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Molecular Studies on the Role of a Root Surface Agglutinin in Adherence and Colonization by *Pseudomonas putida*†

ANNE J. ANDERSON,* POURAN HABIBZADEGAH-TARI, AND CRAIG S. TEPPER
Department of Biology, Utah State University, Logan, Utah 84322-4500

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***Pseudomonas putida* aggressively colonizes root surfaces and is agglutinated by a root surface glycoprotein. Mutants of *P. putida* derived chemically or by Tn*5* insertion demonstrated enhanced or decreased agglutinability. Two nonagglutinable Tn*5* mutants (Agg$^-$) and two mutants with enhanced agglutinability (Agg^s) possessed Tn*5* in unique restriction sites. Agg$^-$ mutants colonized root surfaces of seedlings grown from inoculated seeds, but at levels lower than those observed with the Agg$^+$ parent. In short-term binding studies, Agg$^-$ cells adhered at levels that were 20- to 30-fold less than those for Agg$^+$ parental cells. These data suggest that the agglutination interaction plays a role in the attachment of *P. putida* to root surfaces.**

Pseudomonas putida is a soil bacterium that is associated with increased plant growth and suppression of certain fungi that are root pathogens (17, 18, 21, 24, 26). The bacterium aggressively colonizes root surfaces by mechanisms that are unresolved. In previous studies (3, 5, 14) it has been demonstrated that cells of *P. putida* are precipitated by a glycoprotein, termed an agglutinin, which has been isolated from root surfaces. Consequently, the study described here was initiated to determine whether the recognition between the bacterium and the plant surface agglutinin is important in colonization. We report the isolation of mutants that are altered in agglutination phenotype and compare the colonization potentials of the mutants with that of the parental *P. putida* isolate.

MATERIALS AND METHODS

Isolation of mutants. *P. putida* (Trevisan) Migula isolate Corvallis was selected for resistance to nalidixic acid (Nal^r) and rifampin (Rif^r) as described previously (4). Mutants of *P. putida* (Nal^r Rif^r) were generated by treatment with ethyl methanesulfonate (EMS) or by transposon mutagenesis. Chemical mutagenesis with EMS was done by a previously described procedure (8). An effective killing rate of 85 to 93% was obtained. Selection of agglutinin-deficient (Agg$^-$) mutants involved the removal of agglutinable isolates from the mutagenized population by precipitation with heat-sterilized root agglutinin (3). The bacteria that remained in suspension were grown on minimal medium, and single colonies were isolated and screened for agglutinability.

Transposon mutagenesis involved Tn*5* insertion with the suicide vector system pSUP2021 (Neo^r), which was kindly provided by A. Pühler (23). Cells of *P. putida* (Nal^r Rif^r) and *Escherichia coli* SM10(pSUP2021) (Neo^r), both of which were grown in rich medium (5) to the mid-log phase, were obtained by centrifugation at 10,000 × *g* for 5 min. The cells were suspended to 5×10^{10} CFU/ml. Portions (0.1 ml) of *P. putida* (Nal^r Rif^r) were mixed with 0.1 ml of SM10 (pSUP2021) and transferred to nitrocellulose filters (0.22-μm pore size) which were overlaid on water agar medium. Controls of either SM10(pSUP2021) or *P. putida* (Nal^r Rif^r) alone were prepared in the same manner. After 24 h of incubation at 20°C, cells were suspended in 200 μl of sterile water, and 50-μl portions were plated onto Kings B medium containing 50 μg each of nalidixic acid, rifampin, and neomycin per ml. Recombinants that demonstrated characteristic pseudomonad fluorescence were selected and subjected twice to single-colony isolation. The ability of the recombinants to grow on minimal medium containing the three selective antibiotics was determined on solid medium and in liquid culture. Recombinants that grew on minimal medium were assayed for their agglutinability, sensitivity to bacteriophage, and production of 3-indoleacetic acid (IAA)-like components.

The agglutination assay was done by previously described procedures (3). Agglutinin was obtained from root washes of greenhouse-grown dark-red kidney beans (*Phaseolus vulgaris*; Idaho Seed Bean Co., Twin Falls, Idaho), which were prepared as described by Anderson (3). The root wash was fractionated by passage through Sepharose 6B (Sigma Chemical Co., St. Louis, Mo.) and eluted with water. The void fractions, which contained agglutinin activity, were serially diluted to assess bacterial agglutinability.

