JOURNAL OF BACTERIOLOGY, May 2000, p. 2363–2369 Vol. 182, No. 9
0021-9193/00/$04.00+0

Genetic Analysis of Functions Involved in Adhesion of *Pseudomonas putida* to Seeds

MANUEL ESPINOSA-URGEL,* AMPARO SALIDO, AND JUAN-LUIS RAMOS

Department of Plant Biochemistry and Molecular and Cellular Biology, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, E-18008 Granada, Spain

Received 23 September 1999/Accepted 6 January 2000

Many agricultural uses of bacteria require the establishment of efficient bacterial populations in the rhizosphere, for which colonization of plant seeds often constitutes a critical first step. *Pseudomonas putida* KT2440 is a strain that colonizes the rhizosphere of a number of agronomically important plants at high population densities. To identify the functions involved in initial seed colonization by *P. putida* KT2440, we subjected this strain to transposon mutagenesis and screened for mutants defective in attachment to corn seeds. Eight different mutants were isolated and characterized. While all of them showed reduced attachment to seeds, only two had strong defects in their adhesion to abiotic surfaces (glass and different plastics). Sequences of the loci affected in all eight mutants were obtained. None of the isolated genes had previously been described in *P. putida*, although four of them showed clear similarities with genes of known functions in other organisms. They corresponded to putative surface and membrane proteins, including a calcium-binding protein, a hemolysin, a peptide transporter, and a potential multidrug efflux pump. One other showed limited similarities with surface proteins, while the remaining three presented no obvious similarities with known genes, indicating that this study has disclosed novel functions.

Soil bacteria belonging to the species *Pseudomonas fluorescens* and *Pseudomonas putida* show metabolic versatility and a variety of characteristics that makes them attractive for environmental and agricultural uses (26). They can colonize the surface of plant roots and the surrounding soil regions (rhizosphere) in a mutualistic association in which the bacteria obtain nutrients from root exudates. In turn, some bacterial strains can promote plant growth and have biocontrol potential against certain pathogens (39). Also, some *P. putida* strains have the ability to degrade toxic organic compounds, which are frequently present as contaminants in the environment (26). *P. putida* KT2440, a derivative of the soil isolate mt-2 which has been widely studied in relation to biodegradation processes (17, 21, 28), can also colonize the rhizosphere of agronomically relevant plants at high population densities, making it a suitable candidate for its use in rhizoremediation (20).

Agricultural uses of microorganisms often involve coating seeds with bacterial suspensions. Adhesion to the seed appears as a key element, since it determines the subsequent colonization of the root system. Establishment of the bacterial population in the root and colonization of the rhizosphere are essential for biocontrol efficiency (8). These latter events, root colonization and survival in the rhizosphere, as well as responses to root exudates, are being extensively studied at the molecular level (5, 25, 30, 32, 33), but very little is known regarding the elements that are important for bacterial colonization of seeds. Results obtained by DeFlaun and coworkers have shown that some *P. fluorescens* mutants defective in attachment to soil particles are also defective in attachment to seeds (10, 11). Also, mutants of *P. putida* deficient in lipopolysaccharide have a limited capacity to attach to seeds and are impaired in root colonization (27). However, an exhaustive study of the functions specifically involved in the adhesion of *Pseudomonas* cells to plant seeds had not been carried out. In this paper we report the isolation and the phenotypic and molecular characterization of mutants of *P. putida* KT2440 deficient in adhesion to corn seeds.

* Corresponding author. Mailing address: Department of Plant Biochemistry and Molecular and Cellular Biology, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, E-18008 Granada, Spain. Phone: 34-958-121011. Fax: 34-958-129600. E-mail: mespinos@eez.csic.es.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *P. putida* KT2440 (17) was routinely used except for the mixed attachment experiments, for which KT2442, an otherwise isogenic rifampin-resistant derivative of KT2440 (17), was used. *Escherichia coli* strains and plasmids used for transposon mutagenesis (SM10-λ*pir* harboring pUT-Km; HB101 harboring RK600) have been previously described (12). Plasmid pDLDLUX contains the *luxABE* genes of *Vibrio fischeri* cloned in a shuttle vector, under the control of the *P. putida dld* (encoding D-lactate dehydrogenase) promoter (L. Molina, C. Ramos, and J. L. Ramos, unpublished data).

E. coli strains were grown at 37°C in Luria-Bertani (LB) medium (29). *P. putida* strains were grown at 30°C either in LB or in minimal medium (basal M9 medium [29] supplemented with Fe-citrate, $MgSO_4$, and trace metals [1]) and with benzoate (15 mM) or glucose (0.4%, wt/vol) as a carbon source. Growth rates and doubling times were analyzed on 20-ml cultures growing at 30°C in 100-ml flasks with orbital shaking, measuring the optical density at 600 nm at regular intervals.

When appropriate, antibiotics were added at the following concentrations (in micrograms per milliliter): chloramphenicol, 30; kanamycin, 50; rifampin, 30; ampicillin, 100; and gentamicin, 50. Chloramphenicol was routinely used for selection of KT2440 or its derivatives, since this strain is naturally resistant to this antibiotic.

Mutagenesis. Transposon mutagenesis with mini-Tn*5*-Km, a mini-Tn*5* derivative carrying a kanamycin resistance marker (12), was performed by triparental mating. The recipient (*P. putida* KT2440), donor (*E. coli* SM10-λ*pir* harboring pUT-Km, the suicide vector carrying mini-Tn*5*-Km), and helper (*E. coli* HB101 with RK600) strains were grown overnight in LB with the appropriate antibiotics. After incubation of the recipient at 42°C for 15 min, to temporarily inactivate its restriction systems, 0.7 ml was mixed with 0.2 ml of the donor and 0.1 ml of the helper. Cells were collected by centrifugation, resuspended in 50 μl of fresh LB, and spotted on an LB plate. After overnight incubation at 30°C, cells were scraped off the plate and resuspended in 1 ml of LB, and serial dilutions were plated on selective minimal medium (M9 with benzoate, kanamycin, and chloramphenicol).

