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Development of an Adhesion Assay and Characterization of an Adhesion-Deficient Mutant of *Pseudomonas fluorescens*

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A sand column adhesion assay was developed which distinguishes the adhesion abilities of a number of pseudomonads isolated from fine sandy loam. *Pseudomonas fluorescens* Pf0-1 which adhered at >90% of the total cells added was subjected to transposon Tn*5* insertion mutagenesis. From 2,500 Pf0-1::Tn*5* mutants examined in the sand column assay, two adhesion-deficient Pf0-1 mutants showing <50% attachment were isolated. Marker exchange analysis of one of these mutants, Pf0-5, confirmed that the decreased adhesion was linked to the Tn*5* insertion in the chromosome. The growth rate of Pf0-5 in enriched media and sterile soil was similar to that of the wild type; in minimal medium, however, Pf0-5 grew faster. In a soil column assay, less Pf0-5 than wild-type bacteria were recovered, suggesting a decreased ability to persist in soil. A 34-kilodalton major outer membrane protein present in the wild type was missing in Pf0-5. Transmission electron microscopy of the cell surface revealed that the wild-type possessed polar flagella which were absent in the mutant.

The characteristics that enable microorganisms to survive and colonize the soil environment are diverse and poorly understood. The extreme heterogeneity and complexity of both the biological and physicochemical properties of soil make it a difficult subject for study. Fluctuations in variables such as organic substrates, water content, and oxygen tension on both macro- and microscales have led to high diversity in the microbial community. Consequently, the conditions of in situ soil microhabitats are largely a matter of conjecture.

Microorganisms in soil are apparently nutrient limited; therefore, survival in soil is considered to be linked to traits that enable microorganisms to take advantage of nutrients available only intermittently or in short supply (25). It is not known how important bacterial adhesion is in soil (29); however, the adsorption of simple, soluble carbon sources and organic macromolecules at the surface of soil particles may make it a more nutritionally rich environment for bacteria than the interstices. Attachment of bacteria to soil, therefore, would enhance their ability to obtain these nutrients. Adhesion would also be advantageous in preventing vertical displacement and maintaining aerobes in the topsoil.

The potential use of genetically engineered organisms in environmental applications requires a much greater understanding of their survival traits and the genes which determine them. Survival and persistence characteristics are important factors in assessment of the risk associated with microorganisms considered as candidates for genetic engineering. Pseudomonads are interesting in this respect because of their inherent metabolic flexibility. We chose to examine one aspect of this survival phenotype in pseudomonads, the ability to adhere to and therefore maintain a population in soil.

We developed an adhesion assay which measures the abilities of bacteria to adhere to an inert surface. This assay was used to characterize the adhesive abilities of a number of soil isolates and to select mutant strains whose adhesion capacity has been diminished by Tn*5* insertional mutagenesis. The Tn*5*-containing chromosomal region from one of the adhesion-deficient mutants was cloned and used to establish linkage between the location of the Tn*5* insertion and lack of adhesion.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The *Pseudomonas fluorescens* and *P. putida* strains used in this study were isolated from soil as described previously (10). Pf0-1 is a *P. fluorescens* soil isolate which strongly adheres in the sand column assay. SLH25 is a wild-type *Escherichia coli* fecal isolate which adheres weakly and was used as a negative control in the adhesion assay. The other strains and plasmids used are listed in Table 1.

Media and chemicals. *Pseudomonas* strains were maintained on nutrient agar (Difco Laboratories, Detroit, Mich.) and grown in minimal medium (28). *E. coli* strains were maintained on L agar and grown in liquid L broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 2 g of glucose per liter). The antibiotics used in the media were kanamycin (50 μg/ml), tetracycline (10 μg/ml), and gentamicin (5 μg/ml). Restriction endonucleases were purchased from International Biotechnologies, Inc., New Haven, Conn., and Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used as specified by the manufacturers.

Preparation and analysis of soil. The fine sandy loam used in these assays was collected from the plow layer on a farm in Sherborn, Mass. The preparation of the gamma-irradiated sterile soil used in the development of this assay has been described previously (10). The nutrient and mineral content and textural analyses of the soil were performed in the Soil and Plant Tissue Laboratory of the University of Massachusetts at Waltham. The water-holding characteristics were determined at the Soils Testing Laboratory of the University of Massachusetts at Amherst. The soil was 4.9% (dry weight) organic matter and had a pH of 6.3. The particle size distribution of the soil sample was 43.9% sand, 49.8% silt, and 6.3% clay. Moisture retentions at −0.1, −0.3, −1.0, and −15.0 bars were 35.2, 22.19, 14.52, and 8.91%, respectively; the water-holding capacity was 4.23 ml/10 g. Nutrients in parts per million (micrograms per gram; wt/wt) were as follows: calcium, 1,262; potassium, 94; magnesium, 46;

