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Flagella of a Plant-Growth-Stimulating *Pseudomonas fluorescens* Strain Are Required for Colonization of Potato Roots

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The role of motility in the colonization of potato roots by *Pseudomonas* bacteria was studied. Four Tn*5*-induced flagella-less mutants of the plant-growth-stimulating *P. fluorescens* WCS374 appeared to be impaired in their ability to colonize growing potato roots.

Certain fluorescent *Pseudomonas* isolates are able to increase plant growth (3, 10, 16, 28) or to protect plants against microbial pathogens (17, 25). Our research is focused on strains that can be used to eliminate potato yield reductions as observed in Dutch fields in which potatoes are frequently grown (28). These yield reductions can be abolished by treatment of the seed potatoes with particular *Pseudomonas* strains (3, 10, 27, 28,). Essential for this beneficial effect of the bacteria on plants is the production of fluorescent siderophores, Fe^{3+}-chelating compounds (3, 22) which supposedly enable the *Pseudomonas* cells to scavenge most of the Fe^{3+} ions from the Fe^{3+}-poor soil, thereby depriving deleterious microorganisms of this essential element. A second factor which is supposed to be essential for efficient protection of the roots against deleterious microorganisms is the delivery of siderophores along the whole root system of the plant, which requires efficient colonization of the potato roots. Although root colonization is very important in nature, virtually nothing is known about it at the molecular level. We studied the role of motility of *Pseudomonas* bacteria in the colonization of potato roots.

MATERIALS AND METHODS

Strains and growth conditions. The potato root isolate *Pseudomonas fluorescens* WCS374 (7, 9, 10) is resistant to nalidixic acid (25 μg/ml). Strain JM3741 is a Tn*5*-marked derivative of WCS374 which was isolated by J. Marugg. It does not differ significantly from the parental strain in its root-colonizing ability, its siderophore production, its growth rate in the complex King B medium or in minimal medium, and its motility (P. A. H. M. Bakker, unpublished data). *Escherichia coli* CSH52 harboring the mobilizable plasmid pSUP202 (Ap^r Cm^r Km^r Tc^r) and strain S17-1 harboring pSUP2021 (=pSUP202 with Tn*5* inserted into the gene for tetracycline resistance) were obtained from R. Simon (29). *Pseudomonas* strains were grown at 28°C and *E. coli* strains were grown at 37°C in King B medium (15) under vigorous aeration. For the root colonization assay Tn*5*-labeled strains were cultivated at 28°C for 48 h on King B medium solidified with 1.6% agar and supplemented with kanamycin (50 μg/ml). The Tn*5*-labeled strains were resistant to kanamycin (200 μg/ml) and streptomycin (200 μg/ml) (Tn*5* encodes both kanamycin and streptomycin resistance in *Pseudomonas* species).

Isolation of Tn*5*-induced nonmotile mutants. Tn*5*-induced mutants were obtained by the method of Simon et al. (29) with slight modifications as described by Marugg et al. (22). Briefly, *E. coli* S17-1 harboring pSUP2021 was mated with *P. fluorescens* WCS374 for 3 to 4 h at 28°C. Transconjugants were selected on King B agar plates containing 25 μg of kanamycin per ml and 20 μg of nalidixic acid per ml. Colonies were screened for motility on motility plates consisting of 20-fold-diluted King B medium solidified with 0.3% agar. After spot inoculation, motility was judged after incubation for 16 h at 28°C.

Isolation of flagella. The method described by DePamphilis and Adler (6) for isolation of flagella was slightly modified. Late-logarithmic-phase cells were harvested and gently suspended in 0.1 M Tris hydrochloride (pH 7.8) at a density of 10^{10} cells per ml. Deflagellation with a Sorvall Omnimixer with the speed control dial at position 4 for 15 min at 0°C resulted in loss of motility for 99% of the cells, without loss of viability. The suspension was centrifuged at 12,000 × *g* for 10 min, and the resulting supernatant fluid was centrifuged at 90,000 × *g* for 2 h to harvest the flagella. Flagella to be used for immunization of a rabbit were further purified by isopycnic density centrifugation in cesium chloride. The flagella were suspended in 5 ml of 50% (wt/vol) cesium chloride and layered between 5 ml of 40% and 5 ml of 60% cesium chloride. After centrifugation at 100,000 × *g* for 3 h at room temperature the gradient was fractionated, and the flagellar layer was identified by its turbidity. For density measurements 0.2-ml samples were weighed. Cesium chloride was removed from the flagellar fraction by dialysis.