Seed attachment assays. Corn seeds were surface sterilized by washing twice for 15 min with 70% (vol/vol) ethanol and twice with 20% (vol/vol) bleach, followed by profuse rinsing with sterile deionized water. Prior to the attachment experiments, seeds were routinely hydrated for 12 to 16 h. We have observed that this step increases bacterial adhesion to seeds. For the enrichment in adhesion-

deficient mutants, 25-ml syringes were filled with hydrated, surface-sterilized corn seeds. The bottom end of the syringes was closed, and different dilutions of a pool of transposon mutants were added in 5 ml of M9 basal medium. After their top ends were closed, the syringes were incubated for 1 h with orbital agitation. The syringes were then opened and washed with 5 ml of sterile M9 basal medium, and the whole flowthrough (10 ml) was collected. Dilutions were plated in selective minimal medium.

Qualitative adhesion assays were used as a fast method for the initial identification of putative attachment-deficient mutants. The assays were performed as follows. One microliter of overnight culture grown in LB was inoculated in 1 ml of M9 basal medium, and one seed was added to each bacterial suspension. After incubation for 1 h at room temperature, seeds were removed, washed thoroughly with deionized water, and introduced in tubes containing 1 ml of LB. Tubes were incubated at 30°C, and appearance of turbidity was monitored over time, the rationale being that cells attached to the seeds would start growing and dividing due to the presence of fresh nutrients. Thus, a delay in the appearance of turbidity in a particular strain with respect to the wild type could reflect reduced bacterial attachment to the seeds.

For the quantitative assays, bacterial incubation with the seeds was done as described above except that the number of cells inoculated was determined by plating serial dilutions on LB plates prior to addition of the seeds. After incubation, seeds were removed, washed, and transferred to tubes containing M9 basal medium. The tubes were vortexed for 1 min to remove any bacteria that may not have been tightly attached to the seed surface. Seeds were washed again and disrupted by vortexing with 3-mm-diameter glass beads in M9 basal medium (27). This procedure does not affect bacterial viability. To estimate the percentage of cells attached to the seeds with respect to the number of inoculated cells, dilutions of the resulting suspension were plated on selective medium. Each assay was repeated at least three times.

Attachment of luminescent bacteria was detected as follows. Seeds incubated with the bacterial suspensions and washed as described above were placed on filter paper soaked with 0.01% (vol/vol) *n*-decyl aldehyde (Sigma). Seeds were then covered with plastic wrap, and luminescence was detected after overnight exposure of autoradiographic film (Kodak X-Omat).

Motility and chemotaxis assays, antibiotic sensitivity, and microscopy. Motility was tested in 0.3% (wt/vol) agar plates, in LB or M9 with benzoate. Chemotaxis toward seeds was assayed with a procedure similar to that described by Van Bastelaere et al. (35), namely, 100 μl of each overnight culture was mixed with a cooled (42°C) 0.2% (wt/vol) water agar solution. The mixture was poured onto petri dishes and allowed to solidify. Three seeds were then placed in different spots on the surface of each plate, and the plates were incubated at 30°C. Positive chemotactic responses were observed after 5 h as the appearance of concentric halos around the seeds.

Sensitivity to antibiotics was determined by measuring the inhibition halos on LB plates, with disks (bioMérieux) containing the following amounts (micrograms) of the different antibiotics: ampicillin, 10; tetracycline, 30; carbenicillin, 100; piperacillin, 100; streptomycin, 10; and gentamicin, 10.

Bacterial morphology was visualized under phase contrast on a Zeiss Axioskop light microscope.

Biofilm formation assays. Formation of biofilms on abiotic surfaces was assessed essentially as described elsewhere (22). Cultures were grown overnight and inoculated (1:20 dilution) in 50 μl of LB or M9, supplemented with either benzoate or glucose, in polystyrene microtiter dishes. After 4 h of incubation at 30°C, the wells were washed with deionized water, and 100 μl of a 1% (wt/vol) solution of crystal violet was added to each well. Plates were incubated at room temperature for 15 min and washed thoroughly, and biofilm formation was quantified. For this purpose, the stain was solubilized by adding ethanol (200 μl twice) to each well. This solution was then transferred to an Eppendorf tube, and 600 μl of distilled water was added. Absorbance at 600 nm was then measured in a Perkin-Elmer Lambda 20 spectrophotometer. Similar assays were performed on other surfaces, polypropylene (Eppendorf tubes), and borosilicate (glass tubes).

Analysis of proteins. A fast extraction method was used for preparation of surface proteins by heat treatment (19). One milliliter of each overnight culture was centrifuged, and cells were resuspended in 40 μl of 0.9% (wt/vol) NaCl. After incubation for 15 min at 65°C and centrifugation at 3,000 × *g* for 5 min, supernatants were transferred to clean tubes and immediately frozen. Samples were boiled in the presence of sodium dodecyl sulfate (SDS) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described elsewhere (2).

DNA techniques. Preparation of plasmid and chromosomal DNA, digestion with restriction enzymes (Boehringer), electrophoresis, and Southern blotting were carried out using standard methods (2, 29). Hybridizations were done using the DIG DNA labeling and detection kit (Boehringer) as instructed by the manufacturer. Electroporation of plasmid DNA into *P. putida* was done as described elsewhere (22).

DNA sequences from the transposon mutants were determined using arbitrary PCR (9) with *Taq* DNA polymerase (Pharmacia) on a Perkin-Elmer GeneAmp 9600. A first round of amplification was done by using the chromosomal DNA of the mutants as a template, with an arbitrary primer (ARB1; 5′-GGCACGCGTC GACTAGTACNNNNNNNNNGATAT-3′) and an internal primer of mini-Tn*5*, unique for the right end (TNEXT; 5′-TGATGAATGTTCCGTTGCGCT GCC-3′). The first round was as follows: 3 min at 95°C; 6 cycles of 30 min at 95°C, 30 min at 30°C, and 1 min at 72°C; 30 cycles of 30 min at 95°C, 30 min at 50°C, and 1 min 72°C; and an extension period of 7 min at 72°C. A second round of amplification was done with 5 μl of the first-round reaction as the template as follows: 3 min at 95°C; 30 cycles of 30 min at 95°C, 30 min at 57°C, and 1 min at 72°C; and 7 min at 72°C. Primers used for the second round were those corresponding to the conserved region of ARB1 (ARB2; 5′-GGCACGCGTCGACT AGTAC-3′) and a second internal primer of mini-Tn*5*, closer to the end (TNINT; 5′-GACCTGCAGGCATGCAAGCTCGGC-3′).