Sensitivity to phage was screened by following the procedures described by Anderson (2). Phage specific for *P. putida* (phage 12633-B1) was obtained from the American Type Culture Collection (Rockville, Md.). Mutants of *P. putida* (Nal^r Rif^r) that were resistant to phage were isolated by spontaneous events by the procedure described by Anderson (2).

The production of IAA-like compounds was determined by growing the bacterium on minimal medium containing 10 mg of tryptophan per 100 ml for 48 h. The culture filtrate (100 ml) was concentrated to dryness by lyophilization and suspended in 5 ml of sterile water. The reconstituted solution was tested for IAA-like compounds by use of the Salkowski reagent (11).

Colonization assay. The ability of the bacterium to colonize plant roots was determined by inoculation of dark-red kidney beans. Bean seed was surface sterilized and transferred to 20 ml of sterilized moist vermiculite in a tube (1). Under aseptic conditions, an inoculum of 2×10^3 cells of the *P. putida* isolates that were to be tested was applied. The cells were grown on rich medium (5) and washed twice with sterile distilled water prior to use. Beans were incubated at 26°C for 6 days under constant light, and 3 ml of sterile water was added after 3 days. Plants were harvested and the roots

* Corresponding author.
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were assayed for colonization by using qualitative and quantitative procedures described previously (4). The qualitative assay involved transfer of the root to the surface of an agar plate containing Kings B medium supplemented with nalidixic acid, rifampin, and, where appropriate, neomycin. The plates were examined for bacterial growth after 3 days of incubation at 22°C. The quantitative assay was modified by performing a sequential transfer of the intact root under sterile conditions through 10 washes with water (10 ml for each wash) followed by 1 wash with 10 ml of 5 mM sodium EDTA (pH 6.5). After each transfer the tube was agitated on a Vortex mixer for 60 s. Immediately after the wash with sodium EDTA, 0.3 ml of 1.0 M $MgCl_2$ was added to the tube. In some studies, 5 mM NaCl or 5 mM sodium acetate (pH 6.5) was used to replace EDTA. Portions (0.1 ml) of the washes were diluted and plated onto Kings B medium containing the appropriate antibiotics. Colonies were counted after 3 days of incubation at 22°C. After EDTA treatment, the root was ground in 10 ml of 50 mM potassium phosphate (pH 7.0) and the CFU per milliliter was determined.

Bacterial adherence assay. Roots of intact 8-day-old plants grown under sterile conditions were immersed for 15 min in 30 ml of a suspension containing 10^6 bacteria per ml at 26°C with agitation at 125 rpm. The plants were removed and gently shaken, and the roots were agitated by vortexing them in 10 successive washes of 10 ml of sterile water followed by 10 ml of 5 mM sodium EDTA (pH 6.5). $MgCl_2$ (0.3 ml of a 1.0 M solution) was added immediately after the wash with sodium EDTA. Portions (0.1 ml) were removed from each wash and spread onto a selective medium (Kings B medium containing the antibiotics nalidixic acid, rifampin, and neomycin, as appropriate). The plates were grown at 22°C, and the colonies were scored after 5 days.

Genetic analysis of Tn*5* mutants. *E. coli* SM10(pSUP2021) was maintained on Luria broth (20) solidified with 1.5% agar that contained ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), and neomycin (50 μg/ml). Plasmid DNA was isolated by the cleared-lysate technique (16) from cells of *E. coli* SM10 and *P. putida* (Nal^r Rif^r) following amplification with chloramphenicol and growth in Luria broth. Total DNA was isolated from *P. putida* isolates by the procedure described by Comai and Kosuge (7). The DNA was purified twice by equilibrium centrifugation in cesium chloride-ethidium bromide (20). The ethidium bromide was removed and the DNA was precipitated before the DNA was stored at −20°C (20).

The DNA was digested with *Eco*RI, *Hpa*I, and *Bam*HI, which were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Portions (1 μg) of DNA were digested with 3 to 5 U of restriction enzyme under conditions appropriate for each nuclease.

Plasmid and genomic DNAs were separated by electrophoresis through a horizontal slab gel of 0.7% agarose containing 0.5 μg of ethidium bromide per ml in Tris acetate buffer (40 mM Tris, 10 mM sodium acetate, 1 mM EDTA; pH 7.8) at approximately 1.5 V/cm (20). Plasmid pSUP2021 (0.1 ng) was linearized with *Bam*HI to yield a fragment of 13.6 kilobases (kb) or digested with *Hpa*I to produce two fragments of 5.5 and 8.1 kb. The digested plasmid fragments were used as molecular weight markers for Southern hybridization.