TABLE 1. Bacteria and plasmids

Strain or plasmid	Genotype or phenotype	Reference
E. coli strains		
S17-1	MM294 *recA* IncP *mob*$^+$::Tn*7* Mu	27
BD1527	*met hsdR supE supF rpsL*	8
MM294	*endA1 thi-1 hsdR17 supE44* λ^- F^- Pro^-	23
BC32	MM294 *recA*	14
SLH25	Wild type	20
HB101	*thr leu thi recA*	7
Plasmids		
pLAFR1	IncP Tc^r λ *cos*	16
pPH1JI	IncP Gm^r Str^r	6
pSUP2021	Km^r Amp^r Cm^r pBR325::Tn*5* RP4-*mob*	27
pKan1	Amp^r Km^r Lac^- pUC18 derivative	Cohen et al.; manuscript in preparation
pRK2013	Km^r IncP-*tra*	13

phosphorus, 18; ammonium, 3; nitrate, 15; boron, 0.1; zinc, 1.4; iron, 2.1; manganese, 1.8. There was less than 2 ppm each of extractable arsenic, cadmium, copper, molybdenum, and lead. Total (Kjeldahl) nitrogen as percent nitrogen was 0.177.

Soil studies. Bacteria were added to sterile and nonirradiated soil in 50-ml polypropylene tubes and incubated at 25°C. Growth and persistence under these conditions were monitored over a period of time by subsampling the soil as previously described (10). Bacteria were enumerated on nutrient agar plates with and without 50 μg of kanamycin per ml to distinguish the Tn*5* mutants from the wild-type strain.

Membrane proteins and isolation of flagella. Outer membrane proteins were prepared and visualized by a method modified by Cohen et al. (9), except that separation of the proteins was in a 14% polyacrylamide gel without urea.

Flagella were isolated by a modification of the method described by Martin and Savage (22). Cells were harvested in the late-exponential phase and suspended in 0.1 M Tris buffer (pH 7.8). After deflagellation in a Waring blender, the flagella were purified by three cycles of differential centrifugation. The flagellin was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein bands in the gel were stained with Coomassie blue or silver stain (24).

Transmission electron microscopy. An overnight culture in minimal medium was diluted 1:10 and grown until the optical density (A_{530}) had doubled twice. The cells were then fixed for 20 min by adding glutaraldehyde to a final concentration of 4%. After two washes in buffered saline, the cells were suspended to a density of 10^9 cells/ml and stained in 2% phosphotungstic acid for 20 s.

Adhesion assay. Several column parameters were varied and optimized during development of an assay that would distinguish between and yield the largest difference in percent adhesion between strongly and weakly adhering strains. The physical parameters of the column that were tested included the type of column matrix material, the depth and amount of the column matrix; the length of the attachment period; the number of washes; and the ionic strength of the washes. The cell parameters tested included strain differences, the growth phase and concentration of the bacteria added, and the medium or buffer in which they were introduced.

Three different types of column-packing material were tested: various ratios of sterile soil and sand, coarse sand (20/30 mesh, Ottawa sand standard; Fisher Scientific Co., Pittsburgh, Pa.), and fine sand (60/120 mesh, silicon dioxide; Fisher Scientific). Assays performed with the column matrix composed of fine sand yielded adhesion values that did not vary over a wide range of parameters, indicating that some of the cells were trapped within the column matrix. In contrast, bacterial adhesion to coarse-sand columns varied with physical changes in the columns themselves or in the cells introduced, yet percent adhesion did not vary significantly for a particular strain introduced to the column in a consistent manner. Column assays with soil-sand mixtures distinguished between strain differences; however, these were not as large as with the coarse-sand columns which were selected for subsequent study. The column consisted of either a 20- or a 50-ml polypropylene syringe with a sterile glass wool plug closed off at the tip with a short section of rubber tubing and a Hoffman clamp.

Cells were grown for ~16 h in minimal medium, diluted 1:10 in minimal medium, and then allowed to double twice. The cells were then washed and suspended in buffered saline and introduced to the column in a volume sufficient to saturate the column matrix. After a 5-min equilibration period, the rubber tubing and Hoffman clamp were removed, allowing any excess liquid to drain off the top of the column. After an attachment period that was varied between 1 and 4 h, the columns were washed with a volume of buffered saline equal to that in which the cells were introduced. The washing procedure was tested over a range of one to six washes, and the number of cells in each wash was determined by plate counts.

Motility studies. Cells were grown and washed as described for the sand column assay, except that they were suspended in a volume of buffered saline equal to that of the mid-logarithmic-phase culture. Motility was analyzed by growth of these cultures in stabs of motility test agar (Difco Manual) over a 24-h period at 25°C. The results of the agar test were confirmed by direct microscopic examination at a magnification of ×1,250 under oil immersion.

