Iron Transport-mediated Antagonism between Plant Growth-promoting and Plant-deleterious *Pseudomonas* Strains

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Both plant growth-promoting *Pseudomonas* B10 and its yellow-green, fluorescent iron transport agent (siderophore) pseudobactin enhance potato growth and biologically control certain soil-borne fungal diseases in part by depriving specific root-colonizing endemic microorganisms including phytopathogens of iron(III), thus inhibiting their growth. The present study examines this mode of iron deprivation. The growth inhibition of certain bean-deleterious fluorescent pseudomonads by specific bean-beneficial fluorescent pseudomonads is due in part to the inability of susceptible strains to utilize siderophores from beneficial strains to transport iron(III). Conversely, deleterious strains which were able to utilize siderophores from beneficial strains were not inhibited. The ability of a given pseudomonad to utilize another pseudomonad’s siderophore may depend upon its possessing a specific outer membrane receptor protein for that pseudomonad’s ferric siderophore. Siderophore-mediated competition for iron in microbial systems appears to be a widespread phenomenon.

Specific root-colonizing members of the *Pseudomonas* fluorescent-*Pseudomonas putida* group (1) enhance growth of a variety of crops including radish (1), potato (2), and sugar beet (3). Their plant growth-promoting activity is due in part to reductions in the population of root-colonizing phytopathogenic (4) and deleterious (3, 5) fungi and rhizobacteria. Deleterious rhizobacteria were shown to be a major component of the bacterial microflora of field-grown sugar beet, bean, or lettuce roots (3) and are responsible in part for decreasing the growth of these crops and increasing susceptibility of their roots to infection by fungi (3). The genera of deleterious rhizobacteria include *Pseudomonas*, *Klebsiella*, *Citrobacter*, *Flavobacterium*, *Achromobacter* and *Arthrobacter* (3, 6). Deleterious fluorescent pseudomonads, closely related to phytopathogenic *Pseudomonas syringae*, are of particular interest since they may produce phytotoxins (7), which are responsible in part for disease.

Both plant growth-promoting *Pseudomonas* B10 and its yellow-green, fluorescent siderophore (microbial iron transport agent (8)) pseudobactin (9) enhance potato growth (10) and biologically control the soil-borne fungal pathogens, *Fusarium oxysporum* f. sp. *lini* and *Gaemmamannycyes graminis* var. *tritici* (4). Since the efficacy of pseudobactin or *Pseudomonas* B10 could be eliminated by concomitant addition of iron(III), plant growth-promoting fluorescent pseudomonads appear to exert their beneficial effects in part by producing under iron-limiting conditions extracellular siderophores that efficiently complex environmental iron, making it less available to certain endemic microorganisms, including phytopathogenic fungi, thus inhibiting their growth.

We have begun a study to understand the biochemical basis of the mode of antagonism of plant growth-promoting fluorescent pseudomonads against deleterious fluorescent pseudomonads. We report here that the iron(III) starvation of certain deleterious pseudomonads by specific beneficial pseudomonads or their siderophores is due in part to the inability of deleterious strains to utilize exogenous siderophores from beneficial strains to transport iron, an essential nutrient.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—All yellow-green, fluorescent *Pseudomonas* strains were obtained from Dr. M. N. Schroth, University of California, Berkeley, and were maintained on King’s medium B (KB) plates (11). These included potato growth-promoting strain B10, sugar beet-deleterious strain 7SR1, bean growth-promoting strains A112, A124, A215, A216, B122, B222, and B226, and bean-deleterious strains A214, A225, and B117.

**Isolation of Ferric Siderophores**—An iron-deficient minimal medium consisting of 1 g of KH2PO4, 2 g of Na2HPO4, 0.5 g of NH4Cl, and 10 ml of glycerol/liter, and made 0.05% (w/v) in MgSO4·7H2O and 2% (w/v) in Casamino acids (Difco), was used for the production of pseudobactin A214 from *Pseudomonas* A214. The same medium was used for the production of pseudobactin A112 from *Pseudomonas* A112 and pseudobactin B117 from *Pseudomonas* B117 except that it contained 2.25 g of KH2PO4, 4.5 g of Na2HPO4, and 1 g of NH4Cl/liter. A stock solution of Casamino acids was deferrated with NaH2EDTA as described previously (12).

