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Tn5 Insertion Mutants of *Pseudomonas fluorescens* Defective in Adhesion to Soil and Seeds

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Tn5 insertion mutants of a soil isolate, *Pseudomonas fluorescens* Pf0-1, were selected for decreased ability to adhere to quartz sand in a column assay. Three adhesion-deficient mutants that differed in the location of the Tn5 insertion in the chromosome were isolated and compared with the wild-type strain. One mutant, Pf0-5, was described previously as an adhesion-defective, nonmobile, flagellumless mutant (M. F. DeFlaun, A. S. Tanzer, A. L. McAteer, B. Marshall, and S. B. Levy, Appl. Environ. Microbiol. 56:112–119, 1990). Another insertion mutant, Pf0-10, was also missing flagella and the 34-kDa outer membrane protein that was absent in Pf0-5 but present in the wild-type strain. The third mutant (Pf0-15) had increased amounts of this 34-kDa outer membrane protein and more flagella than the wild-type strain. These mutants also displayed decreased ability to adhere to sterile and natural (live) soil and to a variety of plant seeds. In kinetics studies, the wild-type strain showed an initial rapid binding to seeds followed by a later slow phase of binding. The mutant strains were defective in the initial stages of attachment but did show the later slow binding. The findings indicate that the same mutations that affect binding to sand and soil also affect adhesion to plant seeds.

The fluorescent pseudomonads play important roles in the environment. Their ability to degrade toxic chemicals and their beneficial interactions with plants have made this group of bacteria the subject of considerable study (26). Agronomical applications include their use as biopesticides (18) as well as to improve crop yields by a number of mechanisms (30). The increased interest in using these organisms for genetic engineering and environmental release has encouraged studies to better understand the genetic basis for their survival and spread in soil.

The ability to adhere to surfaces is likely an important survival mechanism for obligate aerobic soil microorganisms and root colonizers such as soil pseudomonads. In order to identify the genes associated with adhesion in this genus, we used transposon Tn5 insertions to create adhesion-deficient mutants of a strongly adhering soil isolate, *Pseudomonas fluorescens* Pf0-1 (9). We have previously described Pf0-5, a Tn5 insertion mutant which was defective in adhesion to sand compared with its parent, Pf0-1. The mutant also showed loss of flagella and motility (9). We have isolated two other adhesion-defective mutants of Pf0-1, Pf0-10 and Pf0-15. Like Pf0-5, Pf0-10 is nonmotile and has no flagella; however, the position of its Tn5 insertion differs from that of Pf0-5. Pf0-15 has increased numbers of flagella and therefore has a presumably unrelated basis for defective adhesion. We have characterized these mutants further and have demonstrated that all three mutants are defective in their ability to adhere to soil and to plant seeds.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The *P. fluorescens* Pf0-1 strain used in this study was isolated from loam soil (3). *Escherichia coli* SLH25 is a wild-type fecal isolate which was used as a negative control in the adhesion assays. Tn5 insertion mutants of Pf0-1 were obtained by filter matings with *E. coli* S17-1 harboring plasmid pSUP2021 (9). Because of its narrow host range, pSUP2021 can be used to introduce Tn5 into the chromosome of pseudomonads with subsequent loss of the vector (28). Tn5 was ideal for this purpose because it confers a resistance (kanamycin) not normally borne by *Pseudomonas* spp., produces polar insertions, and transposes into the chromosome with very little site specificity (2). Auxotrophic mutants were eliminated from this selection by plating transconjugants on minimal medium containing glucose and kanamycin. Bacterial strains were maintained as described previously (9).

Isolation of adhesion-deficient mutants. The sand column method described for the isolation of Pf0-5 (9) was used for isolating Pf0-10 and Pf0-15. This assay involved pooling 100 Tn5 mutants for a combined titer of 10^7 cells and adding them in 3 ml of buffered saline to a 20-ml polypropylene syringe barrel filled with 12 g of quartz sand. The cells were allowed to interact with the column for 1 h, and then the column was washed several times to remove nonadherent cells. The nonadherers were passed over a second and subsequently a third column to obtain a preparation enriched in adhesion-defective Tn5 insertion mutants.