Reaction mixtures were electrophoresed, and the most intense bands were isolated with a Qiagen gel extraction kit and sequenced. Sequencing was done on an ABI PRISM 310 automated sequencer, using oligonucleotide TNINT as a primer. The ~40-bp distance between this primer and the end of the mini-Tn*5* provides an internal control to ensure that the obtained sequence corresponds to the junction between the transposon and the chromosome.

Sequences were analyzed and compared with the GenBank database by using BLAST programs (3). Preliminary sequence data for the *P. putida* and *P. aeruginosa* genomes were obtained from The Institute for Genomic Research (www.tigr.org) and the Pseudomonas Genome Project (www.pseudomonas.com), respectively.

Nucleotide sequence accession numbers. The sequences reported have been deposited in GenBank under accession no. AF182512 through AF182519.

RESULTS

Adhesion of *P. putida* KT2440 to corn seeds: roles of protein synthesis and surface proteins. To begin analyzing the functions involved in the attachment of KT2440 to corn seeds, we tested whether new synthesis of proteins, occurring in the presence of seeds, was required for attachment. Initially, quantitative analyses were performed as described in Materials and Methods, incubating corn seeds with a suspension of overnight grown cells diluted 1:1,000 in M9 basal medium. After 1 h of incubation, the average number of attached bacteria was 0.65% of the number of inoculated cells. To assess the role of newly synthesized proteins, tetracycline was added before incorporating the seeds to the bacterial suspension, in order to block translation. Addition of tetracycline had no effect on viability and did not affect significantly the attachment of KT2440 to corn seeds. The number of attached cells was approximately 89% with respect to the control without tetracycline treatment, suggesting that most of the functions required for efficient bacterial adhesion to the seeds in the conditions of our experiments are already present in the stationary-phase cultures of KT2440 after growth in rich medium.

Outer membrane proteins have been shown to play an important role in the attachment to biotic and abiotic surfaces (15, 22, 32). To determine the involvement of extracellular proteins in the initial steps of seed colonization, adhesion assays were performed after incubation of the bacterial cultures in the presence of 20 μg of proteinase K per ml. In this case, while viability was unaffected, attachment was significantly reduced. The number of attached cells was 20% of the number of cells attached in the control without treatment. This result indicates that one or more surface-located or secreted proteins are involved in the adhesion of bacterial cells to corn seeds.

Screening for mus derivatives of KT2440. To identify functions involved in the initial steps of seed colonization, we designed a screen to isolate mutants showing deficiencies in adhesion to seeds, which we termed "mus" (mutants unattached to seeds). The general procedure (outlined in Fig. 1) is based on the column method described by DeFlaun and coworkers (10) to identify mutants of *P. fluorescens* unable to bind to soil particles. KT2440 was mutagenized by random insertion of mini-Tn*5*-Km (12). A pool of kanamycin-resistant mutants was then generated by collecting ~10,000 colonies, obtained after mutagenesis, and plating in selective minimal medium (thus avoiding the isolation of auxotrophs). Dilutions of this pool were incorporated into syringes filled with surface-sterilized corn seeds and then incubated to enrich in attach-

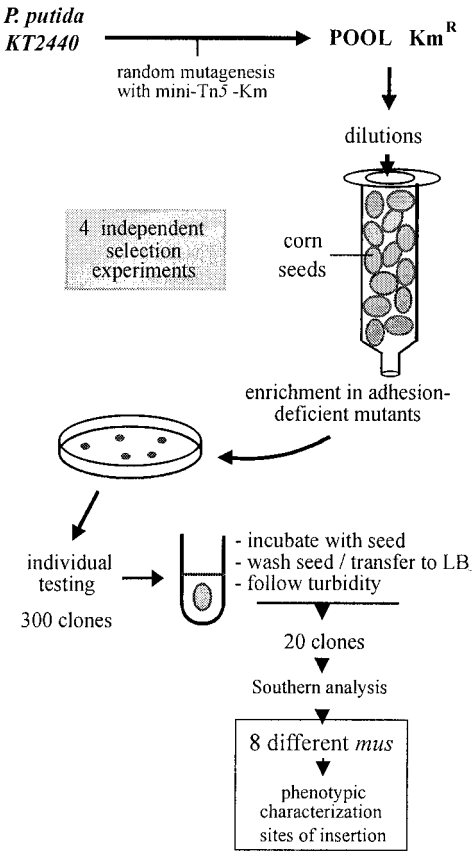

FIG. 1. Summary of the screening strategy used to isolate mus derivatives of *P. putida* KT2440. Details are presented in the text.

ment-deficient mutants described in Materials and Methods. After incubation and collection of the flowthrough (i.e., cells that had not adhered to the seeds), dilutions were plated in selective minimal medium. Four independent selection experiments were performed, in which 7×10^3, 7×10^4, 7×10^5, and 2×10^7 cells were inoculated into the columns; 300 of the colonies obtained were then tested individually for the ability to attach to seeds. For this second round of selection, each clone was grown overnight, and those with obvious growth defects were discarded. A qualitative attachment assay was performed with the rest of the strains as described in Materials and Methods. After incubation with each bacterial suspension, washing, and subsequent incubation of the seeds at 30°C in tubes containing LB medium, appearance of turbidity was monitored after 6 to 10 h. The clones presenting a delay in the appearance of turbidity with respect to the wild type were selected as being potentially defective in attachment and subjected to a second round of a similar selection process. After these rounds of selection, 20 mutants were identified as putative mus clones.

Since we used a pool of colonies for the first selection step, it was possible that some of the mus clones obtained were identical. To eliminate these potential siblings, Southern hybridization was performed using pUT-Km as a probe on chromosomal DNA of the 20 clones digested with *Bst*EII, for which there is no site inside the transposon. Only one band giving hybridization signals appeared in each case, indicating that a single copy of the transposon was present in all the mutants (data not shown). When two or more clones presented a band of the same size, only one of them was selected, the rest being discarded as potential siblings (Table 1), and some of them were confirmed later by arbitrary PCR and sequencing. Thus, eight clones were finally chosen for further analysis.

