cells were grown with or without iron and then tested for the ability to transport ferric citrate. For cells which had been grown without iron, there was an apparent rapid uptake for the first 2 min, followed by a slower, linear uptake over the next 25 min (Fig. 3). In contrast, cells grown in 50 µM iron showed the same apparent uptake of ferric citrate for the first 2 min but then showed no further uptake over the next 25 min (Fig. 3). Uptake of ferric citrate by iron-starved cells incubated on ice or in the uncoupler 2,4-dinitrophenol showed the same pattern of uptake as iron-sufficient cells (data not shown). Prevention of iron uptake after the first 2 min by either treatment indicates that uptake of ferric citrate is an energy-dependent transport process rather than nonspecific adsorption or precipitation of insoluble iron polymers onto the cell surface.

To assess the effect of citrate in the growth medium on the uptake of ferric citrate by iron-starved cells of strain 61A152, cells were grown with or without 1 mM citrate. Rates of iron uptake were similar for cells grown with or without citrate (data not shown).

DISCUSSION

The siderophore produced by strain 61A152 was identified as citric acid. This is the first report of citric acid release in response to iron stress by a rhizosphere bacterium. *Azotobacter chroococcum* has been reported to release citric acid, but not in response to iron limitation (30). There is also another report that *A. chroococcum* acidifies its culture medium when starved for iron, but the identity of the acidifying agent has not been determined (10). A number of soil-inhabiting fungi have been shown to release both citric acid and malic acid; release of organic acids in response to iron stress is well documented for *Neurospora crassa* (51). The acids excreted by these fungi are proposed to interact with iron which has been concentrated at the cell surface, solubilizing the iron and making it available for use by the fungi (51). Although the surface of rhizobia has been the subject of numerous studies because of its proposed role in recognition and attachment of rhizobial cells to host plant cells and its apparent role in nodule formation itself, there are no reports of surface iron deposition per se. It is also possible that the citric acid released by the rhizobial cells in the rhizosphere aids plants (e.g. see reference 7).

Our finding that only 1 of 20 strains tested produced citric acid adds to the growing evidence that rhizobia, like other bacterial species, exhibit strain-to-strain variation in the ability to produce particular siderophores (e.g., see reference 33). Whether the low incidence of siderophore production reported here will turn out to be a property of bradyrhizobial strains in general remains to be determined. Bradyrhizobia as a group may not possess highly developed iron acquisition systems, having evolved in the acid soils of the tropics, where iron is more generally available than in neutral or high-pH soils. It is also possible that the low incidence of bradyrhizobial siderophore production reflects the inadequacies of our current methods. Rhizobia produce

a number of siderophores with novel structures (39, 42); however, the CAS assay, which was used in this study and which is independent of siderophore structure, should have identified such novel compounds.

In addition to fungi, many plants are also known to accumulate and/or release citric acid under iron stress (e.g. 17). Most importantly, from our point of view, soybeans have been shown to transport iron as ferric citrate (46). Soybean xylem fluid contains approximately 5 to 6 μM ferric citrate, with citric acid present in great excess at a concentration of 1 to 2 mM (50). Therefore, it is not surprising to find that *B. japonicum* can utilize ferric citrate as an iron source. It would encounter ferric citrate both in the rhizosphere and in the plant itself. All of the *B. japonicum* strains which we have screened are able to utilize ferric citrate as an iron source (data not shown).

Citrate release by cells by strain 61A152 was correlated with the iron status of the cultures (Table 2). Winklemann had observed a similar inverse correlation of citrate release with the iron status of cultures of *N. crassa* (51). This inverse correlation suggested that ferric citrate should serve as an iron source for this strain. Both the growth and transport assays with ferric citrate confirmed this prediction. In addition, the uptake experiments with radiolabeled iron demonstrated that ferric citrate could serve directly as an iron source.

Ferric citrate can serve as an iron source for a number of bacterial species (2, 6, 12, 19, 20, 23, 49), and in *Escherichia coli* it requires the products of the *fecABCDE* genes and functional *tonB* and *exbB* genes for transport into cells (32, 44). These genes are negatively regulated by iron, as are the other *E. coli* genes involved in siderophore production and transport (27). On the basis of these criteria, ferric citrate is considered to be a siderophore, although its chelating ability is not as high as those of other siderophore compounds (26).