Preparation of flagellum-specific antiserum. A rabbit was immunized intradermally with 100 μg of the purified flagellar preparation emulsified in Freund complete adjuvant. Three booster injections in Freund incomplete adjuvant were given at 2-week intervals. Serum was obtained 2 weeks after the last injection and stored at −20°C. The resulting antiserum contained antibodies reacting (in immunoblots) with the flagellar subunit and with lipopolysaccharide (LPS). To obtain a flagellum-specific antiserum, the LPS antibodies were removed by absorbing the antiserum with cells of the nonmotile mutant LWM74-29. Approximately 10^{10} cells washed with 10 mM phosphate-buffered saline (pH 7.2) containing 1 mg of NaN_3 per ml were suspended in 0.5 ml of antiserum. After incubation for 1 h at 37°C, cells were

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removed by centrifugation. The procedure was repeated with fresh cells. After this treatment the reaction with LPS on immunoblots was no longer observed, while the reactivity toward the flagellar subunit had not been affected.

SDS-PAGE. Samples were solubilized by incubation for 15 min at 95°C in the standard sample mixture and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (18). Cell envelope samples were obtained by differential centrifugation after disruption of the cells by ultrasonic treatment (18). Samples containing 10 to 20 μg protein were applied per lane, and gels were stained with fast green FCF (18). For the analysis of LPS the samples were first treated with proteinase K (13) after which 10-fold dilutions were applied per lane. Gels were stained with silver reagent (31).

Immunoblotting. For immunoblotting the amounts applied per lane were 100-fold less than the amounts applied to gels to be stained with fast green. For analysis of the culture supernatant, 2 μl from a stationary-phase culture (A_{620} = 5 to 6) was applied per lane. Electrophoretically separated proteins were electrophoretically transferred to nitrocellulose with a blotting cell (Bio-Rad Laboratories, Richmond, Calif.). The nitrocellulose sheet was then incubated with the flagellum-specific antiserum, followed by incubation with goat anti-rabbit antiserum-alkaline phosphatase conjugate. The blotting reaction was visualized with fast red TR salt and naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, Mo.) as described by van der Meide et al. (33).

DNA isolation and analysis. DNA of small plasmids was isolated by the method of Birnboim and Doly (4) and purified by isopycnic density centrifugation in cesium chloride. Extraction of total DNA and digestions with restriction endonucleases (*Bam*HI and *Eco*RI) were done as described by Maniatis et al. (20). DNA fragments were transferred from the 0.8% agarose gels to nitrocellulose filters (20). ^{32}P-labeled plasmid pNP520 (23) was used as a Tn*5* probe, and pSUP202 (29) was used as a probe for vector plasmid DNA. Conditions for hybridization with ^{32}P-labeled DNA probes prepared by nick translation were as described by Maniatis et al. (20).

Electron microscopy. Bacterial cells were negatively stained with phosphotungstic acid and examined with a Philips 300 transmission electron microscope.