Phenotypic characterization of mus mutants. To begin the characterization of these eight mus mutants, we compared their growth rates to those of the parental strain in different culture media, to confirm that the delayed appearance of turbidity observed in the qualitative attachment assays was not due to some metabolic defect affecting their growth. All of the mus clones showed doubling times similar to that of the wild type in LB (55 ± 3 min) and in M9 minimal medium with either benzoate (95 ± 2 min) or glucose (99 ± 4 min) as a carbon source.

Quantitative attachment assays were then performed as described above. Results are presented in Fig. 2A. Adhesion of all the selected mutants to corn seeds was significantly reduced with respect to the wild type, ranging from 3.5 to 15 times fewer cells recovered from the disrupted seeds relative to the parental strain. To ensure that these reduced numbers were not simply due to the mutants being unable to survive in basal M9 medium without a carbon source during the period of incubation, the eight clones were incubated in this medium for the same time but in the absence of seeds. No significant loss of viability was observed in any case (data not shown).

A direct adhesion assay was performed to confirm the results obtained from the quantitative assays. For this purpose, KT2440 and the eight mus strains were transformed with pDLDLUX, a plasmid harboring the bioluminescence genes *luxABE* under the control of the *dld* promoter, the expression of which is constitutive in *P. putida* (Molina et al., unpublished data). Attachment assays were performed with these luminescent strains as described in Materials and Methods, and the results (Fig. 2B) were recorded by exposing autoradiographic film to the seeds after incubation with the bacterial suspensions. In all cases, the seeds inoculated with the mus strains showed less luminescence than the seeds incubated with the parental strain, indicating a lower number of cells attached to the seeds. Although there is not an exact correlation between the data from the luminescence assay and the quantitative assay, this result confirms the attachment defects in all of the mus mutants.

Adhesion of each mutant was also tested in coinoculations with KT2442, an otherwise isogenic rifampin-resistant derivative of KT2440 (17). These assays were performed to determine whether the mutant cells were less competitive than the wild type in their adhesion to seeds and whether the presence

TABLE 1. Phenotypes of mus mutants

Strain	No. of siblings[a]	Motility in M9[b]	Chemotaxis toward seed[c]	Cell morphology	Colony morphology
Wild type		+	+	Normal	Normal
mus-5	1 (1)	+/−	+	Normal	Normal
mus-9	1 (1)	+	+	Normal	Normal
mus-13	8 (4)	+/−	+/−	Normal	Normal
mus-20	2 (1)	+	+	Normal	Normal
mus-21	5 (2)	+	+	Normal	Normal
mus-22	1 (1)	+	+/−	Elongated	Normal
mus-24	1 (1)	+	+	Elongated	Small (in LB)
mus-27	1 (1)	+	+	Normal	Small (in LB)

[a] Deduced from Southern hybridization, some confirmed by sequencing (number of sequenced clones is in parentheses).
[b] +, motility or chemotaxis halos undistinguishable from the parental strain; +/−, delay in the appearance of halos with respect to the wild type.

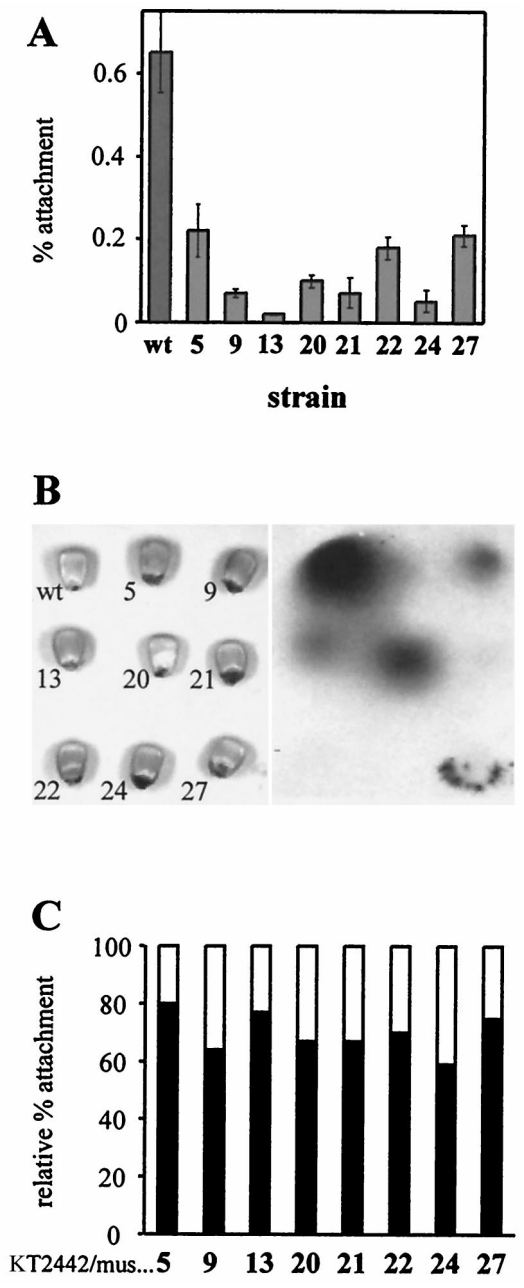

FIG. 2. Adhesion of *P. putida* KT2440 (wild type [wt]) and mus mutants to corn seeds. (A) Quantitation of attachment of *P. putida* KT2440 and eight mus mutants to corn seeds. After 1 h of incubation with each bacterial suspension, seeds were washed and disrupted, and the number of attached cells was estimated as CFU after plating serial dilutions. Results are presented as percentage of attached cells with respect to the number of cells inoculated (average of at least three independent experiments). (B) Adhesion to corn seeds (left) of KT2440 and mus mutants harboring plasmid pDLDLUX, visualized by overnight exposure of film (right). (C) Adhesion of mus mutants (white bars) coinoculated with KT2442 (grey bar). Relative percentages of cells of each strain attached to the seeds (average of three independent assays) are shown.

of the wild-type strain could restore the ability of some mutants to attach to corn seeds. Results are shown in Fig. 2C. In all cases, after coinoculation of seeds with KT2442 and the mutants (1:1 ratio), the number of rifampin-resistant cells recovered was higher than the number of kanamycin-resistant cells, indicating that the mutants have a competitive disadvantage in seed colonization (coincubation in the absence of seeds had no effect on viability of any of the mutants). Yet, in two of the mixtures (with mus-9 and mus-24), the mutants constituted around 40% of the attached population, suggesting that the presence of the wild-type strain may partially complement the defects of these mutants. In these two mutants, the percentage of cells attached doubled with respect to the percentage obtained in the individual assays (data not shown).