Ferric citrate transport in *B. japonicum* has some similarities to, as well as differences from, the systems described in other bacteria. In *E. coli* and *Pseudomonas aeruginosa*, iron transport is inhibited by iron (6, 12), whereas in *Mycobacterium smegmatis* and *Rhodopseudomonas sphaeroides*, the mechanism of iron uptake via ferric citrate appears to be constitutive (20, 23). *B. japonicum* cells which were iron sufficient did not transport ferric citrate, although there was an initial association of radiolabeled ferric citrate, presumably with the cell surface. This has been observed in transport studies with other microorganisms, both for ferric citrate and for other ferric siderophores. For example, in a study of *P. aeruginosa*, Cox (6) found that iron accumulation via ferripyochelin or ferric citrate was composed of at least two steps, an initial association of the ferric complex with the cell surface, followed by a slower rate of energy-dependent transport. Although we tried washing *B. japonicum* cells with thioglycolate, which Cox (6) reported to be effective in distinguishing between iron complexes which were bound to the outside of the cell from those that had been internalized, we were unable to completely eliminate the initial high values which we observed in the transport assay (Fig. 3). *Azotobacter vinelandii* has also been reported to have a biphasic uptake of its own siderophores (16). Unlike the citrate-mediated iron uptake system in *E. coli* (12), the presence of citrate in the growth medium did not induce ferric citrate uptake in *B. japonicum*. *P. aeruginosa* (6) and *M. smegmatis* (20) have also been shown to take up ferric citrate at the same rate, regardless of whether the cells were grown with or without citrate. In *E. coli* (12), *B. japonicum* (this study), and *P. aeruginosa* (6), ferric citrate uptake is an active process; this does not appear to be true for *M. smegmatis* (20).

There are several other studies which have examined the use of citric acid to overcome iron starvation in rhizobia. Smith and Neilands (42) reported that *R. meliloti* D4 could not use citric acid as determined by a bioassay, and Rioux et al. (35) reported that *R. leguminosarum* GF160 could utilize citrate on the basis of faster growth of iron-limited cells in citric acid.

Most commercial *B. japonicum* inocula for soybeans sold in Canada contain strain 61A152, and this strain is now one of two used in commercial inocula for soybeans in the United States (personal communication, Stewart Smith, Nitragin Co., Milwaukee, Wis.). 61A152 has been shown to be an efficient nitrogen-fixing strain on a variety of soybean cultivars (R. Veeraraghavaiah and D. J. Hume, 12th North American Symbiotic Nitrogen Fixation Conference Proc., p. 41, abstr. no. P15, 1989). Whether the iron-scavenging abilities of strain 61A152 contribute to its reported success remains to be determined.

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LITERATURE CITED

1. **Ames-Gottfred, N. P., B. R. Christie, and D. C. Jordan.** 1989. Use of the chrome azurol S agar plate technique to differentiate strains and field isolates of *Rhizobium leguminosarum* biovar *trifolii*. Appl. Environ. Microbiol. **55**:707–710.
2. **Archibald, F. S., and I. W. DeVoe.** 1980. Iron acquisition by *Neisseria menigitidis* in vitro. Infect. Immun. **27**:322–334.
3. **Arnow, L. E.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118**:531–537.
4. **Atkin, C. L., J. B. Neilands, and H. J. Phaff.** 1970. Rhodotorulic acid from species of *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, *Spiridibolus*, and *Sporobolomyces*, and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. J. Bacteriol. **103**:722–733.
5. **Carrillo-Castaneda, G., and J. R. V. Peralta.** 1988. Siderophore-like activities in *Rhizobium phaseoli*. J. Plant Nutr. **11**:935–944.
6. **Cox, C. D.** 1980. Iron uptake with ferripyochelin and ferric citrate by *Pseudomonas aeruginosa*. J. Bacteriol. **142**:581–587.
7. **Crowley, D. E., C. P. P. Reid, and P. J. Szaniszlo.** 1987. Microbial siderophores as iron sources for plants, p. 375–386. *In* G. Winkelmann, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Publishers, New York.
8. **Csaky, T. Z.** 1948. On the estimation of bound hydroxylamine in biological materials. Acta Chem. Scand. 2:450–454.
9. **Enard, C., A. Diolez, and D. Expert.** 1988. Systemic virulence of *Erwinia chrysanthemi* 3937 requires a functional iron assimilation system. J. Bacteriol. **170**: 2419–2426.
10. **Fekete, F. A., L. A. Profenno, L. M. Nadeau, N. F. Ferrala, P. R. Lapp, and G. A. Mabbott.** 1988. Capsular polysaccharide and hydroxamate formation by iron-deficient *Azotobacter chroococcum* B-8. J. Plant Nutr. **11**:945–957.
11. **Fischer, H.-M., T. Bruderer, and H. Hennecke.** 1988. Essential and non-essential domains in the *Bradyrhizobium japonicum* NifA protein: identification of indispensable cysteine residues potentially involved in redox reactivity and/or metal binding. Nucleic Acids Res. **16**:2207–2224.
12. **Frost, G. E., and H. Rosenberg.** 1973. The inducible citrate-dependent iron transport system in *Escherichia coli* K12. Bio-