Root colonization assay. Potato stem cuttings with roots approximately 1 cm long were dipped in bacterial suspensions of 10^8 CFU/ml. After removal of excess suspension by shaking, the stem cuttings were embedded in clay soil in tubes of polyvinyl chloride (4.5-cm diameter; 12 cm long). This soil was collected from a field in which potatoes were grown once every 6 years. The soil moisture content was brought to 25% about 24 h before the soil was transferred into the polyvinyl chloride tubes. Before the stem cuttings were planted, the tubes were placed on a layer of wet vermiculite (Fig. 1), and this system was allowed to equilibrate for 24 h. During the growth of the plants the humidity of the soil was regulated by this layer of vermiculite, so a direct movement of water from top to bottom was avoided. After 12 days of growth in a greenhouse, root samples 1 cm long (total root fresh weight, 0.3 g per sample) were taken from three different depths (0 to 1, 4, and 8 cm) and shaken in 5 ml of 0.1% Proteose Peptone (Difco Laboratories, Detroit, Mich.) with 2.5 g of glass beads (3-mm diameter) in a Vortex mixer at maximum speed. Appropriate dilutions of these suspensions were plated on King B agar plates supplemented with 200 μg of kanamycin per ml and 200 μg of streptomycin per ml. The number of colonies was counted after 48 h of growth. Results were analyzed by the Kruskal-Wallis test followed by nonparametric multiple comparisons by the simultaneous test procedure (30).

Protein determination. Protein was determined by the method described by Markwell et al. (21) with bovine serum albumin as the standard.

RESULTS

Isolation of nonmotile Tn*5* mutants. Mating of *E. coli* S17-1 containing the plasmid pSUP2021 with *P. fluorescens* WCS374 resulted in kanamycin- and nalidixic acid-resistant (Kan^r Nal^r) transconjugants at a frequency of 2×10^{-6} per recipient cell, whereas the frequency was less than 10^{-9} for spontaneous mutants. Kan^r Nal^r mutants of strain WCS374 were screened for motility on semisolid medium. The wild-type strain WCS374 formed swarms that were 2 cm in diameter, while the growth area of nonmotile mutants was restricted to the spot of inoculation. Of 500 Kan^r Nal^r mutants tested, 4 nonmotile mutants were obtained, designated as strains LWM74-4, LWM74-29, LWM74-30, and LWM74-36.

These four mutants were also nonmotile when observed by phase-contrast microscopy. Using electron microscopy, up to nine polar flagella were observed for the wild-type strain WCS374, while no flagella were observed for the nonmotile mutants LWM74-4, LWM74-29, and LWM74-30. Mutant strain LWM74-36 occasionally contained one flagellum per cell (data not shown).

The nonmotile mutant strains were not affected in (i) siderophore production, (ii) growth rate in minimal medium or the complex King B medium, and (iii) membrane protein patterns or LPS ladder patterns as analyzed by SDS-PAGE (data not shown).

Isolation and characterization of flagella. After removal from the cells by shearing, flagella were isolated by differential centrifugation. SDS-PAGE showed that the resulting preparation contained a dominant 58,000-dalton protein as well as a large number of minor protein bands (Fig. 2, lane

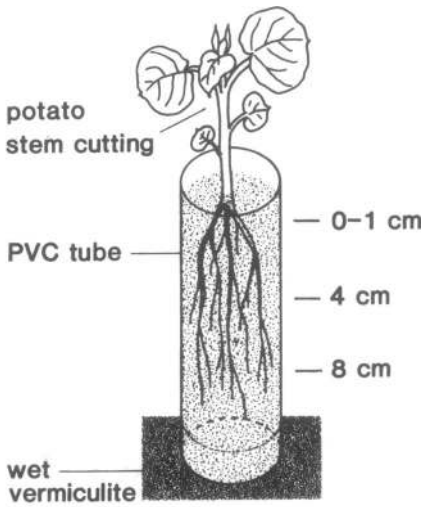

FIG. 1. Schematic representation of the bioassay used to determine root colonization. The potato stem cuttings were inoculated as described in the text, after which they were allowed to grow in a polyvinyl chloride (PVC) tube containing soil which was placed on top of a layer of wet vermiculite. After 12 days of growth, root sections at 0- to 1-, 4- and 8-cm depths were taken to determine the number of viable bacteria present on the different parts of the root.