Motility and chemotaxis have been shown to play an important role in bacterial attachment to both biotic and abiotic surfaces (13, 23, 24, 36). Therefore, we tested the motility of KT2440 and the eight mutants in 0.3% (wt/vol) agar plates, both in LB and in M9 with benzoate. All of the strains were motile and indistinguishable in LB. However, when the assay was performed in M9 supplemented with benzoate, strains mus-5 and mus-13 presented a delay in the formation of a motility halo, which was slightly but consistently smaller than that of the remaining strains (Table 1). We also performed a qualitative chemotaxis assay similar to that described by Van Bastelaere (35). KT2440 and the eight mutants were tested on 0.2% (wt/vol) agar plates for chemotaxis toward corn seeds, identified by the formation of concentric halos around the seeds (see Materials and Methods). Differences were detected only for mus-13 and mus-22, both of which showed a delay in the formation of chemotaxis halos (Table 1).

Colony and cell morphology were also monitored. Mutants mus-24 and mus-27 formed colonies slightly smaller than those formed by the rest of strains on LB plates; mutants mus-22 and mus-24 presented altered morphology, cells being two to three times longer than those of the parental strain (Table 1).

Attachment of mus mutants to abiotic surfaces. Mutants defective in biofilm formation on abiotic surfaces have been isolated and characterized in different bacterial species (22–24). We were interested in the possible correlation between attachment to biotic and to abiotic surfaces and whether the mutants isolated in our screen showed a general adhesion deficiency or were altered in functions specific for seed colonization. We therefore tested biofilm formation of KT2440 and the eight mus clones on different abiotic surfaces. These experiments were performed essentially as described by O'Toole and Kolter (22) on polystyrene (microtiter dishes), polypropylene (Eppendorf tubes), and borosilicate (glass tubes) surfaces. Since biofilm formation can vary depending on the growth medium, we also tested attachment to polystyrene of cultures grown on LB and on M9 supplemented with either glucose or benzoate. Biofilm formation was analyzed after 4 h of growth by staining with crystal violet. The amounts of attached cells were quantified after solubilization of the stain and measurement of optical density at 600 nm. The results obtained in these assays are shown in Fig. 3. Two mutants, mus-22 and mus-24, showed severe defects in biofilm formation in all the tested surfaces, independently of the growth medium. This result indicates that these clones were affected in general functions required for adhesion to both biotic and abiotic surfaces. The remaining mutants presented a slight reduction in biofilm formation in some of the growth conditions or surfaces tested, but the differences with the parental strain were generally not relevant. These results suggest that the interrupted genes are more specifically involved in plant-bacterium interaction.

Molecular characterization of mus mutants. As indicated above, attachment of *P. putida* KT2440 to corn seeds appears to be strongly dependent on surface proteins, since the addition of proteinase K drastically reduces adhesion. Thus, it seemed reasonable that at least some of the mutants isolated presented defects in such proteins. To explore this possibility, surface proteins of KT2440 and the mus clones were analyzed

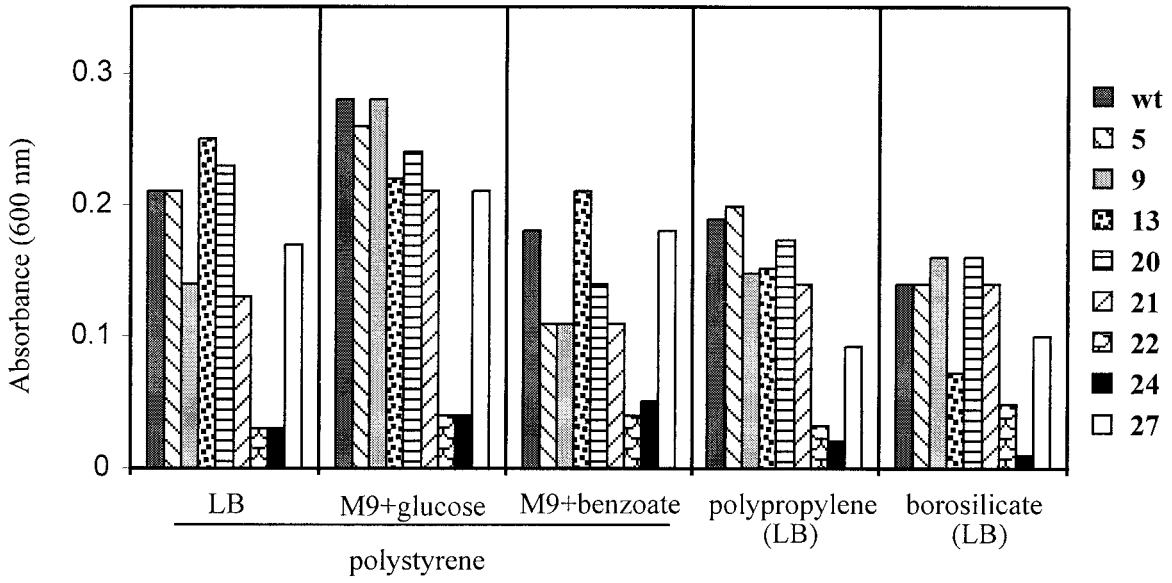

FIG. 3. Biofilm formation on different abiotic surfaces, quantified by staining attached cells with crystal violet and measuring A_{600} after solubilization of the stain with ethanol. Results are the average of four independent assays. wt, wild type.

by SDS-PAGE (Fig. 4). Several clones presented differences with respect to the parental strain, the most obvious being mus-20, mus-22, and mus-24. The latter two mutants showed similar results, with two bands of ~60 and ~29 kDa presenting clearly reduced intensity with respect to the wild type. In mus-22 a third band, of ~40 kDa, was missing. As for mus-20, two bands (~65 and ~45 kDa) of stronger intensity with respect to KT2440 are apparent, while a band of ~35 kDa shows reduced intensity. These results suggest that the attachment defects of some of the mutants could be due to alterations in their surface structures.