Southern hybridization. Purified pSUP2021 plasmid (15 μg) was digested with *Hpa*I, yielding a 5.5-kb Tn*5* probe. This probe was labeled to 1×10^8 to 2×10^8 cpm/μg with [^{32}P]dCTP and [^{32}P]dGTP (800 Ci/mmol) by using a nick-translation kit purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Genomic digests of *P. putida* isolates were transferred from the agarose gel onto GeneScreen Plus (New England Nuclear Corp., Boston, Mass.). Hybridization with the ^{32}P-labeled Tn*5* probe involved incubation in 50% formamide at 42°C for 16 to 20 h. The blots were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C (20). Autoradiography was performed with X-Omat X-ray film (Eastman Kodak Co., Rochester, N.Y.) by using an intensifying screen (Cronex extra life; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

RESULTS

Isolation of mutants altered in agglutination phenotypes. Recombinants that were neomycin resistant were obtained from matings of *P. putida* (Nal^r Rif^r) with *E. coli* SM10 (pSUP2021) (Neo^r) at a frequency of 10^{-7} per recipient cell. No mutants of SM10(pSUP2021) were obtained that would grow on nalidixic acid-rifampin, although neomycin-resistant isolates of *P. putida* were obtained at a frequency of 10^{-10}. Mutants that were unable to grow on minimal medium were obtained in the recombinant population at a frequency of 9 ± 3%. Mutants that grew well on minimal medium and displayed an altered agglutination phenotype were obtained at a frequency of about 0.5%. Certain mutants (3713 and 3923; Agg^s) were agglutinated to a greater degree than the parent (Table 1). Other mutants had various degrees of reduced agglutinability (2521; Agg^i) (Table 1), and some totally lacked the agglutination phenotype (4312 and 5123; Agg$^-$) (Table 1). Six mutants, including mutant 2612, that were originally isolated with an Agg$^-$ phenotype reverted to an Agg$^+$ phenotype after routine transfer. Both Tn*5* Agg$^-$ mutants (4312 and 5123; Table 1) were stable for at least six

TABLE 1. Agglutination of parental *P. putida* (Nal^r Rif^r) and derived mutants by agglutinin isolated from root washes[a]

Isolate[b]	Extent of agglutination with the following amt (U) of agglutinin[c]:			
	100	10	1	0.1
P. putida (Nal^r Rif^r)	+4	+3	+1	−
EMS-derived mutants				
1202 (Agg$^-$)	−	−	−	−
1229 (Agg$^-$)	−	−	−	−
1236 (Agg$^-$)	−	−	−	−
1240 (Agg^i)	+3	+2	+1	−
1213 (Agg^s)	+4	+4	+2	+1
Tn*5*-derived mutants (Nal^r Rif^r Neo^r)				
4312 (Agg$^-$)	−	−	−	−
5123 (Agg$^-$)	−	−	−	−
2521 (Agg^i)	+3	+3	+1	−
3713 (Agg^s)	+4	+4	+1	−
3923 (Agg^s)	+4	+4	+1	−

[a] Mutants were obtained from the parental isolate *P. putida* (Nal^r Rif^r) by EMS or transposon mutagenesis. Agglutination assays were performed with 10^9 cells grown to the log phase in a volume of 1.0 ml that contained agglutinin from bean root washes (3). The agglutinin preparation was serially diluted and initially contained 1,000 U of agglutinin per ml, 0.35 mg of protein per ml, and 1.6 mg of carbohydrate per ml.

[b] Agg$^-$ represents nonagglutinable mutants, Agg^i represents mutants with intermediate agglutinability, and Agg^s represents mutants with enhanced agglutinability.

[c] The extent of agglutination is represented on a scale of +4 for full precipitation of bacteria to − for no agglutination.

monthly transfers onto Kings B medium containing nalidixic acid, rifampin, and neomycin.

Agg^- mutants were obtained by treatment with EMS but at a higher frequency (10%), perhaps because of preselection by using agglutinin precipitation of the wild-type phenotypes (Table 1). Agg^s and Agg^i mutants were also obtained after treatment with EMS (Table 1). The Agg^- EMS-derived mutants were stable during 9 months of regular transfers on Kings B medium containing nalidixic acid and rifampin.