Transposon mutagenesis. Several thousand Tn*5* insertion mutants of *P. fluorescens* Pf0-1 were generated by filter matings with *E. coli* S17-1 harboring plasmid pSUP2021. Plasmid pSUP2021 has a narrow host range and is not stably maintained in pseudomonads; therefore, it can be used to introduce Tn*5* into the host chromosome with subsequent loss of the vector (27). Tn*5* was selected for these studies because it has demonstrated very little site specificity, produces polar insertion mutations (5), and confers an easily identified trait, resistance to kanamycin. Tn*5* insertions which generated auxotrophs were eliminated by selecting transconjugants on minimal medium supplemented with glucose and kanamycin.

Screen for adhesion-deficient mutants. For selection of adhesion-deficient mutants, equal amounts of 100 Tn*5* insertion mutants of Pf0-1 in the logarithmic phase of growth were added to a 12-g sand column in buffered saline at a combined titer of approximately 2×10^7 cells per 3 ml. Cells that passed through the column (i.e., nonadherers) were collected in the saline wash and enumerated on nutrient agar plates containing kanamycin. One hundred of the resulting colonies from the highest-dilution plates were picked, pooled, grown to the logarithmic phase, and passed through a second column to further enrich for adhesion-deficient

mutants. If after three subsequent columns the percentage of cells adhering to the column had not decreased (from >90% to <70%), another set of 100 mutants was selected for screening. Decreasing percentages of adhering cells in columns 2 and 3 indicated that the assay had selected for at least one mutant whose adhesion ability had diminished. Mutants enriched by this protocol were then retested individually in the sand column assay. Adhesion-deficient mutant strains, once identified, were also tested in pairs with wild-type strain Pf0-1 bacteria in the sand column assay. The two strains were mixed in equal numbers for a combined titer of $\sim 2 \times 10^7$ and coinoculated onto columns to test their adhesion under identical conditions.

Analysis of Tn*5* insertion mutants. Total DNA was isolated from each mutant by a modification of the method of Beji et al. (3) using 1.5-ml cultures. DNA was digested to completion with *Sal*I and separated by electrophoresis in a 0.7% agarose gel. The DNA in these gels was transferred to GeneScreen Plus hybridization transfer membrane (Dupont, NEN Research Products, Boston, Mass.) and probed with a Tn*5*-specific probe. The probe was constructed by isolating the *Tth*111I-*Sal*I fragment of Tn*5* contained on the pKan1 vector (generously provided by Seth P. Cohen). It contains IS*50*L and the *neo* gene of Tn*5* up to the *Bam*HI site and 2 kilobases of flanking *E. coli* chromosomal DNA cloned into the *Bam*HI site of pUC18. The fragment was isolated by cutting the appropriate band from low-melting-point agarose and then purifying and concentrating the DNA by a procedure developed for Elutip-d columns (Schleicher & Schuell Technical Bulletin 206; Schleicher & Schuell, Inc., Keene, N.H.). The probe was radioactively labeled with [α-^{32}P]dCTP with a random-primer labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Cloning the Tn*5* containing region. Large fragments (20 to 30 kilobases) from *Eco*RI partial digests of chromosomal DNA from the adhesion-deficient mutant were isolated from low-melting-point agarose as described above. These fragments were then ligated into the *Eco*RI site of the broad-host-range cosmid cloning vector pLAFR1 (Tc^r; 16), which was pretreated with alkaline phosphatase to prevent formation of cosmids without inserts (19). The cosmids were then packaged into bacteriophage λ by using an in vitro packaging system (Packagene; Promega Biotec, Madison, Wis.). These λ phage were used to transduce *E. coli* BD1527, and transductants were selected on plates supplemented with tetracycline. Colonies that grew on tetracycline were replica plated to kanamycin-supplemented plates to select for transductants harboring pLAFR1 with chromosomal DNA insertions containing Tn*5*. Plasmid size was determined by isolation (17) and separation in a 0.7% agarose gel.

Marker exchange. Plasmids from these selected transductants were mobilized into the wild-type strain by triparental mating with helper plasmid pRK2013 in *E. coli* MM294 (13). Introduction of a distinguishable (Gm^r) broad-host-range plasmid (pPH1JI in *E. coli* BC32; 14) incompatible with pLAFR1 was followed by selection for kanamycin and gentamicin resistance and loss of pLAFR1 (loss of Tc^r). Several of these colonies were grown in L broth for >100 generations and then tested for retention of Km^r and loss of gentamicin resistance (loss of pPH1JI). This identified strains in which the homologous Tn*5*-containing DNA had recombined with the host chromosome. Total DNA from the recombinant Pf0-1 was digested with *Sal*I, separated by agarose gel electrophoresis, and hybridized with the probe described above to confirm the presence of the introduced Tn*5*-containing insert. Recombinants were then tested in the adhesion assay to determine whether they had become adhesion deficient.

