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Siderophore Utilization by *Bradyrhizobium japonicum*

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***Bradyrhizobium japonicum* USDA 110 and 61A152 can utilize the hydroxamate-type siderophores ferrichrome and rhodotorulate, in addition to ferric citrate, to overcome iron starvation. These strains can also utilize the pyoverdin-type siderophore pseudobactin St3. The ability to utilize another organism's siderophores may confer a selective advantage in the rhizosphere.**

Rhizobia must be able to persist in the soil in the absence of their host plants. Competition for iron, the availability of which is limited by its insolubility at neutral pH in aerobic environments, may be one factor in determining how successful rhizobia are in maintaining themselves in the rhizosphere (7). Several studies have now addressed the ability of *Bradyrhizobium japonicum*, the soybean symbiont, to produce siderophores (4, 9). Siderophores are ferric ion-specific ligands with high affinity for iron that are taken into cells via specific membrane receptors. To date, surprisingly few strains of *B. japonicum* have been found to produce siderophores when tested either via assays for catechols and hydroxamates, the most common types of siderophores, or via a general assay (chrome azurol S) that is independent of siderophore structure (4, 9). For example, of 20 strains screened, only 1 strain, 61A152, was found to excrete an iron-chelating compound, which was identified as citric acid (9). In field tests, strain 61A152 gives consistently high yields on a variety of soybean cultivars (12), and it is tempting to speculate that its iron-scavenging abilities may contribute to its success. This strain is now being widely used in soybean inoculants in both the United States and Canada.

Because many microorganisms are able to utilize the ferric complexes of siderophores which they themselves do not synthesize, we wanted to investigate whether *B. japonicum* 61A152 could utilize other siderophores, in addition to ferric citrate, which are commonly found in soil. Utilization of siderophores made by other organisms is a sound strategy for iron acquisition because siderophores are excreted into the soil where they are freely available. The majority of soil microorganisms form siderophores containing hydroxamate ligands (2); levels of hydroxamate-type siderophores in soil have been reported to be as high as 10 μM (5), which should be sufficient to support the growth of bradyrhizobia (9). We have focused on three different siderophores: (i) the hydroxamate ferrichrome, which is probably the most commonly produced fungal siderophore; (ii) rhodotorulic acid, another hydroxamate-type siderophore which is synthesized by many yeast and smut fungi; and (iii) pseudobactin St3, which is one of many pyoverdin-type siderophores produced by fluorescent pseudomonads, as well as by various *Azotobacter* and *Azomonas* strains (e.g., 6, 15). We also wanted to extend our observations on the utilization of exogenously supplied siderophores to *B. japonicum* USDA 110, given its use in commercial inocula and its role as the *B. japonicum* strain of choice for molecular genetic studies.

Utilization of siderophores by strains 61A152 and USDA 110. Cells of *B. japonicum* 61A152 (gift of Nitragin Co., Milwaukee, Wis.) and cells of the small colony derivative of *B. japonicum* USDA 110 (8) were cultured initially in yeast extract-mannitol medium (YEM) (21) at 30°C with shaking. Cells were then diluted into minimal medium (9) to which no iron was added and were grown at 30°C with shaking. Mannitol (0.2%) was used as a carbon source for strain 61A152, and xylose (0.2%) was used for strain USDA 110. To minimize the iron content of both the culture vessels and the medium, glassware was washed with acid and plastic culture flasks were filled with 0.5% EDTA, autoclaved, and then rinsed extensively with double-distilled water. After one cycle of growth in minimal medium, cells were once again diluted into minimal medium with no added iron. Cultures then received either rhodotorulic acid (10 μM, final concentration; Sigma Chemical Co., St. Louis, Mo.), ferrichrome (10 μM, final concentration; gift of J. Coulton), pseudobactin St3 (10 μM, final concentration; gift of E. Jurkevitch; see reference 13), sodium citrate (200 μM, final concentration; Sigma Chemical Co.), or ferric chloride (10 μM, final concentration).

The addition of rhodotorulic acid, ferrichrome, or citrate to iron-starved cells of both strains 61A152 and USDA 110 resulted in an increase in the growth rate and final cell density relative to cells which received no additions (Fig. 1 shows a representative set of growth curves). Growth with the addition of any of these three siderophores was equivalent to that seen with ferric chloride. The addition of pseudobactin St3 also resulted in an increase in the growth rate and final cell density for both strains (Fig. 1). However, pseudobactin St3-supplemented cultures had a much longer lag phase than cultures supplemented with other siderophores, and strain USDA 110 did not attain a cell density equivalent to that seen when ferric chloride was added.