1). Subsequent isopycnic density gradient centrifugation in cesium chloride of this flagellar preparation resulted in loss of minor protein bands with higher molecular sizes than the 58,000-dalton protein, but in the middle and lower part of the gel minor protein bands were still visible (data not shown). The fraction containing the 58,000-dalton protein layered at a density of 1.32 to 1.34 g/ml, which is close to the density of flagella of *Bacillus subtilis* and *E. coli* (1.30 g/ml [6]). Therefore, the 58,000-dalton protein most probably represents the subunit of the flagella of strain WCS374.

Cells of the four nonmotile mutants were sheared, and the suspensions were differentially centrifuged. The resulting pellets contained two to four times less protein per volume of cell culture compared with the flagellar pellet of the wild-type strain. The resulting preparations were adjusted to the same protein concentration and analyzed by SDS-PAGE. These preparations of the nonmotile mutants did not reveal the heavy 58,000-dalton protein band which was observed in similar preparations of the wild-type strain, but the minor protein bands were present (Fig. 2, lanes 2 to 5).

Molecular characterization of mutations. Total DNA isolated from the four nonmotile mutants was digested with restriction endonucleases *Eco*RI (for which Tn*5* DNA has no restriction site) and *Bam*HI (which cuts Tn*5* at a site 2.65 kilobases from one end and 2.95 kilobases from the other).

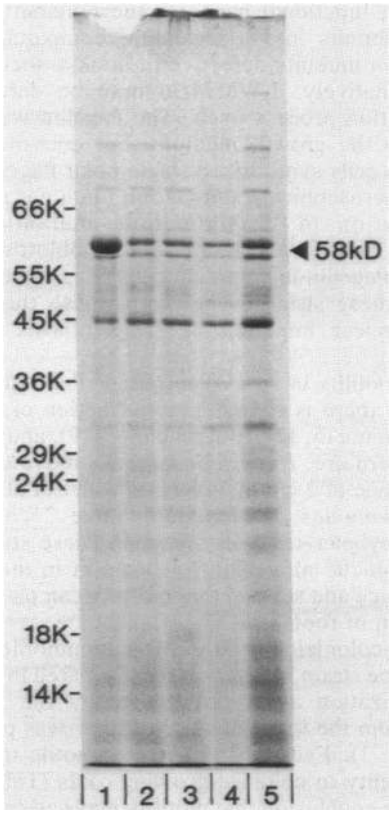

FIG. 2. SDS-PAGE of the flagella of strain WCS374 (lane 1) and of the comparable preparations of the nonmotile mutant strains LWM74-4 (lane 5), LWM74-29 (lane 4), LWM74-30 (lane 3), and LWM74-36 (lane 2). Equal amounts (5 μg) of protein were applied to each lane. The position of flagellin is indicated by an arrow (58kD) at the right, and the positions of molecular weight standard proteins are indicated at the left (k, 10^3). Note that the position of the 58,000-dalton (58kD) protein band in the flagella preparation of the wild type (lane 1) is in between the positions of two proteins present in the preparations of the four nonmotile mutants (lanes 2 to 5).