chim. Biophys. Acta **330**:90–101.

13. **Gill, P. R., and J. B. Neilands.** 1989. Cloning of a genomic region required for a high affinity iron uptake system from *Rhizobium meliloti*. Mol. Microbiol. **3**:1183–1189.
14. **Henderson, N., S. Austin, and R. A. Dixon.** 1989. Role of metal ions in negative regulation of nitrogen fixation by the *nifL* gene product from *Klebsiella pneumoniae*. Mol. Gen. Genet. **216**: 484–491.
15. **Holzberg, M., and W. M. Artis.** 1983. Hydroxamate siderophore production by opportunistic and systemic fungal pathogens. Infect. Immun. **40**:1134–1139.
16. **Knosp, O., M. von Tigerstrom, and W. J. Page.** 1984. Siderophore-mediated uptake of iron in *Azotobacter vinelandii*. J. Bacteriol. **159**:341–347.
17. **Landsberg, E. C.** 1981. Organic acid synthesis and release of hydrogen ions in response to Fe deficiency stress of mono- and dicotyledonous plant species. J. Plant Nutr. **3**:579–591.
18. **Langman, L., I. G. Young, G. E. Frost, H. Rosenberg, and F. Gibson.** 1972. Enterochelin system of iron transport in *Escherichia coli*: mutations affecting ferric-enterochelin esterase. J. Bacteriol. **112**:1142–1149.
19. **Leong, S. A., and J. B. Neilands.** 1981. Relationship of siderophore-mediated iron assimilation to virulence in crown gall disease. J. Bacteriol. **147**:482–491.
20. **Messenger, A. J. M., and C. Ratledge.** 1982. Iron transport in *Mycobacterium smegmatis*: uptake of iron from ferric citrate. J. Bacteriol. **149**:131–135.
21. **Modi, M., K. S. Shah, and V. V. Modi.** 1985. Isolation and characterization of catechol-like siderophore from cowpea *Rhizobium* RA-1. Arch. Microbiol. **141**:156–158.
22. **Moellering, H., and W. Gruber.** 1966. Determination of citrate with citrate lyase. Anal. Biochem. **17**:369–376.
23. **Moody, M. D., and H. A. Dailey.** 1984. Siderophore utilization and iron uptake by *Rhodopseudomonas sphaeroides*. Arch. Biochem. Biophys. **234**:178–186.
24. **Nadler, K. D., A. W. B. Johnston, J. Chen, and T. R. John.** 1990. A *Rhizobium leguminosarum* mutant defective in symbiotic iron acquisition. J. Bacteriol. **172**:670–677.
25. **Nambiar, P. T. C., and S. Sivaramakrishnan.** 1987. Detection and assay of siderophores in cowpea rhizobia (*Bradyrhizobium*) using radioactive Fe (^{59}Fe). Lett. Appl. Microbiol. **4**:37–40.
26. **Neilands, J. B.** 1981. Iron absorption and transport in microorganisms. Annu. Res. Nutr. **1**:27–46.
27. **Neilands, J. B.** 1989. Siderophore systems of bacteria and fungi, p. 141–163. *In* T. J. Beveridge and R. J. Doyle (ed.), Metal ions and bacteria. John Wiley & Sons, Inc., New York.
28. **Neilands, J. B., and S. A. Leong.** 1986. Siderophores in relation to plant growth and disease. Annu. Rev. Plant Physiol. **37**: 187–208.
29. **O'Hara, G. W., M. J. Dilworth, N. Boonkerd, and P. Parkpian.** 1988. Iron deficiency specifically limits nodule development in peanut inoculated with *Bradyrhizobium* sp. New Phytol. **108**: 51–57.
30. **Page, W. J.** 1987. Iron-dependent production of hydroxamate by sodium-dependent *Azotobacter chroococcum*. Appl. Environ. Microbiol. **53**:1418–1424.
31. **Patel, H. N., R. N. Chakraborty, and S. B. Desai.** 1988. Isolation and partial characterization of phenolate siderophore from *Rhizobium leguminosarum* IARI 102. FEMS Microbiol. Lett. **56**:131–134.