Several characteristics that could be important in the survival of the bacteria in the rhizosphere were compared by using mutants and parental isolates. The generation times of the Agg^s and Agg^- mutants on minimal medium were equivalent to that of the parental isolate. The generation time in liquid culture was 85 to 90 min at 22°C. Each mutant secreted a compound that produced a typical IAA reaction with the Salkowski reagent (11) at levels (100 $\mu g/10^{12}$ CFU) which were comparable to those for the wild type. Production of the compound was dependent on the presence of tryptophan in the medium. Each of the EMS- and Tn*5*-derived mutants retained sensitivity to lysis by phage specific for *P. putida*. Twenty mutants of the parental *P. putida* (Nal^r Rif^r) that were selected for resistance to this phage were agglutinable at wild-type levels.

Genetic analysis of Tn*5* mutants. The cleared-lysate technique for plasmid isolation (16) did not reveal the presence of plasmid bands in CsCl-ethidium bromide gradients of the parental isolate or the Tn*5*-derived mutants. Southern hybridization of ^{32}P-labeled Tn*5* probes to *Bam*HI, *Hpa*I, and *Eco*RI digests of parental genomic DNA did not demonstrate any homology to the probe. Hybridization of the Tn*5* probe to similar genomic digests of the Tn*5*-derived mutants revealed the presence of the Tn*5* element. A single insertion of Tn*5* should yield one labeled fragment from an *Eco*RI genomic digest but two labeled fragments from a *Bam*HI genomic digest. This hybridization pattern is predicted because Tn*5* possesses one internal restriction site for *Bam*HI but no sites for *Eco*RI (15). We observed single bands that appeared to have distinct mobilities for the *Eco*RI genomic digest of two of the Agg^s (3713 and 3923) and one of the Agg^- (5123) mutants that were examined (Fig. 1). Two bands were observed in the *Bam*HI genomic digests for one Agg^- (5123) and two Agg^s (3713 and 3923) mutants (data not shown).

Genetic analysis was also performed with mutant 2612 that was isolated with an Agg^- Neo^r phenotype but that had reverted to an Agg^+ Neo^s phenotype after it was subcultured. Southern hybridization of the Tn*5* probe to an *Eco*RI digest of the DNA from the original mutant revealed a single band of hybridization. No bands of hybridization were found after Southern analysis of the Agg^+ revertant 2612.

The presence of other weak bands of hybridization in the *Eco*RI and *Bam*HI genomic digests of the Agg^- mutant 4312 (Fig. 1) indicated a secondary transposition event for all or part of Tn*5*. Independent transposition of IS*50*, which is the terminal inverted repeat of Tn*5*, has been reported in studies of Tn*5* mutagenesis of *Pseudomonas solanacearum* (6, 25). The possible presence of additional IS*50* elements was investigated by using a 5.5-kb probe that was released from pSUP2021 by *Hpa*I digestion, to detect homology with genomic fragments of Tn*5*-derived mutants that were also obtained by *Hpa*I digestion. The *Hpa*I endonuclease was selected because it cuts at the ends of the IS*50* terminal repeats (15). This analysis should produce a single hybridization band of 5.5 kb, corresponding to genomic fragments that contain the complete Tn*5* element. Additional hybridization bands would indicate the presence of fragments that