To determine the chromosomal locations of the transposon insertions and identify the gene disrupted in each case, arbitrary PCR (9) was performed with all mutants as described in Materials and Methods. This technique allowed us to amplify fragments containing 150 to 600 bp of DNA flanking the transposon insertion site. These PCR fragments were gel purified and sequenced. Sequences were compared with the unfinished genome of *P. putida* KT2440. The insertion sites in four mutants (mus-13, mus-21, mus-24, and mus-27) could be unambiguously identified (Table 2). In these cases, sequences of ~2 kb around the insertion point were then used for comparisons with the databases using BLAST programs (3). For the remaining mutants, the sequences obtained from the PCR fragments were used. The results from these analyses are summarized in Table 2. For three loci, *mus-9*, *mus-20* and *mus-22*, no significant match could be found with any sequences in the databases. In the case of *mus-5*, some similarity could be found with a putative adhesion factor of *E. coli* resembling the *prn* gene of *Bordetella pertussis*. The product of this gene, pertactin, is a virulence factor involved in bacterial adhesion to eukaryotic cells (37).

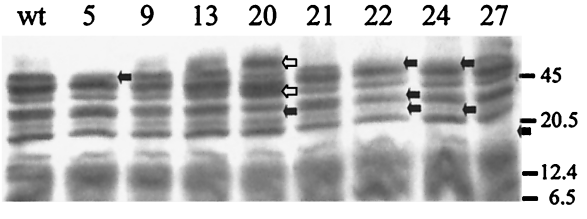

FIG. 4. Surface proteins of KT2440 and mus strains. Surface proteins were analyzed by SDS-PAGE on a 12% polyacrylamide gel and staining with Coomassie brilliant blue. Bands showing less intensity in the mutants than in the parental strain (wild type [wt]) are marked with black arrows; bands with more intensity in the mutants than in KT2440 are indicated with white arrows. Positions of molecular weight markers are indicated in kilodaltons.

In the remaining four mutants analyzed, the insertion had taken place in genes with known homologues in other microorganisms. Of these, *mus-24* showed similarities with various surface proteins, including hemolysins and other toxins, all of which present a repeated motif presumably involved in binding of calcium. Calcium-binding proteins have been identified as important factors in root colonization and nodulation by *Rhizobium* (16, 32). The gene showing the highest similarity with *mus-24* was *expE1* of *Sinorhizobium meliloti* (6), which is in turn closely related to the nodulation factor *nodO* of *Rhizobium*. Locus *mus-27* showed similarities with hemolysins of *Serratia marcescens*, *Proteus*, and the fish pathogen *Edwardsiella tarda* (18). As for *mus-13*, the locus where insertion had taken place presented significant similarities with the *E. coli cstA* gene (coding for carbon starvation protein A [31]) and other *E. coli* homologues of *cstA* which code for putative carbon starvation proteins. CstA is a membrane protein apparently involved in peptide transport (31), whereas the functions of the other homologues are not known. Also *mus-21* shows similarity to membrane proteins, the products of the *kefB* and *kefC* genes of *E. coli* and *Myxococcus*, both of which are glutathione-regulated K^+ efflux pumps (4).

It has been recently reported that a toxin-responsive efflux pump could be important for interaction of a fungal pathogen with rice plants (34). Also, multidrug resistance has been associated with bacterial adhesion to eukaryotic cells (14). We therefore considered the possibility of mus-21 being affected in a gene coding for a multidrug efflux pump. Sensitivity of KT2440 and mus-21 to various antibiotics was tested. In general, mus-21 was significantly more sensitive to all the β-lactam antibiotics tested (ampicillin, carbenicillin, and piperacillin)

TABLE 2. Molecular characterization of mus mutants

Locus	Gene or homologs (organism[s])	Possible function	*P. putida* contig[a]	Homology in *P. aeruginosa*
mus-5	*orf*b1202, *prn* (*E. coli*, *B. pertussis*)	Adhesion factor	ND	Not found
mus-9	No match	Unknown	ND	Contig 54, 90% (in 50 nt)
mus-13	*cstA* (*E. coli*)	Peptide transport	all_930, all_931	Contig 49, 86%
mus-20	No match	Unknown	ND	Not found
mus-21	*kefB*, *kefC* (*E. coli*, *Myxococcus*)	Efflux pump	all_521	Contig 53, 82%
mus-22	No match	Unknown	ND	Not found
mus-24	*expE1* (*S. meliloti*)	Calcium-binding protein	all_2222	Not found
mus-27	*ethA*, *hlyA* (*E. tarda*, *Proteus*)	Hemolysin	all_1784	Not found

[a] Contigs follow the nomenclature provided by the databases. nd, contig containing the relevant sequence could not be unambiguously determined.

than the parental strain, while it did not show significant differences in resistance to tetracycline, streptomycin, and gentamicin. These results suggest that the gene affected in strain mus-21 could in fact code for a multidrug efflux pump involved in transport of β-lactam antibiotics.

The sequences obtained from all the mutants were also compared with the genome of *P. aeruginosa* (Table 2), which is finished but not completely assembled. Only *mus-13* and *mus-21* showed obvious matches with sequences from *P. aeruginosa*, with 86 and 82% identical residues at the nucleotide level, respectively. Relevant similarity was also found for *mus-9*, but only in the last 50 nucleotides of the sequenced fragment (90% identities). Completion of the assembly of the sequences of both the *P. aeruginosa* and *P. putida* genomes will allow us to distinguish which of the genes identified here are present in the two species and which are specific of *P. putida*.