Cells were cultured, and ferric siderophores were isolated as described earlier (13). Ferric pseudobactin B117 was chromatographed isocratically at 4 °C on a column (3.8 × 40 cm) containing CM-Sephadex CM-25, pyridinium form, equilibrated in 200 mM pyridine-acetic acid buffer, pH 5.5. Ferric pseudobactin A214 and ferric pseudobactin A112 were chromatographed at 4 °C on columns (3.8 × 40 cm) containing DEAE-Sephadex A-25, acetate form, equilibrated with 5 mM acetic acid-pyridine buffer, pH 6. Linear gradients (1 liter) from 5 to 700 mM acetic acid-pyridine buffer, pH 6, were used to elute ferric pseudobactin A214 as a single band at approximately 500 mM and ferric pseudobactin A112 as a single band at approximately 350 mM. All ferric siderophores were chromatographed on Bio-Gel P-2 as described previously (9).

Ferric pseudobactin B117 was analyzed by high pressure liquid chromatography (HPLC) on a Varian VISTA 54 HPLC system with an analytical column (4 × 250 mm) containing 5 µm LiChrosorb RP-18 (Merck). Isocratic elution with 99% (v/v) H2O and 1% methanol containing 0.1% (v/v) acetic acid was used for detection. The elution profile of ferric pseudobactin B117 is shown in Figure 1. The abbreviations used are: KB, King’s medium B; HPLC, high-pressure liquid chromatography; EDDA, ethylenediaminetetra[O-hydroxyphenyl]acetate acid.

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Agonism between Plant-beneficial and -deleterious Pseudomonads

at a flow rate of 0.8 ml/min yielded one major peak with a purity greater than 90% as judged by area when detected with a Varian UV-50 ultraviolet and visible detector at 400 nm.

Ferric pseudobactin A112 was further purified as follows. Analysis by HPLC of ferric pseudobactin A112 on the above column with 10 mM tetraethylammonium acetate buffer at 1 ml/min yielded four peaks. The third peak to elute accounted for 64% of the total area; HPLC analysis of cultures of Pseudomonas A112 harvested as a function of time and treated as described above confirmed that this third peak was the major one at early time points (data not shown). Preparative HPLC was performed on a 10-µm LiChrosorb RF-18 column (10 × 250 mm) (Merck) with the same solvent system at 6 ml/min, and the third peak was collected. This ferric pseudobactin A112 was then chromatographed at 4 °C on a column containing DEAE-Sephadex A-25, acetate form, equilibrated with 10 mM tetraethylammonium acetate in 2% acetonitrile at 1 ml/min. This procedure was repeated. The resulting ferric pseudobactin A112 was greater than 90% pure as determined by HPLC analysis.

Pseudobactin B117, Pseudobactin A112, and Pseudobactin A214—Pseudobactin B117, pseudobactin A112, and pseudobactin A214 were obtained by deferration of the corresponding ferric siderophore with 8-hydroxyquinoline as described previously (14).

Pseudobactin B117 was chromatographed at 4 °C on a column containing CM-Sephadex C-25, pyridinium form, equilibrated with 10 mM pyridine-acetic acid buffer, pH 5.5, and eluted with a linear gradient of pyridine-acetic acid buffer from pH 5.5 to 2.0. Pseudobactin B117 was ascertained to be greater than 90% pure by HPLC analysis. After addition of iron(III) to an aliquot of pseudobactin B117, ferric pseudobactin B117 was isolated as described (15) and analyzed by HPLC.

HPLC analysis of pseudobactin A112 on a low iron silica gel-based 4-µm C-18 ion-pair column (10 × 150 mm) (Varian) with 10 mM tetraethylammonium acetate in 3% acetonitrile at 1 ml/min indicated that it was 93% pure when monitoring fluorescence with an LDC Fluoromonitor III. When iron(III) was added back to an aliquot of pseudobactin A112, the resulting ferric pseudobactin A112 was greater than 98% pure by HPLC analysis.

HPLC analysis of pseudobactin A214 was performed as described for pseudobactin A112 and revealed two compounds in similar amounts. Time-course studies of Pseudomonas A214 indicated that the later-eluting compound was predominant at early time points (data not shown) and hence was further purified as follows. Preparative HPLC was performed on a 4-µm low iron silica gel-based C-18 ion-pair column (10 × 150 mm) (Varian) with 10 mM tetraethylammonium acetate in 6% acetonitrile at 2 ml/min, monitoring absorbance at 440 nm, and the second peak was collected. The resulting pseudobactin A214 was chromatographed at 4 °C on a column containing DEAE-Sephadex A-25, acetate form, equilibrated with 10 mM pyridine-acetic acid buffer, pH 5.5, and eluted with a linear gradient of pyridine-acetic acid buffer from pH 5.5 to 2.0. HPLC analysis of this pseudobactin A214 indicated that it was at least 95% pure. When iron(III) was added back to an aliquot of pseudobactin A214, the resulting ferric pseudobactin A214 was greater than 90% pure by HPLC analysis using the Merck analytical column with 10 mM tetraethylammonium acetate in 2% acetonitrile at 1 ml/min. This resulting ferric pseudobactin A214 was used in all subsequent studies.