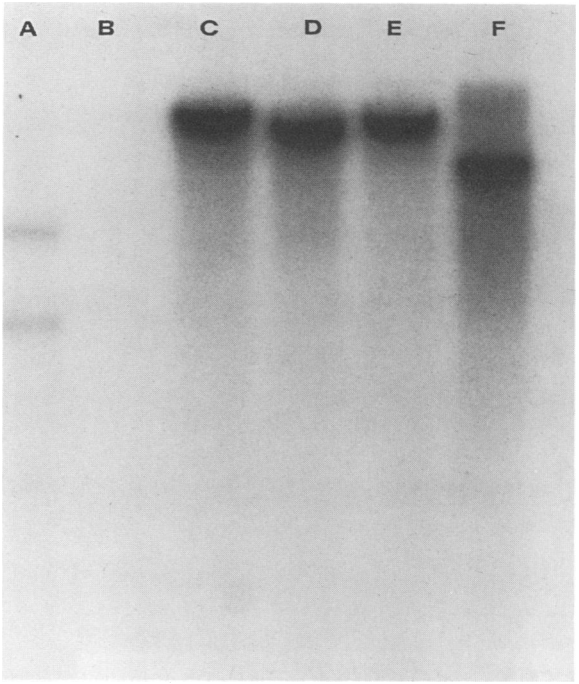

FIG. 1. *Eco*RI-digested total genomic DNA from parental and Tn*5*-derived mutants of *P. putida* hybridized to a ^{32}P-labeled *Hpa*I Tn*5* fragment that was digested from pSUP2021. Lane A, *Hpa*I-digested pSUP2021 DNA yielding a 5.5-kb (lower) band and an 8.1-kb (upper) band; lane B, parental isolate; lane C, mutant 3923 (Agg^s); lane D, mutant 3713 (Agg^s); lane E, mutant 5123 (Agg^-); lane F, mutant 4312 (Agg^-).

contain only IS*50* inserts. Analysis of Agg^- mutant 5123 and Agg^s mutants 3713 and 3923 revealed only a single hybridization band of 5.5 kb (Fig. 2). Mutant 4312 possessed a hybridization band of this molecular size but also possessed two other bands with larger sizes (Fig. 2). Consequently, insertion of Tn*5* and independent transposition of IS*50* elements appear to have occurred in Agg^- mutant 4312. The differences in the intensity of hybridization for the Tn*5* and IS*50* bands (Fig. 2, lane A) might be due to a lower degree of hybridization between the IS*50* element and the probe rather than to the complete Tn*5* structure.

Bacterial colonization of bean roots. Velvet transfer of root imprints of plants inoculated with the parental isolate of *P. putida* (Nal^r Rif^r) demonstrated colonization of the entire root surface. Plate cultures of water washes of roots indicated that the bacterium could be removed from the root surface. The initial wash showed colonization of the root surface at a level of 1×10^6 to 5×10^6 CFU/g of root, and the recovery of bacteria decreased with successive washes. A significant increase in bacterial release was observed on transfer of the root to the EDTA wash (Table 2). No increase in release was obtained by transfer of the root to water, 5 mM NaCl, or 5 mM sodium acetate (Table 2). Bacteria still adhered to the root after treatment with EDTA. Cells were released by grinding the root in buffer at levels which were comparable to those observed in the EDTA wash (Table 3).

The Tn*5*-derived Agg^- and EMS-derived Agg^- mutants also colonized bean roots. The first water washes from the roots demonstrated that the EMS-derived Agg^- mutants colonized the roots at a lower level than did the parental isolate (Table 3). Cells of the Tn*5*-derived Agg^- mutants also

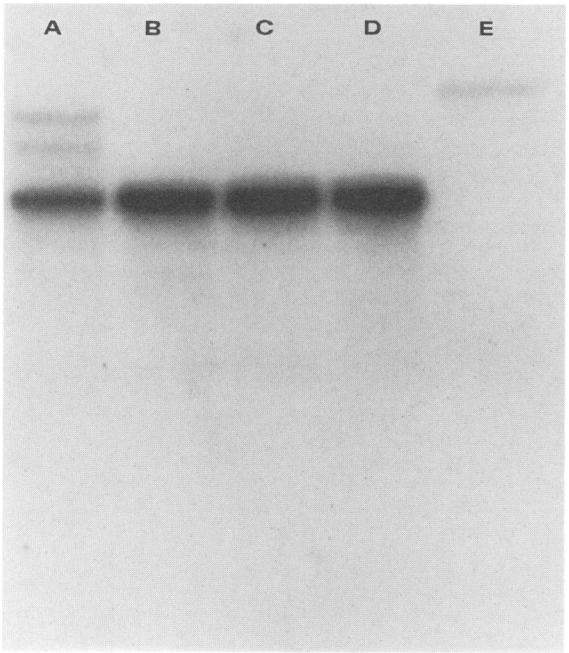

FIG. 2. *Hpa*I-digested total genomic DNA from Tn*5*-derived mutants of *P. putida* hybridized to a ^{32}P-labeled *Hpa*I Tn*5* fragment that was digested from pSUP2021. Lane A, Mutant 4312 (Agg$^-$); lane B, mutant 5123 (Agg$^-$); lane C, mutant 3713 (Agg^s); lane D, mutant 3923 (Agg^s); lane E, *Eco*RI-digested pSUP2021 DNA, which yielded a linear plasmid of 13.6 kb. Note that the only band in lane B, C, or D is the 5.5-kb *Hpa*I fragment of Tn*5*.

