Siderophore Receptor PupA as a Marker To Monitor Wild-Type Pseudomonas putida WCS358 in Natural Environments

JOS M. RAAIJMAKERS,1,* WILBERT BITTER,2 HELMA L. M. PUNTE,1 PETER A. H. M. BAKKER,1 PETER J. WEISEBEEK,2 AND BOB SCHIPPERS1

Section of Plant Pathology, Department of Plant Ecology and Evolutionary Biology, Utrecht University, 3508 TB,1 and Department of Molecular Cell Biology, Utrecht University, 3584 CH2 Utrecht, The Netherlands

Received 8 November 1993/Accepted 2 February 1994

For application of genetically engineered fluorescent Pseudomonas spp., specific markers are required for monitoring of wild-type Pseudomonas strains and their genetically modified derivatives in natural environments. In this study, the specific siderophore receptor PupA of plant growth-promoting Pseudomonas putida WCS358 was used as a marker to monitor wild-type strain WCS358. After introduction into natural soil and rhizosphere environments, strain WCS358 could be recovered efficiently on a medium amended with 300 μM pseudobactin 358. Although low population densities of indigenous pseudomonads (≤107/g of soil or root) were recovered on the pseudobactin 358-amended medium, subsequent agglutination assays with a WCS358-specific polyclonal antisera enabled accurate monitoring of populations of wild-type strain WCS358 over a range of approximately 106 to 107 CFU/g of soil or root. Genetic analysis of the background population by PCR and Southern hybridization revealed that natural occurrence of the pupA gene was limited to a very small number of indigenous Pseudomonas spp. which are very closely related to P. putida WCS358. The PupA marker system enabled the study of differences in rhizosphere colonization among wild-type strain WCS358, rifampin-resistant derivative WCS358rr, and Tn5 mutant WCS358:zylE. Chromosomally mediated rifampin resistance did not affect the colonization ability of P. putida WCS358. However, Tn5 mutant WCS358:zylE colonized the radish rhizosphere significantly less than did its parental strain.

Fluorescent Pseudomonas spp. receive much attention because of possible environmental applications, including use in agri- and horticulture to improve crop production. Suppression of soilborne plant diseases by fluorescent pseudomonads has been demonstrated for several pathogen-host combinations, but results obtained under field conditions have been inconsistent (38, 44). Genetic engineering offers a means of recovering or combining specific properties of applied bacterial strains. One of the major applications of genetic engineering is the introduction of marker genes into fluorescent pseudomonads for monitoring of survival and root-colonizing ability. Specific markers are indispensable for distinguishing a strain of interest from the indigenous microflora. Moreover, a reliable marker has to be stable and should not affect ecologically important traits (2). Various markers have been described for monitoring of particular strains of Pseudomonas spp., like the xylE and lacZY genes and herbicide or antibiotic resistance (2, 12, 16, 17, 19, 35, 45). However, resistance to kanamycin and streptomycin among indigenous Pseudomonas populations hindered monitoring of Pseudomonas fluorescens WCS374 marked by a Tn5 transposon (3). Moreover, spontaneous chromosomally mediated antibiotic resistance can have adverse effects on the ecological competence of the host microorganism (2, 12). Since most traits involved in the rhizosphere competence of fluorescent pseudomonads are unknown, there are difficulties concerning the selection of characteristics of the genetically modified strains that should be compared with those of the parental strain. Therefore, specific markers are required for monitoring of both wild-type Pseudomonas strains and their genetically engineered derivatives in natural environments. Subsequently, differences in survival and rhizosphere colonization between genetically modified strains and their parental strain can be studied.

Analysis of differences in rhizosphere-colonizing competence between genetically modified Pseudomonas spp. and their wild type has usually been limited to sterile systems. Monitoring of wild-type Pseudomonas spp. under natural conditions has been restricted to analysis of cell envelope proteins and lipopolysaccharide (LPS) profiles, which is reliable but very laborious (14). Immunological techniques have specific problems, for example, relative insensitivity (35). Immunofluorescent colony staining, however, is very promising for monitoring of wild-type Pseudomonas spp. (28, 41). Nevertheless, for every strain a different and highly specific antisera is required.