Uptake studies. Exogenously supplied siderophores could be serving directly as iron sources. Alternatively, iron could be transferred via interligand exchange to a chelator produced by the cells, which then serves itself as the immediate source of iron. To try to differentiate between these two possibilities, uptake studies were carried out as described previously (9) with the following modifications. Thirty minutes before the start of the uptake experiments, a solution of $^{55}FeCl_3$ in 0.1 M HCl was added to uptake medium which contained either 1 mM citrate (prepared as a 3:1 mixture of the sodium and potassium salts), 10 μM ferrichrome, 10 μM pseudobactin St3, or 15 μM rhodotorulic acid. The final concentration of ^{55}Fe was 0.4 μCi/ml. Iron-deficient cells of

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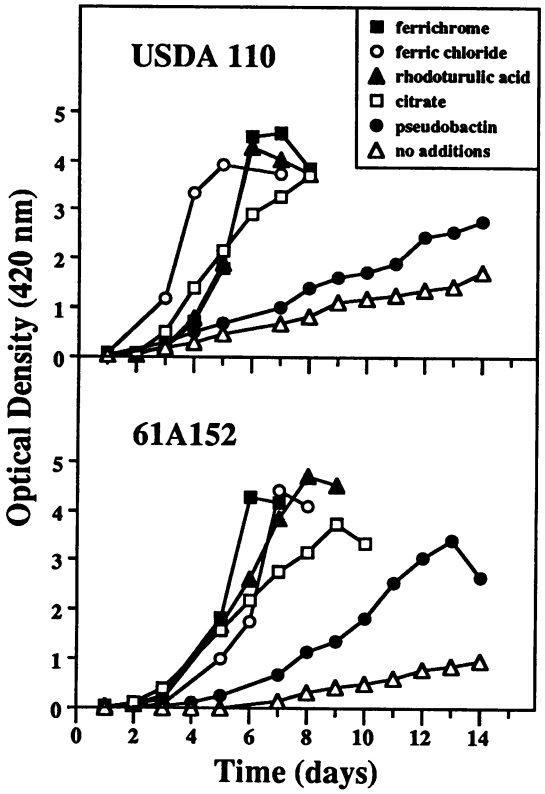

FIG. 1. Growth of strains USDA 110 and 61A152 in minimal medium containing different siderophores. Cells of strains USDA 110 and 61A152 were grown without iron for two cycles in minimal medium and then inoculated into minimal medium containing the indicated supplements.

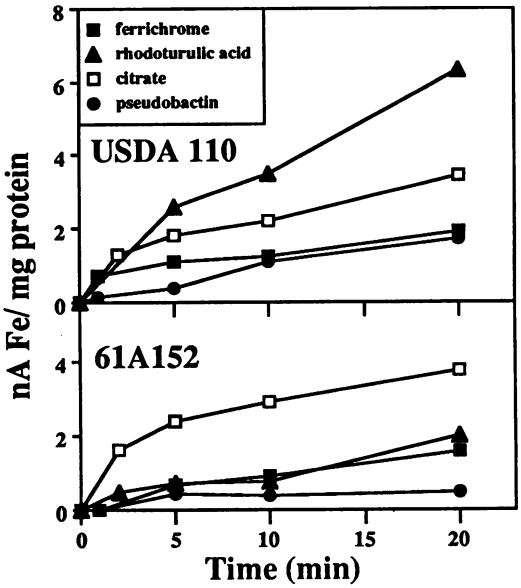

FIG. 2. Uptake of radiolabelled ferric siderophores by iron-deficient cells of strains USDA 110 and 61A152. The values shown are the averages for duplicate samples. Iron-sufficient cells of both strains did not show uptake of any of the siderophores tested (data not shown).