colonized the roots to lesser degrees than did the parental type, but these colonization levels were similar to those observed for the Agg^s mutants. The numbers of cells present in water wash 10 were lower for the Tn*5*- and EMS-derived Agg$^-$ mutants than they were for the parental cells. Release of each isolate was enhanced on treatment with EDTA. Treatment with EDTA stimulated greater release for the Agg$^-$ mutants than for the parental cells (Table 3). Culture of the bacteria released by the EDTA wash from roots inoculated with Agg$^-$ mutants and assay with agglutinin revealed that the cells retained the Agg$^-$ phenotype.

Bacterial adherence. The parental and mutant isolates of *P. putida* (Nal^r Rif^r) differed in their adherence to sterile bean roots in a 15-min study (Table 4). The release of agglutinable isolates required more extensive washing and was enhanced on treatment with EDTA. The Agg$^-$ mutants were removed more readily than the parental or Agg^s mutant cells, and no increase in release on washing with EDTA occurred. The levels of bacteria that were initially washed from the roots were lowest in the two EMS-derived Agg$^-$ mutants. The Tn*5*-derived Agg$^-$ mutants were released at levels comparable to that of the parental isolate. In short-term adherence studies with a mixture of parental and Agg$^-$ mutant cells, binding of the parental cells greatly exceeded that of the Agg$^-$ mutant (Fig. 3).

DISCUSSION

The ability of *P. putida* to be agglutinated by a plant root surface glycoprotein was increased or decreased by chemical or transposon mutagenesis. Stable mutants that lacked agglutinability were obtained by treatment with EMS, whereas several reversions back to agglutinability were

TABLE 2. Effect of different treatments on release of *P. putida* from colonized bean roots[a]

Treatment (wash 11)	CFU (10^5) released/g of root at:	
	Wash 10 (water)	Wash 11 (treatment)
Sodium EDTA	0.9 ± 0.2	6.0 ± 0.5
Sodium acetate	1.6 ± 0.2	1.3 ± 0.3
NaCl	1.1 ± 0.5	1.4 ± 0.5
Water	1.0 ± 0.2	1.0 ± 0.2

[a] Dark-red kidney bean seed was inoculated with 10^3 *P. putida* (Nal^r Rif^r) cells and grown for 6 days at 20°C. Roots were gently removed from vermiculite and transferred to 10 ml of sterile distilled water. The root was agitated by using a Vortex mixer for 60 s. Nine serial dilutions were prepared, and the root was finally transferred to 5 mM sodium EDTA (pH 6.5), 5 mM NaCl (pH 6.5), 5 mM sodium acetate (pH 6.5), or sterile distilled water (wash 11). $MgCl_2$ (0.3 ml of a 1 M solution) was added to wash 11 immediately after vortexing. Portions of water wash 10 and treatment 11 were plated onto Kings B medium containing 50 μg each of nalidixic acid and rifampin per ml. Plates were incubated at 22°C for 3 days, and the CFU were determined.

observed with transposon insertion. Genetic analysis of one Tn*5*-derived mutant (2612) that changed the phenotype from Agg$^-$ Neo^r to Agg$^+$ Neo^s revealed that the change in agglutinability was accompanied by the loss of the Tn*5* element. These observations suggest that the change in phenotype of the Tn*5* mutants to Agg$^-$ is related to Tn*5* insertion. Reversion may reflect the transposability of the active Tn*5* element introduced into the genome (6, 25). Analysis of a stable Tn*5*-derived Agg$^-$ mutant (4312) suggested that independent transposition of the IS*50* element may have occurred.

The Agg^s and Agg$^-$ mutants retained certain wild-type properties that could be important in a soil environment. The mutants displayed wild-type growth rates on minimal medium, indicating that any change in root colonization potential was probably not related to nutritional deficiencies. The production of fluorescent components on Kings B medium suggested that the agglutinin-altered mutants produced siderophores. Production of siderophores has been correlated to the ability of *P. putida* to suppress certain fungal root