In the present report, we describe a novel marker for monitoring of wild-type P. putida WCS358 (5, 38) in natural soil and rhizosphere environments. The marker system is based on the specificity of the ferric siderophore receptor of plant growth-promoting P. putida WCS358 (6, 8, 33). Biosynthesis of siderophores and their receptors is induced by iron limitation (11, 15, 31, 34, 36, 43). Siderophores are low-molecular-weight compounds that strongly bind iron(III) and facilitate its transport into the cell (37). Studies on the specificity of a group of these siderophores, designated pseudobactins or pyoverdines, revealed differences in the utilization spectra of different strains of Pseudomonas spp. (6, 11, 21). P. putida WCS358 produces a siderophore which consists of a dihydroxyquinoline chromophore linked to a linear nonapeptide (40). This siderophore, designated pseudobactin 358 (Ps358), is highly specific as it can be utilized by only 1% of over 105 pseudomonads randomly isolated from potato roots (6). In strain WCS358, ferric Ps358 transport is initiated by binding of this complex to a highly specific outer membrane protein, PupA (pseudobactin uptake protein A) (8). The structural gene of this 85-kDa

* Corresponding author. Mailing address: Section of Plant Pathology, Department of Plant Ecology and Evolutionary Biology, Utrecht University, P.O. Box 800854, 3508 TB Utrecht, The Netherlands. Phone: (030) 537438. Fax: (030) 518366.
PupA protein has been cloned and sequenced (8, 33). Mobilization of the pupA gene into plant growth-promoting P. fluorescens WCS374 enabled this strain to metabolize ferric Ps358 but not other ferric pseudobactins (8, 33).

In this study, PupA was used as a marker for reisolation and monitoring of wild-type P. putida WCS358 introduced into natural soil and rhizosphere environments. Therefore, a low-iron medium was amended with Ps358 to isolate wild-type strain WCS358 selectively. With this medium, rhizosphere colonization by wild-type WCS358 was compared with that of its rifampin-resistant derivative WCS358rr and Tn5 mutant WCS358:xylE. Moreover, this medium enabled us to study the distribution of PupA among naturally occurring Pseudomonas spp.

MATERIALS AND METHODS

Bacterial strains. Wild-type P. putida WCS358 and P. fluorescens WCS374 are plant growth-promoting and disease-suppressing rhizobacteria isolated from potato roots (5, 18, 38). WCS358rr is a spontaneous rifampin-resistant derivative of P. putida WCS358 (19). WCS358:xylE is resistant to tetracycline and harbors the structural genes for catechol-2,3-dioxygenase introduced on a modified Tn5 transposon as described by Kaniga and Davison (23). WCS374pMR is a derivative of strain WCS374 complemented with cosmid pMR, which encodes resistance to tetracycline, and the PupA receptor (8, 33). In strain WCS374pMR47, the introduced pupA gene is disrupted by a Tn5 transposon insertion (33). P. fluorescens B10 and Pseudomonas sp. strain E6 were kindly provided by M. N. Schroth (University of California, Berkeley), and their general properties have been described by Kleoppper and Schroth (25).

Growth media. All strains were cultured on King’s medium B (KB) (24). In soil and rhizosphere studies, population densities of indigenous Pseudomonas spp. were enumerated on KB agar supplemented with 100 μg of cycloheximide per ml, 40 μg of ampicillin per ml, and 13 μg of chloramphenicol per ml (KB+) (18). Soil and rhizosphere populations of wild-type WCS358, WCS358rr, and WCS358:xylE were enumerated on KB “Ps358, which is composed of KB” supplemented with 300 μM Ps358. The pH of KB “Ps358 was adjusted to 6.9 with 37% HCl.