Growth studies. The mutants and the parent strain Pf0-1 were compared for growth in enriched (LB medium) and minimal medium (29). Strains were also tested singly and paired with the wild-type parent in sterile and live soil incubated in 50-ml polypropylene tubes at 25°C. Growth and persistence under these conditions were monitored over a period of time by subsampling the soil and enumerating the bacteria on nutrient agar plates with and without kanamycin. For growth in sterile soil, the bacteria were added at a titer of 10^4 to 10^5 cells per g of soil. Survival in live soil was examined after cells were added at a concentration of 10^7 cells per g of

soil. The preparation of the soil and bacteria, inoculation, and sampling were performed as described before (3).

Analysis of Tn*5* insertion mutants. The location of Tn*5* was determined by probing *Sal*I digests of the chromosomes of each of the three mutants with a Tn*5*-specific probe (9). DNA hybridizations, outer membrane protein separation, flagellum preparations, transmission electron microscopy, motility studies, and marker exchange studies were performed as described previously (9, 23).

Soil adhesion assays. Wild-type and mutant strains were tested for adhesion in columns filled with live soil. In order to detect the wild-type strain Pf0-1 in live soil, a spontaneous rifampin-resistant mutant (Pf0-2) of the wild-type was selected. Pf0-2 was identical to Pf0-1, as judged by its growth in enriched and minimal liquid media as well as in sterile and live soil, its outer membrane proteins, its motility, and its adhesion to sand (1, 3).

In one set of live-soil experiments, 20-ml polypropylene syringes were filled with a bottom layer of 12 g of sand and a top layer composed of 6 g of live soil mixed with 6 g of sand. The columns were inoculated at the top with equal numbers (10^7 CFU) of Pf0-2 and one of the three mutants in a volume of 200 μl. The columns were then watered with a fine-mist sprayer twice a day for a total of 1 ml per day over a period of 3 days. Any effluent from the columns was collected in a sterile tube suspended from the bottom. The number of wild-type and mutant organisms was determined by plating dilutions of the effluent or soil on selective medium.

In a second version of the live-soil minicolumns, 20-ml polypropylene syringes were filled with 12 g of live soil, inoculated at the top with equal numbers (10^7 CFU) of Pf0-2 and one mutant, and sprayed with mist over a period of 3 days as described above. The columns were then extruded and sectioned, and the numbers of wild-type and mutant organisms in each section were determined by selective plating on nutrient agar containing rifampin or kanamycin. In some instances, colonies on the initial rifampin plate were replica plated to medium containing kanamycin.

Plant seed adhesion assay. With minor modifications, the seed adhesion experiments used the same column design and protocol developed for sand (9). The columns in this assay were filled with 12 g of commercially purchased seeds (untreated and originally produced for sprouting and/or human consumption): wheat (*Triticum aestivum* L.), mustard (*Brassica* sp. [*juncea*] L.), mung bean (*Phaseolus vulgaris* L.), alfalfa (*Medicago sativa* L.), and radish (*Raphanus sativus* L.). The inoculum was increased to 6 ml to fully saturate the seed column. The contact time between the culture and the seeds was kept to 20 to 30 min (depending on the seed type) to prevent excessive swelling of the seeds; at that time, the plugged column was filled with 6 ml of a buffered saline wash. The clamp was then removed to drain the column. The inoculum and the wash were serially diluted and plated to determine the viable-cell number per ml.

To evaluate the viability of the bacteria which had adhered to seeds during the assay, sonication, vortexing, or grinding was used to release the cells. For the sonication and vortexing methods, two 1-g aliquots of seeds from the column were weighed into separate tubes. Then, 4 ml of sterile buffered saline was added, and the samples were vortexed at full speed for 2 min or sonicated for 15 s in a Bransonic 12 sonicator. A 3-ml amount of saline supernatant liquid was extracted from the seeds for dilution and plating. This process was repeated for each seed sample two more times for the vortexing method and three more times for the sonication method. The seeds were macerated by manually grinding the total seed volume in 24 ml of saline in a glass grinder and extracting the supernatant liquid for plating.

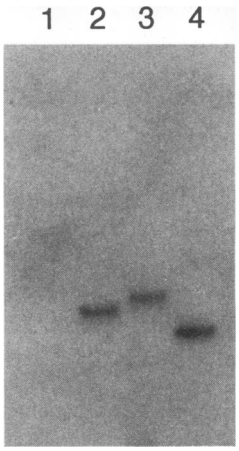

FIG. 1. Location of Tn*5* in the chromosome of Tn*5* insertion mutants. Chromosomal DNA extracted from Pf0-1 (lane 1), Pf0-5 (lane 2), Pf0-10 (lane 3), and Pf0-15 (lane 4) was treated with *Sal*I and subjected to Southern hybridization with a probe for Tn*5*. The hybridization signal was on a different restriction enzyme fragment for each mutant.