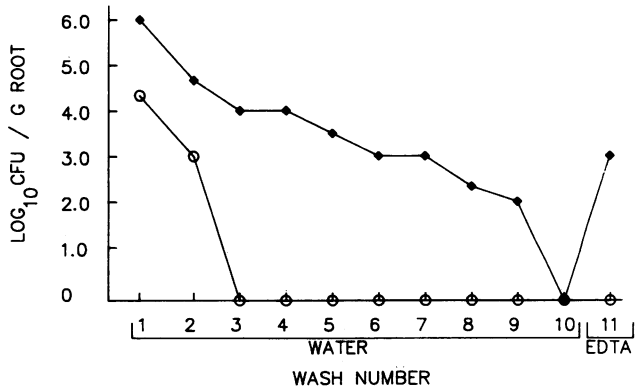

FIG. 3. The intact roots of sterile 8-day-old bean seedlings were immersed into 30 ml of a suspension containing 10^3 CFU of *P. putida* (Nal^r Rif^r) cells per ml (■) or 10^3 CFU of Tn*5*-derived mutant 5123 cells (Agg$^-$) per ml (○). Seedling roots were washed for 15 min, and the washes were plated as described in the text. All cells were detected on medium containing nalidixic acid and rifampin. Only the Tn*5*-derived Agg$^-$ cells were detected on medium containing nalidixic acid, rifampin, and neomycin. Data are the mean values for five roots.

TABLE 3. Extent of colonization of dark-red kidney bean seedling roots by *P. putida* (Nal^r Rif^r) and derived Agg$^-$ or Agg^s mutants

Bacterial isolate	CFU (10^5) released/g of root after the following treatment[a]:			
	Water wash		EDTA wash 11	Extraction
	1	10		
P. putida (Nal^r Rif^r)	47.1 ± 2	0.70 ± 0.2	5.5 ± 0.5	5.2 ± 0.1
Tn*5* mutants				
4312 (Agg$^-$)	39.7 ± 2	0.04 ± 0.01	1.0 ± 0.2	0.9 ± 0.1
5123 (Agg$^-$)	38.1 ± 2	0.07 ± 0.01	0.9 ± 0.2	NA[b]
3923 (Agg^s)	37.6 ± 2	0.10 ± 0.01	1.4 ± 0.3	NA
3713 (Agg^s)	43.1 ± 1	0.17 ± 0.01	3.4 ± 0.5	NA
EMS mutants				
1202 (Agg$^-$)	15.7 ± 1	0.05 ± 0.01	1.8 ± 0.5	1.3 ± 0.2
1236 (Agg$^-$)	20.0 ± 2	0.04 ± 0.01	0.4 ± 0.3	NA

[a] Surface-sterilized bean seeds were inoculated with 2×10^3 CFU of parental or mutant isolates at the time of planting into sterilized vermiculite. Plants were grown for 8 days and were treated as described in the text. Data are the means of three experiments, each of which was done with five roots. Agg$^-$ and Agg^s mutants were obtained from *P. putida* Nal^r Rif^r by EMS or Tn*5* mutagenesis.
[b] NA, Data not available.

pathogens (17, 18, 22). The siderophores may starve these pathogens of iron and, hence, limit their development and pathogenic potential (18). The mutants secreted IAA-like components at wild-type levels. Production of IAA has been reported for other *P. putida* isolates (19), and the external production of this hormone could influence root metabolism. The phage susceptibility of the mutants indicated that the phage recognition site was not altered. The observation that phage-resistant isolates of *P. putida* (Nal^r Rif^r) were agglutinable at wild-type levels supports the hypothesis that agglutinability and phage recognition reside in different structures.

The *Eco*RI and the *Bam*HI genomic digests suggest that two Agg^s and one Agg$^-$ mutant have Tn*5* insertions at different single sites in the host chromosome. The *Hpa*I genomic digest showed that the Agg$^-$ mutant 4312 also contained two independent IS*50* insertion elements. Consequently, the agglutinable phenotype may involve more than one genetic locus or mutations in the regulatory regions of a single locus. This possibility has been suggested from results of an analysis of membrane preparations from mutant and parental isolates. Differences in at least two Coomassie blue-stained bands (V. Acun, F. G. Albert, and A. J. Anderson, unpublished data) were observed when membrane fractions from the Tn*5*- and EMS-derived Agg$^-$ mutants were compared with those from the parental isolate. Alterations in surface polysaccharide structure have been reported for Tn*5* mutants of *Rhizobium trifolii* (12). These mutants adhere more readily than the parental isolate to clover roots and are agglutinated more strongly by the clover lectin (12).