Purification of Ps358. Ps358 preparations were isolated from culture supernatant of strain WCS358 grown in standard succinate medium for 72 h at 24°C (36). Ps358 was extracted from ammonium sulfate-saturated supernatant with phenol-chloroform (1:1, vol/vol), followed by column chromatography with PD-10 columns (Pharmacia) as described previously (40). The concentration of Ps358 was determined by its molar extinction coefficient of 14,000 M⁻¹ cm⁻¹ at 400 nm and pH 7.1 (39). The purity of extracted Ps358 was at least 95%. Crude Ps358 is cell-free lyophilized culture supernatant of strain WCS358 grown for 72 h at 24°C in standard succinate medium. Selectivity of KBPs358 medium in vitro studies. KB was amended with purified or crude Ps358 to a final concentration of 25, 50, 150, 300, or 400 μM. Purified Ps358 was (i) added prior to autoclaving (121°C, 20 min) or (ii) filter (0.2-μm pore size; Sartorius) sterilized after autoclaving of the growth medium. Crude Ps358 was added prior to autoclaving. Bacterial cultures were grown for 48 h on KB at 27°C, harvested in 0.1 M MgSO₄ · 7H₂O, and washed twice by centrifugation (4,000 × g, 5 min). Appropriate dilutions of suspensions of Pseudomonas spp. were plated (about 200 CFU per plate) on KB, KBPs358, and KBPs358 supplemented with Fe²⁺, as FeCl₂ · 6H₂O, equimolar to the concentration of Ps358. CFU were counted after 40 h of incubation at 27°C.

Recovery of P. putida WCS358 from soil. Wild-type WCS358 was introduced into nonsterile sandy soil (28) at four different densities of approximately 10³, 10⁴, 10⁵, and 10⁶ CFU/g of soil, respectively. In the control treatment, no bacteria were introduced. Each treatment consisted of three replicates. Directly after introduction into soil, suspensions were prepared by shaking 2 g of soil for 30 s in glass test tubes containing 9 ml of sterile 0.1 M MgSO₄ · 7H₂O and 2.5 g of glass beads (0.17-mm diameter). Recovery rates for WCS358 were determined on KB “Ps358.

Rhizosphere colonization studies. (i) Potatoes. Potato (Solanum tuberosum cv. Bintje) stem cuttings (4) with roots approximately 1 cm long were dipped into sterile 0.1 M MgSO₄ · 7H₂O (control) or into a suspension of WCS358 or WCS358rr in 0.1 M MgSO₄ · 7H₂O (10⁵ CFU/ml). Treated stem cuttings were planted in polyvinyl chloride tubes (16 cm high and 5 cm in diameter) containing a soil frequently used to grow potatoes (4, 5) and grown for 7 days under controlled conditions with a 16-h light period (irradiance, 40 μW m⁻²) at 21°C and 70% relative humidity followed by an 8-h dark period at 16°C and 85% relative humidity. Each treatment consisted of 10 replicates. Rhizosphere suspensions were prepared from root segments (0.3 to 0.5 g) sampled at different depths from the stem base. Therefore, polyvinyl chloride tubes were carefully emptied and root segments present in soil sections at depths of 0 to 2, 2 to 6, and 6 to 10 from the stem base were selected at random. Excess soil particles were removed gently with a soft artist’s brush. These segments were suspended in 5 ml of sterile 0.1 M MgSO₄ · 7H₂O and shaken vigorously for 30 s in glass test tubes containing 2.5 g of glass beads (0.17-mm diameter). Aliquots of the same suspension were dilution plated (CU Spiral Plater; Spiral Systems, Inc.) on selective media. Enumeration was done after 40 h of incubation at 27°C.

(ii) Radishes. For rhizosphere studies with radish (Raphanus sativus cv. Saxa Nova) plants, seeds were surface disinfected with 0.5% NaHClO (5 min), rinsed three times with sterile distilled water, and coated with 1% methyl cellulose (control) or with a suspension of WCS358, WCS358rr, or WCS358:xylE in 1% methyl cellulose (10⁵ CFU/ml). The coated seeds were air dried for 24 h at room temperature. The final density of the applied bacterial strain was approximately 10⁶ CFU per seed. Two radish seeds were planted in a polyvinyl chloride tube containing nonsterile sandy soil collected from a commercial greenhouse (28). Each treatment consisted of 10 replicates. Radish plants were grown for 10 days under controlled conditions as described previously. Segments (0.3 to 0.5 g) of the taproot and lateral roots were selected at random from a depth of 0 to 8 cm from the stem base as described previously. Rhizosphere suspensions were prepared as described previously and dilution plated on selective media. Enumeration was done after 40 h of incubation at 27°C.