Adhesion kinetics study. A column of alfalfa seeds was used to examine the kinetics of binding by the wild type and the adhesion-deficient mutants. A series of columns were inoculated, and the effluent was drained after 5 min. One column was then washed every 5 to 10 min over a 50-min period. The percentage of the inoculum adhering to the seeds was quantified by comparing plate counts made from the inoculum and wash.

RESULTS

Isolation of adhesion-deficient mutants. From a total of 3,500 Pf0-1::Tn*5* mutants tested in the sand column assay, 3 mutants that were defective in adhesion were isolated. One, Pf0-5, has been described before (9). Each was shown to be unique by the location of Tn*5* in the chromosome (Fig. 1). The adhesion-deficient phenotype was linked to the Tn*5* insertion in the chromosome by marker exchange (14). After the Tn*5*-containing region of the chromosome of the mutant was cloned, it was transferred into the wild type on plasmid pLAFR1 (15) and recovered on the chromosome after homologous recombination. The wild-type strain bearing Tn*5* acquired the adhesion-deficient phenotype, demonstrating that the transposon has interrupted a structural or regulatory gene involved in adhesion.

Growth of mutants in media and sterile soil. The three adhesion-deficient mutants showed differences in growth in enriched and minimal media; however, none grew more slowly than the parental Pf0-1 (Table 1). Pf0-15 doubled at the same rate as the wild type in enriched (LB broth) and minimal media. Pf0-5, however, grew faster than Pf0-1 in minimal medium, and Pf0-10 grew faster in LB broth.

When added to sterile soil, either alone or paired with the wild type, the mutants colonized the soil at the same rate as Pf0-1, reaching a titer of 10^8 CFU/g of soil after 24 h and maintaining that level over a 6-day period (data not shown). The rifampin-resistant derivative of Pf0-1, Pf0-2, also colonized sterile soil at the same rate when inoculated alone or paired with wild-type Pf0-1.

TABLE 1. Characteristics of wild-type and mutant *P. fluorescens* strains

Strain	% Adhesion to sand column[a]	Doubling time[b] (min)		Characteristics[c]		
		L broth	Minimal medium	Flagella	Motility	34-kDa outer membrane protein
Pf0-1	91 ± 4	40	85	+	+	+
Pf0-5	43 ± 14	40	65	−	−	−
Pf0-10	46 ± 8	30	85	−	−	−
Pf0-15	55 ± 14	40	85	++	±	++

[a] ± standard deviation.
[b] Values are representative of three experiments for which results differed by less than 5%.
[c] +, present or positive; −, absent or negative; ++, value higher than for the wild type; ±, positive, but less than Pf0-1.

Membrane and cell surface studies. Previously, we identified a major 34-kDa protein in the outer membrane fraction of the wild-type strain which was missing in Pf0-5 (9). This was shown to correspond to flagellin (9). Pf0-10 is also missing this protein, while Pf0-15 appears to have more of this protein than the wild-type strain (Fig. 2).

Flagellum preparations from Pf0-1 and Pf0-15 produced this 34-kDa band, while the same preparations from Pf0-5 and Pf0-10 did not yield any detectable protein band of this size (Fig. 2). Flagellum preparations from the same number of cells resulted in a much wider, darker band at 34 kDa for Pf0-15 than for Pf0-1, again suggesting that there is more flagellin in the former strain (Fig. 2). Photographs obtained by transmission electron microscopy also supported the evidence that Pf0-15 has more flagella than the wild type (data not shown).

The results of motility studies in motility test agar and by direct microscopic examination indicated that Pf0-15 was motile, although less so than Pf0-1 (17); Pf0-10, like Pf0-5, was nonmotile.

Survival in live soil. In live soil, Pf0-2, a rifampin-resistant derivative of Pf0-1 that is phenotypically similar to its parent, was used to facilitate detection by selective plating. Pf0-5, Pf0-10, and Pf0-1 all decreased in number approximately 10-fold every 8 or 9 days. Pf0-15, however, decreased at a faster rate, approximately 10-fold every 5 days (data not shown).