Paper Electrophoresis—Paper electrophoresis was performed as described earlier (9). Standards included pseudobactin (+1 ionic charge) (9), ferric pseudobactin (neutral) (9), ferric pseudobactin 7SR1 (1-ionic charge) (13), and ferrichrome A (3-ionic charge) (15). Spots were visualized as described previously (9). Ionic charges were assigned to each siderophore and its ferric siderophore complex by comparison of their migrations upon paper electrophoresis at pH 6.5 with those of the above standards.

Determination of Extinction Coefficients of Siderophores and Ferric Siderophores—After the visible absorption maximum of a siderophore solution in 0.1 M sodium acetate, pH 5.2, was determined, the solution was spectrophotometrically titrated with 1.0 mM ferrous ammonium sulfate at 440 nm. The visible absorbance maximum of the resulting ferric siderophore solution was recorded.

Pattern I of Antagonism—About 40 µl of an overnight culture of a pseudomonal grown in KB medium was spotted in the center of a KB agar plate and a KB plate containing 200 µM ferric chloride, and the resulting plates were incubated at room temperature for 48 h. After a suspension of the pseudomonal to be challenged (106 cells/ml) was sprayed over each plate, the plates were incubated at room temperature for 24–72 h and examined for the presence of clear zones of growth inhibition.

Siderophore Antibiosis—KB plates and KB plates containing 200 µM iron(III) were seeded with the pseudomonal to be challenged in 2.5 ml (106 cells/ml) of soft agar (0.7% w/v) agar, 0.5% NaCl). After 48 h, paper discs (BBL Microbiology Systems) containing 20 µl of 1 mM siderophore or ferric siderophore solutions were placed on the seeded plates, the plates were incubated at room temperature for 24–72 h and examined for the presence of clear zones of growth inhibition about the discs. Concentrations of siderophore and ferric siderophore solutions were calculated from their extinction coefficients.

Plate Bioassay for Siderophore Utilization—A literature procedure describing reversal of iron starvation by siderophores was followed (16).

RESULTS

We previously isolated and structurally characterized the siderophores pseudobactin from potato-growth-promoting Pseudomonas B10 (9) and pseudobactin 7SR1 from sugar beet-deleterious Pseudomonas 7SR1 (13). As part of an effort to compare chemical structures of siderophores from both plant-beneficial and plant-deleterious fluorescent pseudomonads, we isolated the siderophore pseudobactin A214 from bean-deleterious Pseudomonas A214 in this study and are presently determining its structure. The structure of pseudobactin A214 is different from those of pseudobactin and pseudobactin 7SR1.5. Pseudobactin A214 displayed properties typical of a siderophore, including complete repression of production in various culture media containing micromolar levels of iron(III) (data not shown). Furthermore, pseudobactin A214 and ferric pseudobactin A214, both at 10 µM, were about equally effective in reversing iron starvation of strain A214 induced by the synthetic ferric complexing agent ethylenediaminedi-(o-hydroxyphenyl)acetic acid (EDDA). The iron of which is not utilized by the cells in the plate bioassay (17, 18). This stimulation of growth was evidenced by a halo of single colonies surrounding the paper discs containing the above compounds. In contrast, FeCl3·6H2O at 10 mM was apparently required to saturate the EDDA in the medium, thereby producing similar-sized growth halos as the above compounds. Hence, Pseudomonas A214 could utilize pseudobactin A214 to transport iron(III). Some physical properties of yellow-green, fluorescent pseudobactin A214 and red-brown ferric pseudobactin A214 are listed in Table I.

Since enhanced plant growth by beneficial fluorescent pseudomonads is accompanied by inhibition of and reduced root colonization of phytopathogenic and deleterious microorganisms, we wanted to determine if the growth inhibition of certain deleterious fluorescent pseudomonads by specific beneficial fluorescent pseudomonads could be accounted for by iron starvation of the former by the siderophore of the latter. Since siderophores were readily available from Pseudomonas strains B10, 7SR1, and A214, we first examined organismal antagonism in pairwise combinations with this set of strains (A, see below). We then tested purified siderophores from each strain for antagonism (B, see below). Finally, we investigated exogenous siderophore utilization using the plate bioassay (C, see below).