DISCUSSION

To understand the functions and processes involved in bacterial colonization of plant seeds, we have developed a screen to isolate mutants of *P. putida* defective in adhesion to corn seeds. The first step of the screen, passing a pool of insertional mutants through a seed column, was an efficient system to enrich in mus derivatives of KT2440, although the possibility of having missed mutants defective in cell-to-cell interactions cannot be discarded as a potential disadvantage of this selection procedure. This caveat notwithstanding, out of 300 clones obtained from four independent enrichment experiments, tested individually, 20 presented the desired phenotype consistently. Several of these proved to be possible siblings, based on Southern analysis, some confirmed by sequencing. Thus, eight different mutants were finally identified. All of them show clear defects in the ability to adhere to corn seeds, both by themselves and when inoculated with the parental strain. The fact that identical insertion mutants were isolated in independent selection experiments validates the screen and may give an idea of the most important functions required for the attachment to seeds under the conditions used here. Extracellular proteins seem to play an important role in attachment, as suggested by the decrease in adhesion when KT2440 is incubated with proteinase K and by the fact that several of the mutants show alterations in their patterns of surface proteins.

The eight mus clones have been further characterized, and the insertion sites have been sequenced. All correspond to genes that have not been described before in *P. putida*. Three of them gave no significant match with sequences in the databases, indicating that they could code for novel functions related to seed colonization. Of the remaining five, an interesting finding is that two loci, *mus-5* and *mus-27*, have similarities with genes of other organisms coding for factors involved in pathogenesis (pertactin and hemolysins). These factors are involved in the early steps of bacterial colonization of host tissues. Another locus, *mus-21*, codes for a putative multidrug efflux pump. Such an efflux pump has been recently identified as a pathogenicity factor in *Magnaporthe grisea*, the fungus responsible for rice blast disease (34). The function of such transporters would be to protect the microorganism against toxic compounds produced by the plant as a defense response. Finally, *mus-24* shows a putative calcium-binding motif present in nodulation proteins as well as in virulence factors and other surface proteins. All of these data suggest that there are common mechanisms involved in the early stages of colonization of host tissues by pathogenic and nonpathogenic microorganisms.

A noticeable result is the fact that the mutants identified in our screen were not affected in other functions previously known to play a role in adhesion, such as flagella, pili, or other aggregation factors (7, 13, 15, 24). Motility has been shown to play an important role in the attachment of *Pseudomonas* and other bacteria to biotic and abiotic surfaces (23, 24, 36). However, none of the mus clones were nonmotile, although two of them showed a slight defect in minimal medium. We also expected to find mutants deficient in chemotaxis, another important factor in bacterial attachment (24, 36). Again, two mutants showed a slight defect but not a null phenotype. It could be that in our experimental conditions the relative importance of these functions was diminished. Incubating the bacteria in a column filled with seeds, with constant agitation, probably facilitates adhesion without the need for cells to move toward the seeds. The situation may be different in natural settings, where the bacteria have to find the seeds. It is worth noticing, however, that mus-13 showed defects both in motility in minimal medium and in chemotaxis. Transposon insertion in this clone had taken place in a locus very similar to a family of *E. coli* genes including *cstA*, the product of which seems to be involved in peptide transport (31). It is possible that peptides and amino acids, which are relevant components of root exudates (25), act as chemoattractants toward the seeds, being recognized and utilized as nutrients by the bacterial cells. The fact that mus-13 shows a strong defect in seed attachment, and that siblings of this mutant (up to eight) were isolated in all four independent screens, suggests that this gene plays a key role in seed colonization.

We have also investigated whether mus clones were generally defective in adhesion, although none of the genes identified here correspond to previously identified genes involved in attachment of *Pseudomonas* to abiotic surfaces (22). Two of the mutants, mus-22 and mus-24, appear to be severely affected in biofilm formation on abiotic surfaces; the rest do not show appreciable defects. Thus, two types of mutants seem to have been isolated, some with general defects in adhesion to solid surfaces and others which appear to be more specifically deficient in seed attachment. It has been proposed that bacterial biofilm formation proceeds through divergent pathways de-

pending on whether the surface can be a source of nutrients or not (38). Our results may support that idea, although there are common elements involved in both, exemplified by mutants mus-22 and mus-24.

The results presented here give an overview of the important functions for seed colonization by *P. putida*. Although a more detailed analysis of the identified genes and their specific roles will be required, several conclusions can be drawn from this study. First, the isolation of genes of unknown function and with no obvious similarities with previously described genes indicates that we may be unraveling novel elements of the physiology and genetics of *Pseudomonas*. Second, there seem to be somewhat divergent processes involved in bacterial adhesion to biotic and abiotic surfaces, with some aspects common to both. Finally, the similarities of several of the genes identified here with virulence factors of pathogenic microorganisms suggest that initial colonization of host tissues proceeds via similar or common pathways. Thus, as suggested by previous findings (15, 23), there seems to be a thin line between pathogenesis and mutualistic association of bacteria with eukaryotic organisms.

ACKNOWLEDGMENTS

We thank A. Hurtado for DNA sequencing and G. O'Toole for suggestions and helpful discussions. Preliminary sequence data were obtained from The Institute for Genomic Research and the Pseudomonas Genome Project.

This work was supported by grant BIO4-CT97-2313 from the European Union.