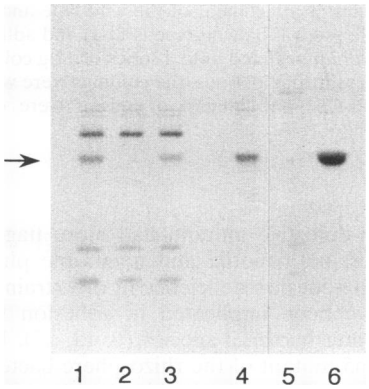

FIG. 2. Outer membrane proteins of wild-type and mutant pseudomonads analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with Coomassie blue. Lanes 1 to 3, strains: 1, Pf0-15; 2, Pf0-10; 3, Pf0-1. Lanes 4 to 6, flagellum preparations (from equal amounts of cells): 4, Pf0-1; 5, Pf0-10; 6, Pf0-15. The arrow points to the 34-kDa protein missing in Pf0-10 (and Pf0-5).

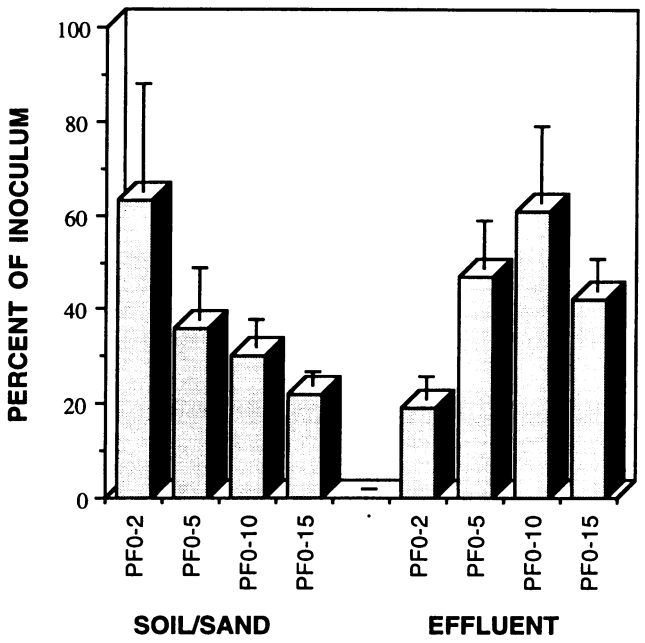

FIG. 3. Distribution of wild-type and mutant strains in live-soil plus sand column. Wild-type (Pf0-2) and mutant (Pf0-5, Pf0-10, and Pf0-15) strains of *P. fluorescens* were inoculated in pairs in similar numbers (10^7 CFU each) onto a column consisting of 12 g of a 50:50 mixture of live soil and sand over 12 g of sand. After being watered by fine spray (1 ml/day) for 3 days, the number of organisms in the effluent and soil-sand layers was determined. Error bars signify standard deviation.

Adhesion of mutants to live-soil minicolumns. Tests for adhesion to live soil were performed over a period of 3 days. Tests in columns containing a live soil plus sand mixture over a sand layer showed retention of about 64% of the inoculated wild-type organisms in the soil layer; 19% washed through the column and were present in the effluent. This relative distribution was reversed for the adhesion-deficient mutants. In the soil plus sand layer, Pf0-5, Pf0-10, and Pf0-15 were retained at averages of 35, 30, and 21%, respectively, while 47, 61, and 43%, respectively, were lost in the effluent (Fig. 3).

In the soil-only columns, Pf0-1 was found in the top 1.5 cm at an average of 61% ± 11% of the inoculum, while Pf0-5, Pf0-10, and Pf0-15 were present at only 36, 47, and 23%, respectively, after 3 days.

Seed adhesion studies. Greater than 90% of the wild-type Pf0-1 inoculum bound to the seeds within 20 to 30 min. The adhesion mutants showed lower values for adhesion to seeds, similar to the adhesion levels obtained in the sand column assay (Fig. 4).