The following three patterns of antagonism and utilization were observed on KB plates.

Pattern I—Strain 1 inhibited the growth of strain 2, but strain 2 did not inhibit the growth of strain 1. (a) Organismal antagonism: When strain A214 was established on KB plates, it inhibited strain B10. However, when strain B10 was established on KB plates, it inhibited strain A214. (b) Siderophore antagonism: When strain A214 was established on KB plates, it inhibited strain B10. However, when strain B10 was established on KB plates, it inhibited strain A214.
Organismal antagonism: Strain A214 did not inhibit the growth of strain B10, but pseudobactin did not inhibit the growth of strain A214. Pseudobactin A214 inhibited the growth of strain B10. The siderophore used by each organism inhibited the other organism. The siderophore used by one organism inhibited the growth of the other organism.

Siderophore utilization: Strain B10 could not utilize pseudobactin A214 or ferric pseudobactin A214 as siderophores, but strain A214 could utilize pseudobactin or ferric pseudobactin for iron transport.

Pattern II—Strains 1 and 2 were mutually growth inhibitory. (a) Organismal antagonism: When either strain B10 or strain 7SR1 was established first, the established strain inhibited the other strain. (b) Siderophore antagonism: Each siderophore inhibited the growth of the other strain. (c) Siderophore utilization: Strains B10 and 7SR1 could not use each other’s siderophore or ferric siderophore to transport iron.

Pattern III—No growth inhibition between strains 1 and 2 was seen. (a) Organismal antagonism: Strain A214 did not inhibit strain 7SR1, and vice versa. (b) Siderophore antagonism: Each strain was not inhibited by the other’s siderophore. (c) Siderophore utilization: Strains A214 and 7SR1 were both able to use each other’s siderophore or ferric siderophore.

The above results suggest that iron-repressible antagonism, i.e. growth inhibition which did not occur in the presence of iron, between fluorescent pseudomonads was due in part to the inability of the susceptible strain to utilize the other strain’s siderophore to transport iron, an essential nutrient. In order to ascertain if this mechanism could be operative in the rhizosphere, bean-beneficial fluorescent pseudomonads were screened for antagonism against bean-deleterious fluorescent pseudomonads (data not shown). Since beneficial strain A112 inhibited more deleterious strains (see below) than any other beneficial strain, we focused our studies on it. Siderophores were isolated and purified from strains A112 and B117 in addition to strain A214 to ascertain if these siderophores could mimic the behavior of their producing strains. Pseudobactin A112 and pseudobactin B117 exhibited properties typical of siderophores as discussed above for pseudobactin A214. Some physical properties of these siderophores are given in Table 1.

### TABLE 1

<table>
<thead>
<tr>
<th>Siderophore</th>
<th>λ max (10^-4)</th>
<th>Ionic charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudobactin A214</td>
<td>381 (1.6)</td>
<td>1–</td>
</tr>
<tr>
<td>Pseudobactin A112</td>
<td>379 (1.8)</td>
<td>0–</td>
</tr>
<tr>
<td>Pseudobactin B117</td>
<td>380 (1.2)</td>
<td>+1</td>
</tr>
<tr>
<td>Ferric pseudobactin A214</td>
<td>405 (2.3)</td>
<td>2–</td>
</tr>
<tr>
<td>Ferric pseudobactin A112</td>
<td>398 (2.4)</td>
<td>1–</td>
</tr>
<tr>
<td>Ferric pseudobactin B117</td>
<td>400 (2.0)</td>
<td>0–</td>
</tr>
</tbody>
</table>

The four possible patterns of antagonism and utilization between beneficial and deleterious strains are as follows.

**Pattern I**—Beneficial strain inhibited the growth of deleterious strain, but not vice versa. (a) Organismal antagonism: Beneficial strain A112 inhibited deleterious strains A214 and A225, but not vice versa. (b) Siderophore antagonism: Pseudobactin A112 inhibited strains A214 and A225, but pseudobactin A214 did not inhibit strain A112. (c) Siderophore utilization: Strain A112 could utilize pseudobactin A214 and ferric pseudobactin A214 for iron transport, but strains A214 and A225 could not utilize pseudobactin A112 or ferric pseudobactin A112.