Soil columns. The persistence of strain Pf0-1 and the adhesion-deficient mutant was tested in four different soil and sand columns constructed within 20-ml polypropylene syringes. Column A contained a 12-g top layer containing equal parts of sterile soil and sand and a bottom layer of 12 g of sand. Column B was composed of a 12-g soil-sand top layer at a ratio of 1:2; the bottom layer was composed of 12 g of sand. Columns C and D had the same ratio of soil-sand in the top and bottom layers as columns A and B, but the total weight of each layer was 6 g. Each column was inoculated in the center of the top soil layer with equal numbers of Pf0-1 and mutant bacteria in a total volume of 200 μl. The columns were sprayed with a fine mist for a total of 4 ml/24-g column per day and 2 ml/12-g column per day over a period of 5 days. The wild-type and mutant Pf0-5 in the top soil layer and bottom sand layer were enumerated at the end of this 5-day period.

RESULTS

Bacterial adhesion to sand columns. In the initial column assays with soil-and-sand mixtures, *P. fluorescens* Pf0-1 and *E. coli* SLH25 had the highest and lowest adhesion values, respectively. Therefore, the sand column assay was optimized to yield the greatest difference in percent adhesion for these two strains.

Pf0-1 bacteria grown in minimal medium to the logarithmic phase were washed and suspended in either minimal medium or buffered saline and added to three columns each at identical titers. The cells that were added in minimal medium adhered at percentages ranging from 46 to 82%. Cells suspended in buffered saline, however, adhered at 93 to 95% of the total introduced. Growth of Pf0-1 in L broth, as opposed to minimal medium, with subsequent washing in buffered saline, yielded lower adhesion values ($\bar{x}$ = 77%, n = 5). Adding Pf0-1 to the column in buffered saline with two and three times the NaCl content only slightly decreased the percentage that adhered to the column.

The effect of the growth phase on adhesion of the inoculum was tested by comparing Pf0-1 in the logarithmic phase, the stationary phase (18-, 24-, and 30-h cultures), and a starvation state (i.e., suspension in buffered saline for 48 h). All cultures were prepared by washing and suspending the cells in buffered saline before addition to the column. While logarithmic-phase cells consistently adhered at >90% (93 ± 3%, n = 6), starved cells adhered less well (73 ± 5%, n = 6) and stationary-phase cultures adhered the least (52 ± 16%, n = 6).

Column parameters affecting adhesion. Pf0-1 bacteria (10^7) added to a 12-g sand column adhered at >90% whether the sand was contained in a 20- or 50-ml syringe, indicating that the depth of the sand in the column was not a determining factor. Increasing the attachment over a period of 1 to 4 h, with samples taken every hour, also did not affect the total number of cells that adhered (92 ± 1.6%). The number and salinity of the washes applied to each column were also tested. Columns containing either *P. fluorescens* Pf0-1 or *E. coli* SLH25 were washed with six 3-ml samples of buffered saline, and each wash was counted separately. For the Pf0-1 columns, all nonadhering cells came off in the first three washes (6% of the total cells were removed). With SLH25, 88% of the cells were removed in the first three washes. The greatest difference in adhesion between the two strains occurred after four washes, with Pf0-1 adhering at 94% and

TABLE 2. Adhesion of soil pseudomonads and *E. coli* in the sand column assay

Strain	Mean % adhesion ± SD	No. of expts
P. fluorescens Pf0-1	91 ± 4	21
P. fluorescens Pf1-1	39 ± 12	19
P. fluorescens Pf2-1	50 ± 24	5
P. putida Pp1-1	58 ± 3	4
E. coli SLH25	8 ± 4	9
E. coli HB101	22 ± 8	10

SLH25 adhering at 10%. Doubling and tripling the amount of sodium chloride contained in the buffered saline used to wash the columns had little effect on the amount of strain Pf0-1 bacteria that adhered to the sand column. After four 3-ml washes, the column washed with buffered saline had 94% adhesion of Pf0-1, while the columns treated with the higher-salt washes both had adhesion values of 92%.

Pf0-1 and SLH25 were also tested together in the same column. In six separate experiments, Pf0-1 adhered at 93 ± 4% while SLH25 adhered at only 9 ± 7%.

Under conditions yielding the greatest difference in adhesion between *P. fluorescens* Pf0-1 and *E. coli* SLH25, the percent adhesion values of different strains were determined (Table 2). Other pseudomonads adhered at 40 to 60%, whereas *E. coli* HB101 adhered at about 20%.

Isolation of adhesion-deficient mutants. From a total of 2,500 Pf0-1::Tn*5* mutants tested in the sand column assay, we isolated two adhesion-deficient mutants (Pf0-5 and Pf0-10) which consistently adhered at <50%. The position of the Tn*5* insertion in chromosomal digests of Pf0-5 was different from that of Tn*5* in digests of Pf0-10 and Pf0-6, a Tn*5* insertion mutant that adhered at >90% (Fig. 1). We selected Pf0-5 for further study.