32. **Pressler, U., H. Staudenmaier, L. Zimmermann, and V. Braun.** 1988. Genetics of the iron dicitrate transport system of *Escherichia coli*. J. Bacteriol. **170**:2716–2724.
33. **Reigh, G., and M. O'Connell.** 1988. Siderophore production is strain specific in *Rhizobium*, p. 826. *In* H. Bothe, F. J. DeBruijn, and W. E. Newton (ed.), Nitrogen fixation: hundred years after. Gustav Fischer, New York.
34. **Rioux, C. R., D. C. Jordan, and J. B. M. Rattray.** 1983. Colorimetric determination of catechol siderophores in microbial cultures. Anal. Biochem. **133**:163–169.
35. **Rioux, C. R., D. C. Jordan, and J. B. M. Rattray.** 1986. Iron requirement of *Rhizobium leguminosarum* and secretion of anthranilic acid during growth on an iron-deficient medium. Arch. Biochem. Biophys. **248**:175–182.
36. **Roessler, P. G., and K. D. Nadler.** 1982. Effects of iron deficiency on heme biosynthesis in *Rhizobium japonicum*. J. Bacteriol. **149**:1021–1026.
37. **Schacterle, G. R., and R. L. Pollack.** 1973. A simplified method for the quantitative assay of small amounts of protein in biologic material. Anal. Biochem. **51**:654–655.
38. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**:47–56.
39. **Schwyn, B., and J. B. Neilands.** 1987. Siderophores from agronomically important species of the Rhizobiaceae. Comments Agric. Food Chem. **1**:95–114.
40. **Skorupska, A., A. Choma, M. Derylo, and Z. Lorkiewicz.** 1988. Siderophore containing 2,3-dihydroxybenzoic acid and threonine formed by *Rhizobium trifolii*. Acta Biochim. Pol. **35**: 119–130.
41. **Skorupska, A., M. Derylo, and Z. Lorkiewicz.** 1989. Siderophore production and utilization by *Rhizobium trifolii*. Biol. Metals **2**:45–49.
42. **Smith, M. J., and J. B. Neilands.** 1984. Rhizobactin, a siderophore from *Rhizobium meliloti*. J. Plant Nutr. **7**:449–458.
43. **Smith, M. J., J. N. Shoolery, B. Schwyn, I. Holden, and J. B. Neilands.** 1985. Rhizobactin, a structurally novel siderophore from *Rhizobium meliloti*. J. Am. Chem. Soc. **107**:1739–1743.
44. **Staudenmaier, H., B. Van Hove, Z. Yaraghi, and V. Braun.** 1989. Nucleotide sequences of the *fecBCDE* genes and locations of the proteins suggest a periplasmic-binding-protein-dependent transport mechanism for iron(III) dicitrate in *Escherichia coli*. J. Bacteriol. **171**:2626–2633.
45. **Stookey, L. L.** 1970. Ferrozine—a new spectrophotometric reagent for iron. Anal. Chem. **42**:779–781.
46. **Tiffin, L. O.** 1970. Translocation of iron citrate and phosphorus in xylem exudate of soybean. Plant Physiol. **45**:280–283.
47. **Verma, D. P. S., and S. Long.** 1983. The molecular biology of *Rhizobium*-legume symbiosis. Int. Rev. Cytol. Suppl. **14**:211–245.
48. **Vincent, J. M.** 1970. A manual for the practical study of root nodule bacteria. International Biological Program handbook 15. Blackwell Scientific Publications, Oxford.
49. **Wee, S., S. Hardesty, M. V. V. S. Madiraju, and B. J. Wilkinson.** 1988. Iron-regulated outer membrane proteins and non-siderophore-mediated iron acquisition by *P. denitrificans*. FEMS Microbiol. Lett. **51**:33–36.
50. **White, M. C., F. D. Baker, R. L. Chaney, and A. M. Decker.** Metal complexation in xylem fluid. II. Theoretical equilibrium model and computational computer program. Plant Physiol. **67**:301–310.
51. **Winklemann, G.** 1979. Surface polymers and hydroxy acids. A model of iron supply in sideramine-free fungi. Arch. Microbiol. **121**:43–51.