**Pattern II**—Deleterious strain inhibited the growth of beneficial strain, but not vice versa. (a) Organismal antagonism: Deleterious strain B117 inhibited beneficial strain A112, but not vice versa. (b) Siderophore antagonism: Pseudobactin B117 inhibited strain A112, but pseudobactin A112 did not inhibit strain B117. (c) Siderophore utilization: Strain B117 utilized pseudobactin A112 and ferric pseudobactin A112 as siderophores, whereas strain A112 could not utilize pseudobactin B117 or ferric pseudobactin B117.

**Pattern III**—Beneficial and deleterious strains were mutually growth-inhibitory.

**Pattern IV**—No growth inhibition between beneficial and deleterious strains was seen.

No examples were found for patterns III and IV, presumably due to the small number of strains in our collection.

These results suggest that the same mechanism of antagonism occurs with bean-beneficial and -deleterious strains as in the round-robin study. Beneficial strains inhibited the growth of deleterious strains in part by depriving them of iron because these deleterious strains could not utilize the siderophore from the beneficial strain. In contrast, deleterious strains resistant to beneficial strains appeared to use the beneficial strains’ siderophore for iron transport.

### DISCUSSION

A favored hypothesis on how plant growth-promoting fluorescent pseudomonads enhance plant growth is that aggressive colonization of the root system results in displacement or exclusion of deleterious components of the microflora along roots. Specifically, plant growth promotion is related in part to reduced infection and colonization by specific pathogenic and deleterious fungi and bacteria. The present study provides further insights on how yellow-green, fluorescent siderophores from beneficial fluorescent pseudomonads enhance plant growth and biologically control certain soilborne fungal diseases. The growth inhibition of certain deleterious fluorescent pseudomonads by specific beneficial fluorescent pseudomonads is due in part to the inability of susceptible strains to utilize siderophores from beneficial strains to transport iron. Conversely, deleterious strains which are able to utilize the siderophore from a beneficial strain, will be resistant. It is conceivable that following inoculation of a crop with a beneficial strain, the rhizosphere-population density of any microorganism that can utilize the siderophore from the beneficial strain will actually increase. Another case of interest is that in which natural populations of beneficial and deleterious strains cannot use each other’s siderophores, resulting in mutual antagonism. In this instance, factors such as the relative equilibrium binding constants for iron(III) of their siderophores and the amount of siderophore production and efficiency of the iron assimilation systems of each strain might determine which species predominates in an iron-limiting environment.

Other modes of antagonism besides iron deprivation of
deleterious microorganisms could contribute to plant growth promotion by beneficial strains. For example, antagonism between some strains occurred even in the presence of iron(III) (data not shown), implicating antibiotics or bacteriocins in a second mode of action. Loper and Schroth suggested that the growth-inhibitory behavior of certain beneficial fluorescent pseudomonads against deleterious microorganisms under iron-sufficient conditions is due to their production of bacteriocins (19).

Competition for iron via siderophores appears to be a widespread phenomenon in microbial systems. Blue-green algae belonging to the genus *Anabaena* produce under iron-limiting conditions the hydroxamate siderophore schizokinen (20), which inhibits the growth of the alga *Chlamydomonas reinhardtii* but not the alga *Chlorella vulgaris* (21). During blue-green algal blooms associated with *Anabaena* species, the growth inhibition of other algae was attributed to production of hydroxamate siderophores by *Anabaena* (22).

*Escherichia coli* elaborates under iron-limiting conditions the catecholate siderophore enterobactin (23, 24), whereas strains harboring the ColV plasmid synthesize the hydroxamate siderophore aerobactin (25). In addition, *E. coli* has iron transport systems for the fungal siderophores ferrichrome (26), coprogen (27), and rhodotorulic acid (28, 29). Presumably, these other iron uptake systems enable *E. coli* to survive outside the intestine under iron-limiting conditions in environments inhabited by fungi that produce the above siderophores.

*E. coli* has separate outer membrane receptor proteins for the various ferric siderophores that it utilizes (30), suggesting that fluorescent pseudomonads may also have specific outer membrane receptor proteins for different ferric siderophores. Hence the ability of a given pseudomonad to utilize an exogenous siderophore from another pseudomonad may depend upon its possessing a specific outer membrane receptor protein for that pseudomonad’s ferric siderophore. In the accompanying paper we describe cloning of the gene coding for the putative outer membrane receptor protein for ferric pseudobactin. Transfer of this gene into fluorescent pseudomonads, whose growth was inhibited by *Pseudomonas* B10 or pseudobactin, rendered them no longer susceptible to iron deprivation by pseudobactin because they were now able to utilize pseudobactin as a siderophore.

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