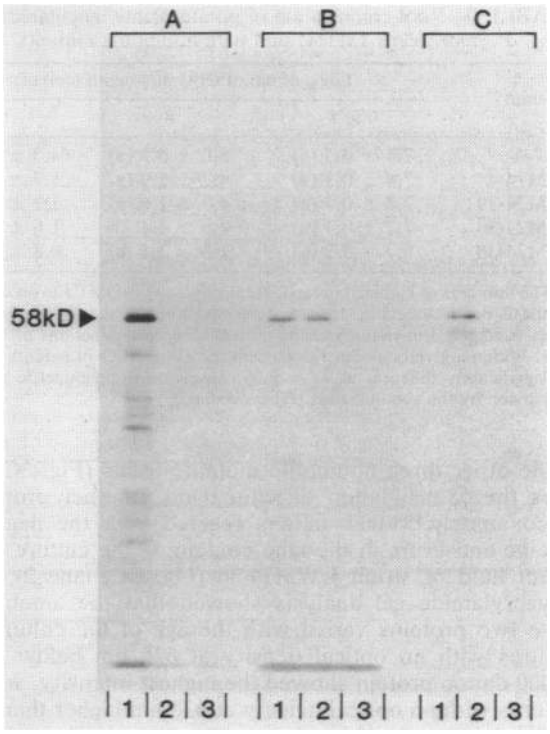

FIG. 3. Immunoblots of resuspended flagellar pellets (A), whole cells (B), and culture supernatant fluids (C). Lanes 1, wild-type strain WCS374; 2 and 3, nonmotile mutants LWM74-36 and LWM74-30, respectively. Mutant strains LWM74-4 and LWM74-29, had behavior identical to that of strain LWM74-30 in immunoblots. The position of flagellin is indicated by an arrow (58kD).

Digests were fractionated by electrophoresis, blotted to nitrocellulose, and hybridized with the ^{32}P-labeled Tn*5* probe. The resulting autoradiogram showed that in all *Eco*RI digests only one band hybridized with Tn*5* (data not shown). Moreover, fractionated *Eco*RI and *Bam*HI digests of the four mutants showed different bands for each of the mutant strains. Hybridization with the ^{32}P-labeled pSUP202 probe (=pSUP2021 without the Tn*5*) did not reveal any bands on the autoradiogram. These results show that the four Tn*5* mutations are independent, that they are located in different restriction nuclease fragments, and that no vector DNA was transferred to the mutants.

The flagellar preparation of the wild-type strain and the comparable preparations of the mutant strains were subjected to SDS-PAGE, and the flagellin subunit was detected by immunoblotting with a flagellum-specific antiserum. In the flagellar preparation of the wild-type strain WCS374 the flagellar subunit was detected. In addition, several proteins with lower molecular weights (Fig. 3A, lane 1) were detected. In the lanes with the "flagellar preparations" of the four nonmotile mutants no reaction was observed except for a faint band at the gel front (Fig. 3A, lanes 2 and 3).

Using the flagellum-specific antiserum in an immunoblot, flagellin was not only detected in whole cells of the wild-type strain WCS374, but also in those of mutant strain LWM74-36 (Fig. 3B). No flagellin was detected in cells of the other three nonmotile mutants. Flagellin appeared to be present in substantial amounts in the culture supernatant of strain LWM74-36 (Fig. 3C, lane 2), while only a small amount was detected in the culture supernatant of the wild-type strain WCS374. No flagellin was found in the culture supernatants

TABLE 1. Root colonization of potato plants inoculated with *P. fluorescens* JM3741 and with nonmotile mutants[a]

Strain	Log_{10} of no. of CFU at a depth (cm) of:		
	0 to 1	4	8
JM3741	7.4 ± 0.5 (a)	5.0 ± 0.5 (a)	4.5 ± 0.4 (a)
LWM74-4	7.4 ± 0.3 (a)	4.7 ± 1.9 (a)	1.7 ± 2.2 (b)
LWM74-29	7.2 ± 0.7 (a)	4.3 ± 1.6 (a)	1.2 ± 2.0 (b)
LWM74-30	7.2 ± 0.4 (a)	2.9 ± 2.4 (b)	1.6 ± 2.0 (b)
LWM74-36	6.7 ± 0.2 (a)	1.6 ± 2.1 (c)	0.8 ± 1.8 (b)

[a] The numbers of kanamycin- and streptomycin-resistant CFU on the roots at various depths (see Fig. 1) were determined. The values given are mean values ± standard deviations of log-transformed determinations of 10 replicates. Within a given column values with the same letter in parentheses are not significantly different at $P = 0.05$, based on nonparametric multiple comparison by the simultaneous test procedure.

of the other three nonmotile mutant strains (Fig. 3C). Besides the 58,000-dalton flagellin band, another protein of approximately 55,000 daltons reacted with the flagellum-specific antiserum in the lane containing the culture supernatant fluid of strain LWM74-36 (Fig. 3C, lane 2). SDS-polyacrylamide gel analysis showed that the amounts of these two proteins varied with the age of the cultures. In cultures with an optical density at 620 nm below 4, the 55,000-dalton protein showed the highest intensity, while in cultures with an optical density at 620 nm higher than 5 the 58,000-dalton protein band was dominant (data not shown). The flagellin in the culture supernatant of the wild-type strain appeared to be sedimentable by ultracentrifugation (90,000 × *g* for 2 h) as expected for flagellar fragments, whereas the flagellin of mutant strain LWM74-36 remained present in the supernatant fluid under these conditions (data not shown), indicating that it was present as nonpolymerized subunits.