Comparison of outer membrane proteins in mutant and wild-type strains. The outer membrane proteins of strains Pf0-1 (wild type), Pf0-6, and Pf0-5 were compared. Pf0-1 and Pf0-6 showed indistinguishable membrane protein profiles. Both contained a major protein at ~34 kilodaltons (kDa) that was not present among the outer membrane proteins of Pf0-5, the adhesion-deficient mutant (Fig. 2). The outer membrane proteins of Pf0-1 cultures in logarithmic, stationary, and starved conditions were identical (data not shown).

Transmission electron microscopy. Electron microscopic studies of strain Pf0-1 revealed long, thin (~10 nm), multitrichous polar flagella extending from the cell. Strain Pf0-5, however, lacked these cell surface structures (Fig. 3). Purified flagellin from Pf0-1 migrated as a 34-kDA protein in sodium dodecyl sulfate-polyacrylamide gels (data not shown), indicating that the membrane protein missing in Pf0-5 is attributable to the absence of flagella in the mutant.

Motility studies. Growth of Pf0-1 in the motility test agar extended throughout the tube, while Pf0-5 grew only along the stab line. This result was confirmed by microscopic observation of these cells, which indicated that Pf0-1 was motile while Pf0-5 was nonmotile.

Growth of mutant in media and sterile soil. Growth of Pf0-1 and Pf0-5 was compared in liquid culture. In L broth, both doubled in 40 min; in minimal medium, however, Pf0-5 doubled in only 65 min, in contrast to the 85-min doubling time of Pf0-1 (Fig. 4).

In previous studies (10; B. J. Al-Achi, E. Platsouka, and S. B. Levy, submitted for publication), sterile-soil assays have been used to assess the competitive fitness of mutants compared with the parent strain. When Pf0-1 and the adhesion-deficient mutant Pf0-5 were added individually to sterile soil, both reached titers of 4×10^8 CFU/g of soil after 48 h and maintained that level over a period of 5 days (data not shown). When added together into sterile soil, both reached titers of $\sim 1 \times 10^8$ to 2×10^8 CFU/g of soil after 48 h and maintained that level over the course of the experiment (5 to 7 days; data not shown).

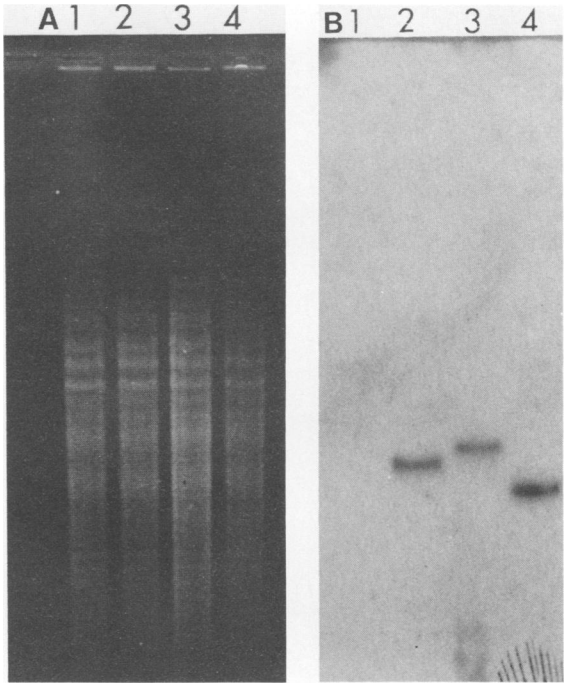

FIG. 1. (A) *SalI* digestion of chromosomal DNAs. Lanes: 1, Pf0-1; 2, Pf0-6; 3, Pf0-10; 4, Pf0-5. (B) Autoradiogram resulting from hybridization with the ^{32}P-labeled *Tth*111I-*Sal*I fragment of Tn*5*.

Adhesion of mutant to soil columns. Strain Pf0-1 and Pf0-5 bacteria were added in equal numbers to sterile soil-sand columns and watered for a period of 5 days. Subsequent analysis showed that the top layers of soil and sand contained 45 to 71% (58 ± 12%, $n = 4$) less Pf0-5 than Pf0-1, with

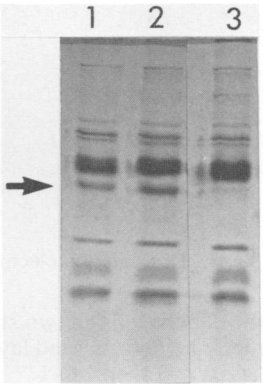

FIG. 2. Coomassie blue-stained sodium dodecyl sulfate–14% polyacrylamide gel electrophoresis of outer membrane proteins. Lanes: 1, Pf0-1; 2, Pf0-6; 3, Pf0-5. The arrow indicates the protein missing in the mutant.