Agglutination assay. Immunoglobulin was prepared against whole cells of P. putida WCS358 in a rabbit at the Research Institute for Plant Protection, Wageningen, The Netherlands, as described by Vrugink and Maas-Geeesterman (42) and was kindly provided by J. van de Wolf and J. W. van Vuure. Agglutination of randomly isolated colonies with polyclonal antiserum Ab9059 (dilution, 1:400 in 10 mM phosphate buffer [pH 7.0]) was tested on 10-well multistest slides (Flow Laboratories).

EDDA assay. Ps358 utilization by Pseudomonas spp. was determined in a plate bioassay based on reversal of iron starvation induced by EDDA [ethylenediaminedi(o-hydroxyphenylacetic acid)] (11). Bacterial suspensions were added to
molten KB agar (40°C) at a concentration of 1,000 CFU/ml and sufficient EDTA to inhibit growth completely. The concentration of EDTA in KB agar plates was 1 mg ml\(^{-1}\) for WCS358 and varied for indigenous pseudomonads between 0.5 and 2 mg ml\(^{-1}\). After solidification in petri dishes, filter paper discs containing 4 μl of Ps358 (100 μM) were placed on the surface of the agar. The plates were incubated at 27°C and examined for bacterial growth after 24 h.

**Southern hybridization and PCR.** Total DNAs were isolated from *Pseudomonas* strains by Sarkosyl-pronase lysis as described previously (7). Digestion with restriction enzymes, agarose gel electrophoresis, and Southern hybridization were performed as described by Maniatis et al. (32). For Southern hybridization, a 1.7-kb fragment of the *pupA* structural gene was excised from plasmid pPU1 (8) by EcoRV digestion and labelled with [α-\(^{32}\)P]dCTP by nick translation (32). Southern hybridization was performed under the following low-stringency conditions: prehybridization for 1.5 h at 50°C, hybridization for 12 h at 50°C, washing twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)—0.1% sodium dodecyl sulfate (20 min, 20°C), and washing once with 1× SSC—0.1% sodium dodecyl sulfate (30 min, 50°C) (32). For PCR analysis, two-mer oligonucleotides (sequences: 5’-CAACGTGGATTGCAGCGTTCG-3’ and 5’-GAGTCCAGTAAACCTCCACC-3’) homologous to specific regions within the *pupA* structural gene (8, 9, 27) were synthesized and used as primers. PCR was performed in a total volume of 50 μl containing 5 ng of genomic DNA, 1 U of Taq polymerase (Sphaero Q) 100 μM dNTPs, 200 μM of each primer per ml. Amplification was performed with 30 cycles of 94°C for 1.5 min for melting, 57°C for 1 min for annealing and 72°C for 2 min for primer extension.

**Statistical analysis.** Populations of *Pseudomonas* spp., introduced on seed or planting material, approximate a logarithmic distribution along growing roots, with the highest numbers near the inoculum source and decreasing numbers towards the root tips (30). Therefore, enumerated values were submitted to logarithmic transformation prior to statistical analysis. Transformed values were analyzed by analysis of variance followed by Student’s t test. Data obtained in recovery studies were analyzed by linear regression analysis (SAS Institute Inc., Cary, N.C.). Experiments were performed at least twice.

## RESULTS

**Selectivity of KPBs358 medium in vitro studies.** In *P. putida* WCS358, incorporation of iron complexed to Ps358 is initiated by binding of this complex to the highly specific receptor PupA (8). Addition of different concentrations of Ps358 to a low-iron medium (KB) did not affect the growth (expressed as the number of colonies per plate) of strains with a functional *pupA* gene (WCS358, WCS358rr, WCS358::xyE, and WCS374pMR), except at the highest concentration (400 μM) of Ps358 (Table 1). Disruption of the *pupA* gene in derivative strain WCS374pMR47 inhibited growth completely at Ps358 concentrations of 50 μM and higher, the same inhibitory concentrations as observed for strains WCS374, E6, and B10 (Table 1). No differences in selectivity were observed between additions of purified or crude Ps358. Moreover, autoclaving of Ps358 did not affect the selectivity of KPBs358, which implies heat stability of this siderophore (Table 1). The selectivity was completely suppressed by addition of Fe\(^{3+}\) equimolar to the concentrations of Ps358 (Table 1).