It was of interest to determine whether the lack of recovery of viable organisms from the seeds was due to binding or loss of viability. Three methods were used to release the adherent cells from the seeds. Sonication and vortexing were the most efficient and recovered ~38 to 50% of the cells calculated to be attached to the seeds. Grinding recovered somewhat less (~30%). Seeds which had been sonicated or vortexed were placed on nutrient agar plates to evaluate residual bacteria. Growth appeared around all the inoculated seeds but rarely appeared around uninoculated control seeds. This finding indicated that the test seeds still contained additional viable

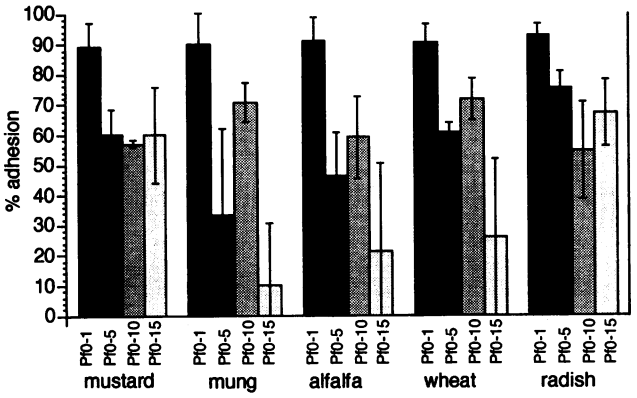

FIG. 4. Binding of parental strain and Tn*5* insertion mutants to seeds. Wild-type strain Pf0-1 and mutants were inoculated on top of a 12-g column of different plant seeds. After 20 to 30 min, the column was washed, and the number of organisms retained was determined as a percentage of the original inoculum. Error bars signify standard deviation.

Pseudomonas cells which were not released by these manipulations. Thus, the loss of organisms from the inoculum was due to binding to the seeds, not to loss of viability.

Adhesion kinetics. In order to determine whether the difference in binding between the wild-type and mutant strains occurred early or late during the contact with the seeds, kinetic experiments were performed with alfalfa seeds. These studies demonstrated a dramatic difference between the wild-type strain and the mutants. Five minutes after the inoculum was added to the column, the effluent was collected and the concentration of inoculated organisms was determined. The number of wild-type Pf0-1 per milliliter of effluent (~1 ml) was only 40 to 50% of that in the inoculum. In contrast, the mutants showed little or no depletion in inoculum concentration. After incubation of the column for an additional 25 min, the column was washed and the numbers of wild-type and mutant organisms which were removed were determined. In all cases, the adhesion of Pf0-1 was 1.5- to 2.0-fold higher than that of the mutants.

The results of these studies suggested that there may be two phases to bacterial binding: one which occurred within 5 min, and another which was prolonged. To evaluate this further, we examined the binding of bacteria to seeds during early time points. As shown in Fig. 5, the wild type showed rapid binding to all seeds tested that reached a plateau within 15 min. In contrast, the three mutants failed to demonstrate any significant attachment to the seeds within the first 5 to 10 min but showed a gradual increase in association over time. Even after 60 min, the attachment by the mutant never reached the level of that by Pf0-1 (Fig. 5).