REFERENCES

1. **Abril, M. A., C. Michán, K. N. Timmis, and J. L. Ramos.** 1989. Regulation and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J. Bacteriol. **171:**6782–6790.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
3. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
4. **Bakker, E. P., I. R. Booth, U. Dinnbier, W. Epstein, and A. Gajewska.** 1987. Evidence for multiple K^+ export systems in *Escherichia coli*. J. Bacteriol. **169:**3743–3749.
5. **Bayliss, C., E. Bent, D. E. Culham, S. MacLellan, A. J. Clarke, G. L. Brown, and J. M. Wood.** 1997. Bacterial genetic loci implicated in the *Pseudomonas putida* GR12-2R3–canola mutualism: identification of an exudate-inducible sugar transporter. Can. J. Microbiol. **43:**809–818.
6. **Becker, A., S. Ruberg, H. Kuster, A. A. Roxlau, M. Keller, T. Ivashina, H. P. Cheng, G. C. Walker, and A. Puhler.** 1997. The 32-kilobase *exp* gene cluster of *Rhizobium meliloti* directing the biosynthesis of galactoglucan: genetic organization and properties of the encoded gene products. J. Bacteriol. **179:**1375–1384.
7. **Buell, C. R., and A. J. Anderson.** 1992. Genetic analysis of the *aggA* locus involved in agglutination and adherence of *Pseudomonas putida*, a beneficial fluorescent pseudomonad. Mol. Plant-Microbe Interact. **5:**154–162.
8. **Bull, C. T., D. M. Weller, and L. S. Thomashow.** 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. Phytopathology **81:**954–959.
9. **Caetano-Annoles, G.** 1993. Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Appl. **3:**85–92.
10. **DeFlaun, M. F., A. S. Tanzer, A. L. McAteer, B. Marshall, and S. B. Levy.** 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **56:**112–119.
11. **DeFlaun, M. F., B. Marshall, E.-P. Kulle, and S. B. Levy.** 1994. Tn*5* insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. Appl. Environ. Microbiol. **60:**2637–2642.
12. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
13. **De Weger, L. A., C. I. M. Van der Vlugt, A. H. M. Wijfjes, P. A. H. M. Bakker, B. Schippers, and B. J. J. Lugtenberg.** 1987. Flagella of a plant growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. J. Bacteriol. **169:**2769–2773.
14. **Di Martino, P., D. Sirot, B. Joly, C. Rich, and A. Darfeuille-Michaud.** 1997. Relationship between adhesion to intestinal Caco-2 cells and multidrug resistance in *Klebsiella pneumoniae* clinical isolates. J. Clin. Microbiol. **35:**1499–1503.
15. **Dörr, J., T. Hurek, and B. Reinhold-Hurek.** 1998. Type IV pili are involved in plant-microbe and fungus-microbe interactions. Mol. Microbiol. **30:**7–17.
16. **Economou, A., W. D. O. Hamilton, A. W. B. Johnston, and J. A. Downie.** 1990. The *Rhizobium* nodulation gene *nodO* encodes a Ca^{2+}-binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. EMBO J. **9:**349–354.
17. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta*-cleavage pathway. Proc. Natl. Acad. Sci. USA **78:**7458–7462.
18. **Hirono, I., N. Tange, and T. Aoki.** 1997. Iron-regulated haemolysin gene from *Edwardsiella tarda*. Mol. Microbiol. **24:**851–856.
19. **Khan, A. S., and D. M. Schifferli.** 1994. A minor 987P protein different from the structural fimbrial subunit is the adhesin. Infect. Immun. **62:**4233–4243.
20. **Molina, L., C. Ramos, E. Duque, M. C. Ronchel, J. M. García, L. Wyke, and J. L. Ramos.** Survival of *Pseudomonas putida* KT2440 in soil and in the rhizosphere of plants under greenhouse and environmental conditions. Soil Biol. Biochem., in press.
21. **Nusslein, K., D. Maris, K. N. Timmis, and D. F. Dwyer.** 1992. Expression and transfer of engineered catabolic pathways harbored by *Pseudomonas* ssp. introduced into activated sludge microcosms. Appl. Environ. Microbiol. **58:**3380–3386.
22. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
23. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295–304.
24. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30:**285–293.
25. **Rainey, P. B.** 1999. Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. Environ. Microbiol. **1:**243–257.
26. **Ramos, J. L., E. Díaz, D. Dowling, V. de Lorenzo, S. Mølin, F. O'Gara, C. Ramos, and K. N. Timmis.** 1994. The behavior of bacteria designed for biodegradation. Bio/Technology **12:**1349–1358.
27. **Rodríguez-Herva, J. J., D. Reniero, E. Galli, and J. L. Ramos.** 1999. Cell envelope mutants of *Pseudomonas putida*: physiological characterization and analysis of their ability to survive in soil. Environ. Microbiol. **1:**479–488.
28. **Ronchel, M. C., C. Ramos, L. B. Jensen, S. Mølin, and J. L. Ramos.** 1995. Construction and behavior of biologically contained bacteria for environmental applications in bioremediation. Appl. Environ. Microbiol. **61:**2990–2994.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1987. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Sarniguet, A., J. Kraus, M. D. Henkels, A. M. Muehlchen, and J. E. Loper.** 1995. The sigma factor σ^S affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. Proc. Natl. Acad. Sci. USA **92:**12255–12259.
31. **Schultz, J. E., and A. Matin.** 1991. Molecular and functional characterization of a carbon starvation gene of *Escherichia coli*. J. Mol. Biol. **218:**129–140.
32. **Smit, G., S. Swart, B. J. J. Lugtenberg, and J. W. Kijne.** 1992. Molecular mechanisms of attachment of *Rhizobium* bacteria to plant roots. Mol. Microbiol. **6:**2897–2903.
33. **Stockwell, V. O., L. W. Moore, and J. E. Loper.** 1993. Fate of *Agrobacterium radiobacter* K84 in the environment. Appl. Environ. Microbiol. **59:**2112–2120.
34. **Urban, M., T. Bhargava, and J. E. Hamer.** 1999. An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. EMBO J. **18:**512–521.
35. **Van Bastelaere, E., M. Lambrecht, H. Vermeiren, A. Van Dommelen, V. Keijers, P. Proost, and J. Vanderleyden.** 1999. Characterization of a sugar-binding protein from *Azospirillum brasilense* mediating chemotaxis to and uptake of sugars. Mol. Microbiol. **32:**703–714.
36. **Vande Broek, A., M. Lambrecht, and J. Vanderleyden.** 1998. Bacterial chemotactic motility is important for the initiation of wheat root colonization by *Azospirillum brasilense*. Microbiology **144:**2599–2606.
37. **Van den Berg, B. M., H. Beekhuizen, R. J. Willems, F. R. Mooi, and R. van Furth.** 1999. Role of *Bordetella pertussis* virulence factors in adherence to epithelial cell lines derived from the human respiratory tract. Infect. Immun. **67:**1056–1062.
38. **Watnick, P. I., K. J. Fullner, and R. Kolter.** 1999. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. J. Bacteriol. **181:**3606–3609.
39. **Weller, D. M.** 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26:**379–407.