**Recovery of *P. putida* WCS358 from soil.** From noninoculated natural soil, an indigenous *Pseudomonas* population of approximately \(10^6\) CFU/g was recovered on KB+Ps358, which accounts for 1.6% of the total indigenous population recovered on KB\(^+\). From soil treated with WCS358, the numbers of pseudomonads recovered on KB+Ps358 were correlated linearly with the numbers of strain WCS358 organisms introduced initially into the soil (Fig. 1). Subsequent serological identification of colonies randomly isolated from KB+Ps358 was used to determine the recovery rates for wild-type WCS358. Agglutination assays revealed that the WCS358 antiserum was very specific, as only 2.8% of the background population cross-reacted (Table 2). Consequently, recovery rates for wild-type WCS358 on KB+Ps358 approximated 100% for population densities of \(10^4\) CFU/g of soil or higher. For

<table>
<thead>
<tr>
<th>Strain</th>
<th>Filter sterilized</th>
<th>Autoclaved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>Crude</td>
</tr>
<tr>
<td></td>
<td>Fe(^{3+}) added</td>
<td>Fe(^{3+}) added</td>
</tr>
<tr>
<td>WCS358</td>
<td>400</td>
<td>&gt;400</td>
</tr>
<tr>
<td>WCS358rr</td>
<td>400</td>
<td>&gt;400</td>
</tr>
<tr>
<td>WCS358::xyE</td>
<td>400</td>
<td>&gt;400</td>
</tr>
<tr>
<td>E6</td>
<td>50</td>
<td>&gt;400</td>
</tr>
<tr>
<td>B10</td>
<td>50</td>
<td>&gt;400</td>
</tr>
<tr>
<td>WCS374</td>
<td>50</td>
<td>&gt;400</td>
</tr>
<tr>
<td>WCS374pMR</td>
<td>400</td>
<td>&gt;400</td>
</tr>
<tr>
<td>WCS374pMR47</td>
<td>50</td>
<td>&gt;400</td>
</tr>
</tbody>
</table>

*a* MICs represent the minimal concentrations of Ps358 added to KB which inhibited growth of \(10^2\) to \(10^6\) bacteria per plate completely. Purified Ps358 was added to the medium prior to autoclaving or after filter sterilization. Crude Ps358 was added to the medium prior to autoclaving, Fe\(^{3+}\) equimolar to the concentration of Ps358 was added. Concentrations of Ps358 tested were 25, 50, 150, 300, and 400 μM.

**Number of bacteria per plate reduced from 200 to an average of 100 to 50.**

![FIG. 1. Recovery of wild-type *P. putida* WCS358 from natural sandy soil on KB+Ps358 medium.](http://aem.asm.org/Downloaded from http://aem.asm.org/ on October 29, 2017 by guest)
TABLE 2. Percentage of pseudomonads reisolated from soil on KB+ Ps358 which reacted positively in an agglutination assay with a polyclonal antiserum directed against P. putida WCS358

<table>
<thead>
<tr>
<th>Initial density of wild-type WCS358 (CFU/g)</th>
<th>Positive agglutination with WCS358 antiserum (%)</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.8</td>
<td>36</td>
</tr>
<tr>
<td>$3 \times 10^3$</td>
<td>88</td>
<td>50</td>
</tr>
<tr>
<td>$3 \times 10^4$</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>$3 \times 10^5$</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>$3 \times 10^6$</td>
<td>100</td>
<td>50</td>
</tr>
</tbody>
</table>

n*, number of KB+ Ps358-grown colonies tested in agglutination assays.

recovery of a WCS358 population density of $3 \times 10^3$ CFU/g, use of KB+Ps358 alone overestimated the actual population density of wild-type WCS358, as 88% of the colonies were serologically identified as WCS358 (Table 2).