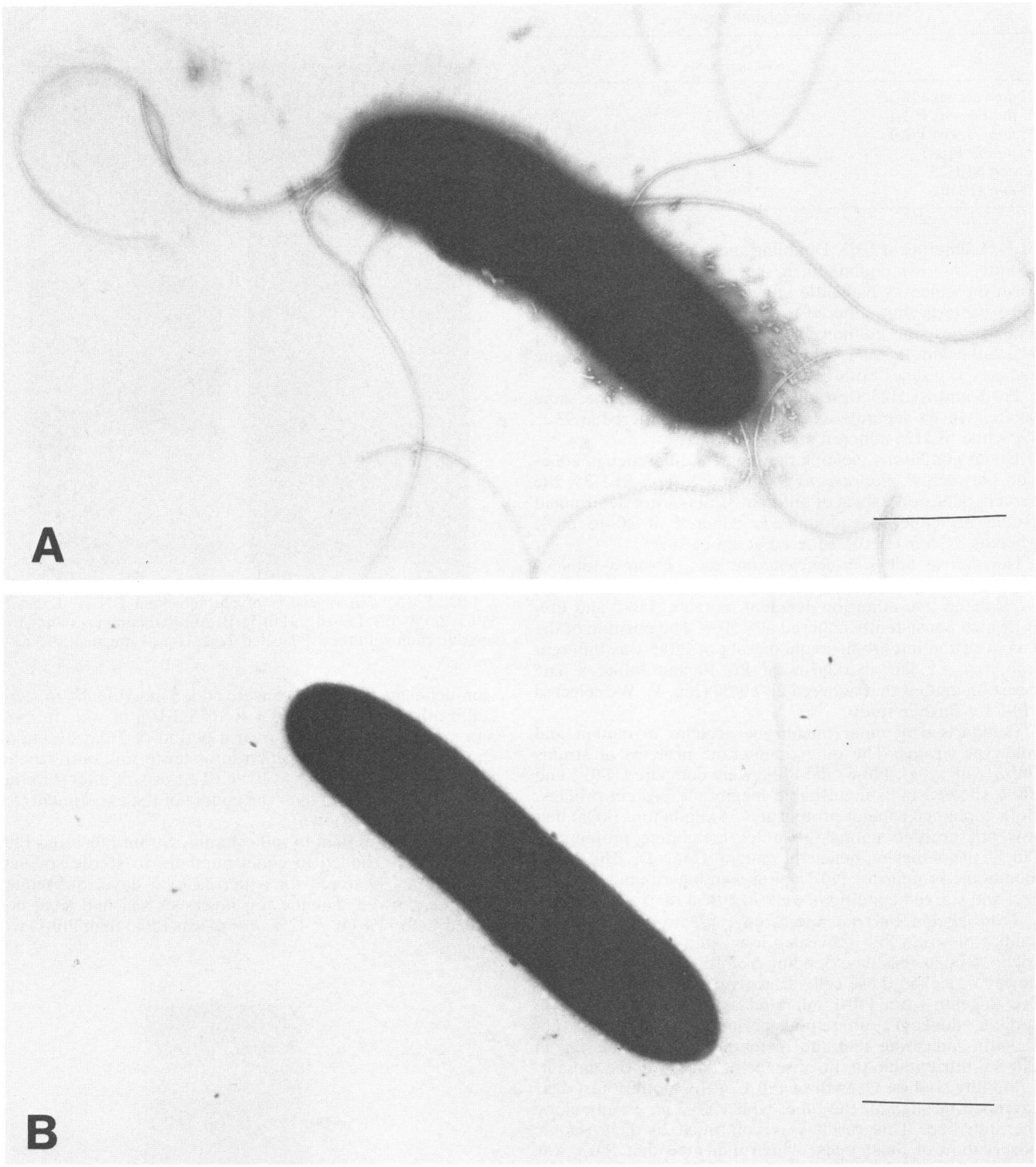

FIG. 3. Transmission electron micrographs of negatively stained Pf0-1 (A) and Pf0-5 (B). Bar, 1 μm.

the greatest differences between the two strains found in the 6-g soil-sand columns. The bottom sand layers had 68 to 84% (75 ± 7%, $n = 4$) less Pf0-5 than Pf0-1.

Site identified by Tn*5*. Two plasmids of different sizes (pMFD1 and pMFD2) were selected from the kanamycin- and tetracycline-resistant *E. coli* BD1527 that resulted from transfection with cosmid-containing phage λ. These two plasmids were mated into wild-type Pf0-1 for subsequent recombination of the Tn*5*-containing insert into the chromosome. Thirty-nine percent of the Km^r Tc^s Gm^r colonies grown for >100 generations lost pPH1JI (loss of Gm^r). The two different recombinant Pf0-1/5a and Pf0-1/5b (Km^r Tc^s Gm^s) strains tested in the adhesion assay adhered at 48 and 32%, respectively.