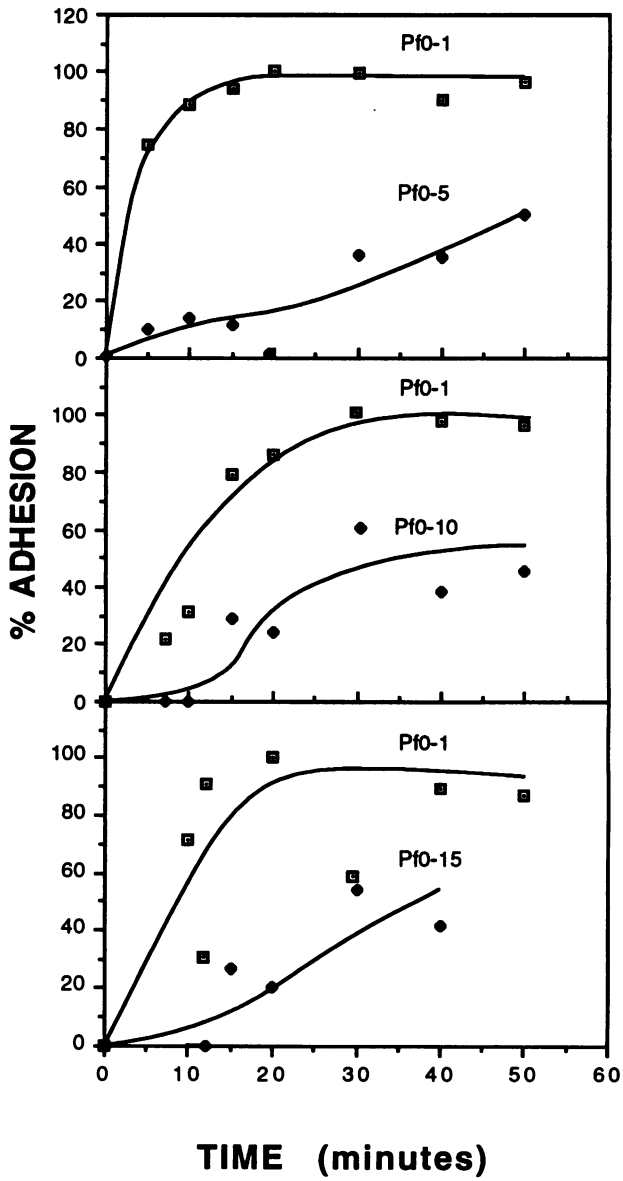

FIG. 5. Kinetics of attachment of the wild type and Tn*5* insertion mutants to alfalfa seeds. The wild-type (Pf0-1) and adhesion-deficient mutant strains were inoculated onto a series of 12-g columns of alfalfa seeds. After different time periods, the columns were washed, and the numbers of wild-type and mutant organisms were determined by selective plating.

DISCUSSION

Adhesion is a major characteristic of bacteria sharing a heterogeneous ecosystem, whether it be the gastrointestinal tract or soil (4, 5, 7, 11–13, 16, 19, 24, 31, 32). Our interest in bacterial survival in complex environments led us to focus on this trait in order to identify genes associated with this function. Three adhesion-deficient Tn*5* insertion mutants of a *P. fluorescens* soil isolate have been identified by using a sand column assay. Two of the mutants, Pf0-5 and Pf0-10, lost flagella concomitantly with loss of adhesion, suggesting a role for flagella in the ability to adhere to sand. However, Pf0-15, a third adhesion-defective mutant, had more flagella than the wild-type. Thus, both motile and nonmotile phenotypes are associated with adhesion deficiency in this strain.

Flagella have been implicated in adhesion for both soil isolates and other bacterial species (6, 12, 27). Loss of polar flagella in a Tn*5* mutant of the rhizosphere bacterium *Azospirillum brasilense* Sp7 also resulted in the loss of adsorption to wheat roots (7). This wheat root adhesin was identified as a protein component of the polar flagellum (6). The fact that attachment was due to an adhesin protein on the flagellum and not the flagellum itself may explain how Pf0-15, which has flagella, can be nonadhesive; Pf0-15 may lack the adhesin but

not the flagella. This type of arrangement has been described for other bacterial species as well as for other cell surface structures. In certain strains of *E. coli* which bind by pili, most of the pilus is not involved in adhesion; only an adhesin protein at the tip of the pilus is essential. Nonpiliated bacteria which retained the adhesin protein were still able to adhere (21). An adhesin carried on the flagella was postulated to be the mechanism for adhesion of a *Vibrio cholerae* strain in its interaction with mammalian cells. As with Pf0-5 and Pf0-10, loss of motility in this *Vibrio* strain was associated with loss of adhesive ability (19). The adhesin for *V. cholerae* is reported to be a flagellar sheath protein similar to those found in *Campylobacter pylori* and several other species, including a *Pseudomonas stizolobii* strain (16).

Although Pf0-15 had polar flagella, the fact that their numbers exceeded those possessed by the wild type suggested another mechanism for the adhesion-deficient phenotype of this strain. It was possible that the extra flagella interfered with adhesion because their concentration caused them to adhere to themselves rather than to surfaces. This interference mechanism is unlikely, however, since inoculating a sand column or a seed column with a mixture of Pf0-15 and Pf0-1 did not affect the binding of Pf0-1 (Fig. 5 and unpublished data). It is more likely that, although more abundant, these flagella lack an adhesin on their surface.