Rhizosphere colonization studies. (i) Potatoes. From the control treatment, the numbers of pseudomonads recovered on KB+Ps358 were approximately $5 \times 10^5$ CFU/g of root for all depths (Fig. 2), which accounts for 0.6% of the total indigenous Pseudomonas population recovered on KB+. This background population density was very low compared with the number of pseudomonads recovered on KB+Ps358 from roots treated with WCS358, except for a depth of 6 to 10 cm (Fig. 2). Subsequent agglutination assays revealed that all of the colonies recovered on KB+Ps358 from roots treated with WCS358 or WCS358rr reacted positively with a WCS358-specific antiserum, whereas no reaction was observed in the control treatment at this depth (data not shown). These results clearly demonstrate the usefulness of KB+Ps358 for monitoring of wild-type WCS358 in rhizosphere environments. Comparison between wild-type strain WCS358 and its derivative WCS358rr revealed no difference in colonization of the potato rhizosphere (Fig. 2). Both strains were lognormally distributed along the root system (Fig. 2).

(ii) Radishes. From the control treatment, an indigenous Pseudomonas population of approximately $3 \times 10^2$ CFU/g of root was recovered on KB+Ps358 (Fig. 3), which accounts for 0.02% of the total indigenous Pseudomonas population recovered on KB+. Again, this background population density was very low compared with the numbers of pseudomonads recovered on KB+Ps358 from radish plants treated with WCS358 (Fig. 3). Moreover, subsequent agglutination assays revealed that 100% of the colonies recovered on KB+Ps358 from roots treated with WCS358 reacted positively with a WCS358-specific antiserum, whereas only 13.3% of the background population cross-reacted. Comparison between wild-type WCS358 and WCS358rr revealed no significant population density differences in the radish rhizosphere, but Tn5 mutant WCS358::xyIE established significantly lower population densities than parental strain WCS358 (Fig. 3).

Distribution of PupA among indigenous Pseudomonas spp. Growth of small populations of indigenous Pseudomonas spp. on KB+Ps358 enabled us to study the distribution of PupA among naturally occurring rhizosphere pseudomonads. Therefore, pseudomonads indigenous to the potato rhizosphere (depth, 0 to 6 cm) and the radish rhizosphere (depth, 0 to 8 cm) were recovered on KB+Ps358 as described previously. Approximately 30 colonies, randomly selected from both the potato and radish rhizospheres, were purified and cultured on KB. Surprisingly, subsequent EDDA assays revealed that 20 to 30% of the selected isolates were not able to utilize ferric Ps358. However, most isolates (70 to 80%) were able to utilize ferric Ps358 and were used for further studies. By analysis of their LPS profiles (14), these isolates from the potato and radish rhizospheres were clustered into five and eight different groups, designated PTR1 and RDR1 groups, respectively (data not shown). Isolates of LPS groups PTR1 and RDR1 have an LPS pattern identical to that of strain WCS358. From all 13 LPS groups, one representative isolate was used for PCR analysis and Southern hybridization.
Because of the structural diversity among pseudobactin siderophores of fluorescent Pseudomonas spp., most strains can metabolize iron only via a limited number of these compounds (6, 11). P. putida WCS358 is remarkable in this respect, as it exploits a large variety of heterologous siderophores (6), whereas its own siderophore, designated Ps358, can be utilized by only a very small number of indigenous Pseudomonas spp. (6). In P. putida WCS358, ferric Ps358 transport is initiated by binding of this complex to a highly specific outer membrane protein designated PupA (8). In the present study, the siderophore receptor PupA was tested for usefulness as a marker for monitoring of wild-type strain WCS358 introduced into natural soil and rhizosphere environments. Although various markers have been described for strains of Pseudomonas spp. (16, 17, 20, 35, 45), detection of wild-type strains is usually limited to sterile systems. In view of recent developments in genetic engineering, specific markers for monitoring of wild-type strains of Pseudomonas spp. in natural environments are needed to study possible differences in survival and rhizosphere competence between genetically modified pseudomonads and their parental strains. To develop such a detection method for wild-type WCS358, a low-iron medium was amended with 300 μM Ps358. In vitro studies clearly demonstrated that this medium, designated KBPs358, is highly selective for strains harboring a functional PupA receptor. The selectivity of KBPs358 is due to iron deprivation, as it could be overcome by addition of Fe³⁺ (Table 1). Surprisingly, autoclaving of Ps358 did not affect the selectivity of KBPs358, which implies heat stability of this siderophore (Table 1). Heat stability has also been demonstrated for ferrioxamine B (9) and for siderophores used by Salmonella typhimurium (1).