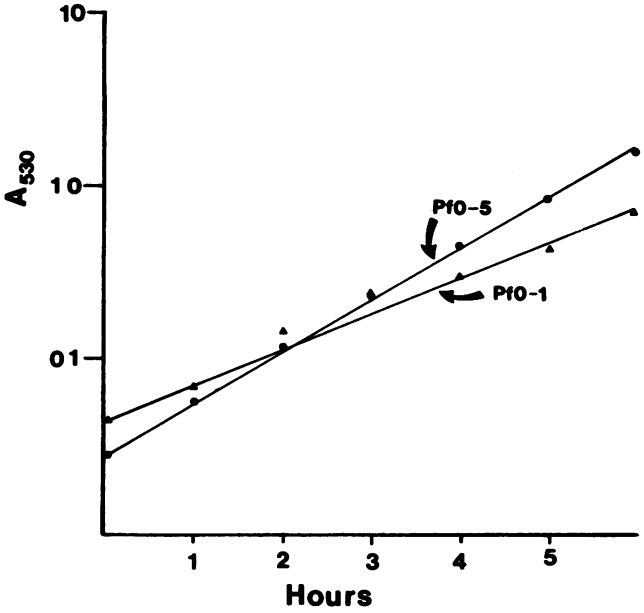

FIG. 4. Growth of Pf0-1 and Pf0-5 in minimal medium containing 1% glucose.

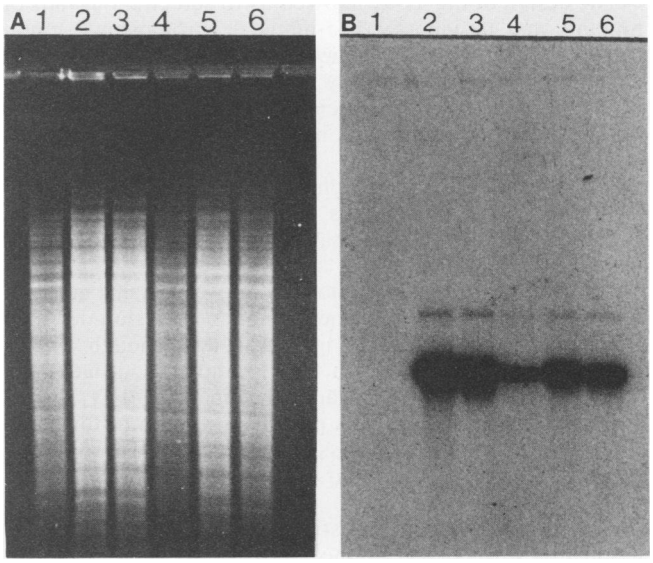

FIG. 6. (A) *SalI* digestion of chromosomal DNAs. Lanes: 1, Pf0-1; 2, Pf0-5; 3 and 4, Pf0-1/5a; 5 and 6, Pf0-1/5b. (B) Autoradiogram showing the position of the Tn*5* insertion in the chromosomes of the above-listed strains.

Outer membrane protein preparations of these two recombinants revealed a profile identical to that of Pf0-5 (Fig. 5). Probing *SalI* chromosomal digests with the *Tth*111I-*SalI* fragment of Tn*5* indicated that Tn*5* was in the same position in the recombinant Pf0-1 strains as in Pf0-5 (Fig. 6).

DISCUSSION

The reproducibility of the adhesion data obtained in the sand column assay indicates that the assay can distinguish between strains which adhere differentially to an inert matrix. Soil pseudomonads and two *E. coli* strains demonstrated a wide range of adhesive abilities. All soil isolates adhered in higher percentages than the enteric strains.

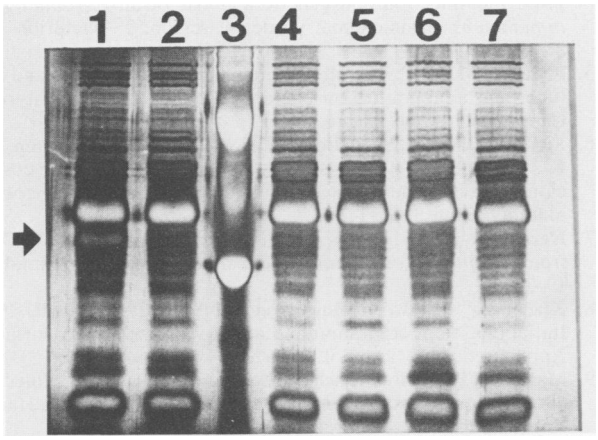

FIG. 5. Separation of outer membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (silver stain). Lanes: 1, Pf0-1; 2, Pf0-5; 3, molecular weight standards; 4 and 5, Pf0-1/5a; 6 and 7, Pf0-1/5b. The arrow indicates the outer membrane protein in the wild type which is absent in Pf0-5 and the mutants of Pf0-1 derived by marker exchange.