both strains showed uptake of radiolabelled iron when supplied with ferric rhodotorulate, ferric citrate, or ferric ferrichrome (Fig. 2), demonstrating that the cells can probably use these chelates directly as iron sources. Cells of strain 61A152 showed little, if any, uptake of radiolabelled iron when supplied with ferric pseudobactin St3 over the time course of the experiment. However, as can be seen in Fig. 1, when grown with pseudobactin St3, strain 61A152 attained a high cell density after a prolonged lag phase. The long lag phase and the lack of uptake of radiolabelled iron when supplied with ferric pseudobactin together suggest that strain 61A152 is not utilizing pseudobactin St3 directly as an iron source but rather may be relying on the exchange of iron between pseudobactin St3 and its own siderophore. Such interligand exchange has been documented for other organisms. For example, in cocultures of *Agrobacterium tumefaciens* and *Azotobacter vinelandii*, *A. tumefaciens* did not use the siderophores of *A. vinelandii* directly but rather used them as a source from which to transfer iron to agrobactin, the *Agrobacterium* siderophore (17). Interligand exchange is slow (as discussed in reference 18) and, hence, is consistent with the long lag phase observed when 61A152 was grown with pseudobactin St3. We have previously documented that strain 61A152 released citric acid under iron-deficient growth conditions and that this strain could utilize ferric citrate as an iron source (9). In contrast, no release of citric acid or of any other siderophore was detected for USDA 110 (9). This may explain why USDA 110 did not grow as well as 61A152 on pseudobactin St3.

Implications. The ability to utilize the siderophores of another organism is obviously of great selective advantage during iron-limited growth in the presence of a variety of competing microorganisms. *B. japonicum* must persist in the soil in the absence of its legume host, and persistence is presumably a function of its competitiveness with other soil organisms for available nutrients. The growth assays showed that both strains can utilize exogenously supplied siderophores. Our observations of pseudobactin St3 utilization are especially noteworthy as Loper and Buyer (16), in a recent review of microbial interactions on plant surfaces, stated that utilization of pyoverdins by organisms other than pseudomonads had not been demonstrated. Of course, the ability to utilize pseudobactin St3 does not mean that bradyrhizobia will necessarily be able to utilize all pseudobactins. Although the pseudobactins examined to date have similar structures, there is enough diversity (especially in the peptide portion of the molecules) that many bacteria which may utilize one particular pseudobactin cannot utilize another with a similar yet distinct structure (e.g., 11). This is presumably due to specificity in the uptake systems for the various ferric pseudobactins (e.g., 1).

To put the ability of bradyrhizobia to utilize exogenous siderophores in perspective, Jurkevitch et al. (13) have determined that about 20% of the total CFU from the rhizospheres of four plant species (maize, sorghum, melon, and bean) were able to use ferrioxamine B and another 12% were able to use ferrichrome. They also looked at three pseudobactins, including the one used in this study (pseudobactin St3), and found that 10% or less of the rhizosphere CFU could utilize these pseudobactin compounds. Thus, the ability of bradyrhizobia to utilize the siderophores tested in this study indicates that they do belong to a rather select group of rhizosphere inhabitants.

Only a few rhizobial species have been examined for their ability to utilize various siderophores as iron sources. Cells of *Rhizobium meliloti* DM4 can use the hydroxamate-type

siderophores ferrichrome and ferrioxamine B (23). However, it is not clear whether this strain utilizes these siderophores directly, because utilization was assessed via bioassay, and *R. meliloti* DM4 does produce its own siderophore, rhizobactin (23). Skorupska et al. (22) have reported that seven of eight strains of *Rhizobium trifolii* (*R. leguminosarum* biovar trifolii) screened for siderophore utilization did not utilize ferrioxamine B, citrate, or pseudobactin. However, the one remaining strain, which did make a siderophore, grew better in the presence of any of the three chelators. Ferric anthranilate, although not a true siderophore, is able to serve as an iron source for *R. leguminosarum* GF160 (20); this strain was shown to release anthranilic acid under iron starvation (19).

In *Escherichia coli*, different siderophores are taken into the cell via different outer membrane receptors. Ferrichrome is imported via FhuA, ferric rhodotorulate is taken into the cell via FhuE, and ferric citrate enters via FecA (as reviewed in reference 3). Ferrioxamine B, a siderophore made by actinomycetes which is also likely to be available in the rhizosphere, appears to be imported via FhuE, the same receptor used for ferric rhodotorulate (10). A ferric pseudobactin receptor, PupA, which shows considerable similarity to the FhuE receptor of *E. coli*, has been identified in *Pseudomonas putida* WCS358 (1). PupA seems to be a specific high-affinity receptor for pseudobactin 358 (1). We know that *B. japonicum* 61A152 and USDA 110 induce a number of new outer membrane proteins under iron-deficient conditions which are probably receptors for various siderophores (7, 14), but we have yet to assign a specific function to each of the induced proteins.

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