The use of PupA as a marker for monitoring of wild-type strain WCS358 introduced into natural environment was tested for both soil and plant rhizospheres and depends on the selectivity of KB⁺ Ps358 medium. Low population densities of indigenous Pseudomonas spp. of approximately 10⁵ CFU/g of soil or root were recovered on KB⁺ Ps358 (Fig. 2 and 3) and accounted for only 0.02 to 0.6% of the total indigenous Pseudomonas population recovered on KB⁺. This background level of indigenous Pseudomonas spp. is equal to or even lower than the ones described for resistance to rifampin or nalidixic acid (10⁵ to 10⁶ CFU/g of soil) and for lacZY (16, 20). For most of the indigenous isolates selected on KB⁺ Ps358, subsequent EDDA assays (11) confirmed their ability to utilize ferric Ps358. However, 20 to 30% of the selected isolates were not able to utilize ferric Ps358. It is assumed that utilization of metabolites excreted by other colonies initiated growth of these isolates, as suggested by Jurkevitch et al. (22). Surprisingly, subsequent genetic analysis revealed that only two groups of isolates, PTR1 and RDR1, harbored the pupA gene.
(Fig. 4 and 5). Moreover, their LPS profile is identical to that of *P. putida* WCS358 and they reacted positively in an agglutination assay with a WCS358-specific antiserum. These results strongly indicate that these two specific groups are very closely related to *P. putida* WCS358. The other selected indigenous isolates apparently incorporate iron complexed to P358 by other receptors which have a very low degree of sequence homology with the *pupA* gene. Disparity between sequence homology and substrate specificity has also been demonstrated for receptors of *Escherichia coli* and *Yersinia enterocolitica* (26).

Despite the background levels of indigenous *Pseudomonas* spp., the efficiency of recovery of wild-type strain WCS358 on KB*+P*358 from natural soil is close to 100% for densities of 10^3 CFU/g and higher (Fig. 1 and Table 2). Background populations recovered on KB*+P*358 from rhizosphere environments were negligible compared with population densities of wild-type WCS358 (Fig. 2 and 3). However, for monitoring of low population densities of wild-type WCS358 (10^2 CFU/g) in soil and rhizospheres, the use of *PupA* as a marker is limited because of the background population (Table 2 and Fig. 2). Subsequent agglutination assays with a WCS358-specific antiserum, however, enabled accurate monitoring of small populations of wild-type strain WCS358 in soil and rhizospheres. Since a large number of bacterial isolates has to be screened in agglutination assays, future research will focus on the combination of KB*+P*358 with immunofluorescence colony staining (28, 41) for easier monitoring of small populations of wild-type strain WCS358.

By using *PupA* as a marker, rhizosphere colonization by wild-type WCS358 was compared with that of its rifampin-resistant derivative WCS358rtrn and Tn5 mutant WCS358:xyIE. No differences in the ability to colonize both potato and radish rhizospheres were observed between wild-type strain WCS358 and its rifampin-resistant derivative (Fig. 2 and 3). Changes in ecologically important traits due to introduced antibiotic resistance and Tn5 mutagenesis have been reported for *Pseudomonas* spp. (2, 12, 13) and *Rhizobium* spp. (10, 29). These studies demonstrated that chromosomally mediated rifampin resistance does not affect the colonizing ability of strain WCS358, confirming the results of Glandorf et al. (19). However, Tn5 mutant WCS358:xyIE colonized the radish rhizosphere significantly less well than did its parental strain (Fig. 3). Because the site of integration of the Tn5 transposon is unknown, it remains speculative whether the Tn5 transposon itself is the cause of the reduced rhizosphere-colonizing competence of WCS358:xyIE.

In conclusion, it can be stated that the specific siderophore receptor *PupA* enabled monitoring of a wild-type *Pseudomonas* sp. in natural soil and rhizosphere environments. Because of the structural diversity among pseudobactins produced by fluorescent pseudomonads, receptors generally exhibit a high degree of specificity (21). Dependent on their distribution among indigenous bacteria, these receptors may be exploited to monitor wild-type strains of other fluorescent *Pseudomonas* spp.

**ACKNOWLEDGMENT**

This work was supported by a Biotechnology Action Programme from the European Communities (BAP-0406).

**REFERENCES**