The effects of the Agg$^-$ mutant phenotype in the colonization and short-term adherence studies suggest that agglutination is involved in the bacterial-root surface interaction. Results of the root dip studies showed that unlike agglutinable isolates, the Agg$^-$ mutants were more readily washed from the root surface with water and release was not enhanced after treatment with EDTA. Washes with EDTA were used to determine whether bacteria were bound through divalent metal bridges. In a previous study by James et al. (13), it was demonstrated that Ca^{2+} and Mg^{2+} play a role in promoting the adherence of *P. putida* isolates to plant roots within 5 min. The need for a divalent metal ion in agglutination (5) suggests that bacteria that are bound by an agglutinin molecule would be released by EDTA. The EDTA-enhanced release of agglutinable parental and Agg^s isolates, but not the Agg$^-$ mutants, is consistent with a role of the agglutinin in short-term adherence. This possibility is

TABLE 4. Short-term binding of *P. putida* (Nal^r Rif^r) and derived Agg$^-$ and Agg^s mutants to roots dipped into bacterial suspensions

Root wash[a]	CFU (10^4)/g of root for the following bacterial isolate[b]:						
	P. putida (Nal^r Rif^r)	3923 (Tn*5*; Agg^s)	3713 (Tn*5*; Agg^s)	4312 (Tn*5*; Agg$^-$)	5123 (Tn*5*; Agg$^-$)	1202 (EMS; Agg$^-$)	1236 (EMS; Agg$^-$)
1	143	152	208	163	150	20.7	14.3
2	10.1	43	30.1	4.6	2.3	4.2	1.8
3	2.9	6.7	2.8	1.1	0.17	0.56	0.5
4	1.5	2.5	0.77	0.3	0.4	0.5	0.0
5	1.0	0.6	0.05	0.03	0.02	0.02	0.0
6	1.0	0.36	0.01	0.01	0.01	0.01	0.0
7	0.95	0.31	0.01	0.01	0.01	0.01	0.0
8	0.66	0.30	0.00	0.00	0.00	0.01	0.0
9	0.31	0.18	0.00	0.00	0.00	0.01	0.0
10	0.08	0.03	0.00	0.00	0.00	0.00	0.0
11	0.28	0.23	0.12	0.00	0.00	0.00	0.0

[a] Roots of 8-day-old seedlings grown under sterile conditions were immersed in bacterial suspensions containing 10^6 CFU/ml. After being shaken for 15 min in the bacterial suspensions, roots were washed 10 times in 10 ml of water (washes 1 to 10), followed by one wash with EDTA (wash 11), as described in the text.
[b] Portions (0.1 ml) of the washes were plated to determine the number of bacteria released from root surfaces. Data are the means of two experiments, each of which was done with five roots.

strongly supported by the 20- to 30-fold-greater ability of parental cells compared with Agg^- cells to bind to the root surface. These data suggest that the agglutination phenotype provides a competitive edge in short-term attachment.

A role for the agglutinin interaction in long-term colonization was observed. Under monoculture conditions, the inoculum of Agg^- mutants colonized the seeds. EMS-derived Agg^- mutants were less effective as colonizers than the Tn*5*-derived Agg^- mutants for reasons we currently cannot explain. Release of both EMS- and Tn*5*-derived Agg^- mutants was increased on treatment with EDTA. This stimulation in cell release was not due to the selection of agglutinable revertants during the colonization process, because cells that were recovered from roots inoculated with Agg^- mutants retained their nonagglutinable phenotype. Failure of washes with sodium chloride or sodium acetate to increase bacterial release in the colonization studies suggests that the effects of the washes with EDTA were independent of changes in ionic strength or pH. The attachment, although sensitive to EDTA, presumably is agglutinin independent because it occurred with the Agg^- mutants. Also, additional cells of both the Agg^- and Agg^+ phenotypes remained associated with the root after EDTA treatment and were released by homogenization. These firmer attachments may involve bacterial or plant components that are induced as a result of their interactions, a phenomenon similar to the production of specific glucans by *Agrobacterium tumefaciens* on colonization of the plant surface (10). In *Rhizobium* species, different phases of attachment are proposed to occur with long-term association involving fibrils of bacterial origin (9). Although such a secondary attachment phase may occur with *P. putida*, recognition involving an interaction with agglutinin may be important in securing the initial niche, after which other processes required for colonization can proceed. It is presumed that formation of additional attachment structures in the *P. putida*-root surface interaction is independent of the agglutination phenotype.

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