In general, the initial stage of bacterial attachment has been reported to be a weak, reversible phase governed by physicochemical interactions as described by colloid chemical theories. These colloid theories include Van der Waals, electrostatic, and steric effects on attachment. Delayed, "irreversible" attachment is usually a function of a physical attachment by pili, flagella, or an extracellular polymer (31). However, it has been shown that flagella may adhere to surfaces during the initial reversible attachment phase (20). The kinetic studies with seeds suggest that the adhesion mutants of Pf0-1 are defective in some aspect of the initial stages of attachment. The wild-type strain Pf0-1 showed attachment within the first 5 min, as evidenced by the lower concentration of the cells in the effluent than in the inoculum. The mutants, however, had the same concentration of cells in the effluent and in the inoculum, indicating that there was minimal, if any, attachment during that time period. The finding suggests that there are two phases to wild-type strain attachment, a very rapid initial binding followed by a slower mode. Only the slower mode of irreversible binding occurs with the mutants. Alternatively, these differences may only reflect a much slower overall binding by the mutants. This difference does not appear to be the result of a change in hydrophobicity or cell surface charge, since hydrophobicity and electrophoretic motility studies of the three Tn*5* insertion mutants showed no change from the wild-type parent (33).

The residual attachment found for the mutants may be due to an alternative attachment mechanism, such as an extracellular polysaccharide or protein that is present in both the mutants and the wild type. It is also possible that there were other differences in the membrane proteins of the mutants and the wild type that were not detected in the protein gels.

A biphasic type of binding behavior has also been described in studies of the attachment and desorption of *Streptococcus sanguis* from artificial pellicle (saliva-coated hydroxyapatite [4]). After more than 2 h of adsorption, desorption had a single rate constant, while after a shorter period of adsorption, desorption was described by biphasic kinetics (4). This finding indicates that only a part of the population was able to bind irreversibly after a short period of attachment, while the remainder of the population bound irreversibly only after a long period of exposure.

The delayed attachment found for the mutants is similar to that described for Tn*5* insertion mutants of a plant pathogen, *Agrobacterium rhizogenes* (5). The binding of the nonpathogenic *A. rhizogenes* mutants to plant cells was delayed by several hours compared with that of the pathogenic wild type. It is not known whether the number of attached *P. fluorescens* adhesion mutants would ever approach that attained within 15 min by the wild-type strain Pf0-1. After 1 h of exposure to the seeds, only about 50% of the mutant cells had bound.

All three of the adhesion-deficient mutants identified in the present study are similar to a previously described class of mutants of the rhizosphere bacterium *A. brasilense* Sp7, which were adsorption defective and anchoring proficient (Ads^- Anc^+). The biphasic nature and independence of these two mechanisms were identified by the two classes of mutants characterized (Ads^- Anc^+ and Ads^+ Anc^-). The identification of these two modes of attachment required measurement of the strength of the attachment as well as determination of a physical difference between the two classes of mutants, i.e., loss of a particular surface polysaccharide (25). Although in our study the three mutants are distinguishable by the position of Tn*5* in the chromosome, they all seem to be defective in the same phase of attachment.

Soil columns were not used to select adhesion-deficient mutants because they filtered the bacteria rather than measured attachment (9). Used as small microcosms over a longer time period, however, they could demonstrate differences between the mutants and the parent; adhesion mutants failed to remain in the upper portion of soil column microcosms (Fig. 3).

One aspect of bacterial adhesion which is important in agronomical applications is the use of rhizosphere bacteria to combat plant pathogens. Seed-borne biological control agents, such as rhizobacteria, have been used successfully in the control of a number of crop-destroying pests and as growth promoters (18). *P. fluorescens* establishes itself preferentially in the rhizosphere rather than the bulk soil (13). *P. fluorescens* has also been used as a seed inoculum to combat the take-all fungus that affects wheat growth (30). An understanding of the genetic basis of adhesion to seeds and roots will allow the manipulation of this important colonization feature in the development of improved biological control agents.

While adhesion may be an important survival trait for soil pseudomonads, lack of adhesion may be beneficial for those degradative strains being considered for treatment of hazardous wastes in situ. One of the technical hurdles to overcome in introducing bacteria into the subsurface involves finding organisms which do not adhere, the cause of plugging of the formation immediately around the well. Therefore, degradative bacteria which are also adhesion deficient may be an important tool for in situ treatment of recalcitrant contaminants. By the same approach described here, adhesion-deficient mutants of trichlorethylene-degrading bacteria have been selected. These strains demonstrated an enhanced ability to travel through sand in a model groundwater aquifer (8).

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