Although use of soil as the column matrix would have been more similar to the environment from which these strains were isolated, we found that soil columns filtered rather than measured adhesion of the bacteria. This feature of soil columns may partially explain results of others who studied soil adsorption of *Azospirillum brasilense* and recovered no cells from washed soil columns (2). In this work, however, *A. brasilense* applied to a sand column did not adhere and was almost completely removed by the wash procedure. In contrast, *P. fluorescens* in our assay remained attached to the sand column despite repeated washes. This result indicates a relatively strong mechanism of adhesion by these pseudomonads.

In optimizing the assay, we found that logarithmically growing cells adhered in higher percentages than stationary-phase cultures. However, minimal medium was a better growth medium for attachment than was L broth. These findings suggest that growth phase and nutrient state affect the attachment potential of these cells. Moreover, adhesion was best if cells were washed free of growth medium before inoculation. These results agree with previous reports that bacterial attachment to surfaces is one response to conditions of nutrient limitation (18, 21). Similar findings have been reported for marine bacteria (11, 15). Logarithmically growing marine pseudomonads adhered to polystyrene better than those in the stationary phase (15), and vibrios inoculated in salt solution rather than rich medium adhered more readily (11).

Increasing the ionic strength of a suspending buffer also decreases the electrostatic repulsion between two surfaces of like charge. If both the bacterial cell and the attachment surface are negatively charged, purely on the basis of physicochemical properties, increasing ionic strength should increase adhesion (30). In our assay, however, increasing the salt concentration in either the buffer in which the cells were added or in the wash buffer did not significantly affect adhesion. These results suggest that electrostatic repulsion is not important in adhesion of Pf0-1 to sand.

The design of the assay allowed us to screen thousands of mutants and to identify Tn*5*-induced chromosomal mutations in *P. fluorescens* which caused reduced ability to adhere to sand. Preliminary evidence suggests that this screening process is also efficient for *P. putida* soil isolates, although an adhesion mutant has not been verified from this group (unpublished data).

The adhesion deficiency phenotype suggested an alteration in the cell surface. This was confirmed by membrane protein profiles. A 34-kDa major protein in the outer membrane of the wild-type Pf0-1 strain and in the Tn*5* mutants which retain wild-type adhesion ability was missing in Pf0-5. Preliminary studies of the second adhesion-deficient Pf0-1::Tn*5* mutant, Pf0-10, indicate that although Tn*5* is located at a different site on the chromosome, it lacks the same 34-kDa outer membrane protein as Pf0-5. This is additional evidence that this protein is important for adhesion in this strain. Transmission electron microscopy and protein purification identified the wild-type protein as flagellin.

Although pili or fimbrial structures are more commonly associated with bacterial adhesion, flagella have been implicated in the adhesive abilities of certain *Vibrio* strains (1, 4). Adhesion of *P. fluorescens* to a soil amoeba has also been attributed to polar flagella as revealed by light microscopy. Electron microscopy suggested that this attachment was not confined to the flagellar tip but involved other surfaces of these appendages (26). Flagella of *P. fluorescens* were found to be essential for colonization of potato roots; this was attributed to lack of motility in flagellumless mutants (12). Our findings are the first to link a defined mutation site with the absence of this motility structure in *P. fluorescens* and reduced adhesion to an inert surface such as sand.

The molecular weight of the flagellum protein in *P. fluorescens* seems to vary with the strain. The flagella from a *P. fluorescens* isolate from potato roots which were purified by the same method that we used had a molecular mass of 58 kDa on a polyacrylamide gel (12), which is much larger than the 34-kDa molecular mass that we found for our purified flagellin. An approximate molecular weight obtained by sedimentation coefficients for *P. fluorescens* flagella was 38 kDa, although this was thought to be an underestimate (31). Antisera produced against this purified flagellin did not react with the flagella or flagellin of all of the *P. fluorescens* strains tested, indicating that there are indeed subspecies differences in flagella (31).

The residual adhesive ability of Pf0-5 (40 to 50%), which adhered at a higher percentage than the *E. coli* strains tested (8 to 22%), indicates that other proteins may be responsible for attachment. Although Pf0-5 does not have flagella, such adhesins may still be present on the cell surface and account for the residual attachment observed in this strain.

Linkage between the Tn*5* insertion and the adhesion-deficient phenotype of Pf0-5 was demonstrated by the marker exchange technique. Placement of Tn*5* into the same chromosomal site in unmutated strain Pf0-1 caused the adhesion deficiency phenotype of Pf0-5. The Tn*5* marker thus affords a means for identifying and cloning the gene(s) responsible for flagellar synthesis and understanding its role in adhesion.

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