Colonization of potato roots. The abilities of the wild-type strain and the nonmotile mutants to colonize potato roots were compared with potato stem cuttings inoculated with the motile Tn*5*-labeled strain JM3741 or with one of the nonmotile mutants. After 12 days of growth in soil, the number of Tn*5*-carrying CFU on various parts of the root system (Fig. 1) was determined (Table 1). The numbers of Tn*5*-containing CFU on root samples taken from the 0 to 1-cm depth did not differ significantly among the plants. This was to be expected since inoculation took place at this part of the root system. However, in root samples taken from a part of the root system formed after inoculation (e.g., root samples taken from the 8-cm depth), the number of Tn*5*-containing CFU was drastically reduced in the plants inoculated with nonmotile mutants when compared with that in plants inoculated with the wild-type strain (Table 1). For mutant strains LWM74-30 and LWM74-36 the number of Tn*5*-containing CFU was also significantly reduced on root samples taken from the 4-cm depth. The results show that motility is required to colonize growing roots successfully.

DISCUSSION

Flagellin of *P. fluorescens* WCS374. The plant-growth-stimulating strain WCS374 is a motile *P. fluorescens* strain with up to nine polar flagella per cell (data not shown). Of 500 Tn*5*-induced mutants of this strain, 4 nonmotile mutants were isolated. A large number of genes are involved in the formation of functional flagella of gram-negative bacteria such as *E. coli* (2), *Salmonella typhimurium* (24), and *Pseudomonas aeruginosa* (32). The high frequency with which the nonmotile mutants of this *P. fluorescens* strain were found as well as the observation that the insertions of Tn*5* in the four nonmotile mutant strains are located on four different DNA restriction fragments are consistent with the large number of genes required for the synthesis and assembly of flagella.

The flagellar preparation of the wild-type strain showed a dominant 58,000-dalton protein band and minor protein bands with lower molecular sizes (Fig. 2). This 58,000-dalton protein most likely represents the flagellar subunit flagellin since (i) it copurifies with particles with a buoyant density of 1.32 to 1.34 g/ml, which is close to the buoyant density of flagella of *B. subtilis* and *E. coli* (1.30 g/ml [6]); (ii) it is the major protein in purified flagella; and (iii) it is absent in comparable preparations of the nonflagellated mutants (Fig. 2 and 3). The identity of the other protein bands in the flagellar preparations (Fig. 2) is not known. At least some of these most likely represent degradation products of flagellin since they were detected by immunoblotting with flagellum-specific antiserum in the flagellar preparation of the wild type (Fig. 3).

Characterization of defects of nonmotile mutants. In one of the four nonmotile mutants (LWM74-36), the 58,000-dalton flagellin protein was present in intact cells and as nonpolymerized subunits in the cell culture supernatant fluid (Fig. 3). In this mutant strain, the impairment in biosynthesis of flagella may be in the basal body or hook structure, thus not supplying a functional basis for the polymerization of the flagellar subunits into a flagellum, comparable to the *S. typhimurium* mutants defective in hook-associated proteins (14). Alternatively, LWM74-36 may be defective in the polymerization process itself. The flagellin was usually secreted into the growth medium, but occasionally mutant LWM74-36 cells synthesized single polar flagella as seen by electron microscopical examination (data not shown).

Examination of the nonmotile mutants LWM74-4, LWM74-29, and LWM74-30 by immunoblotting showed no detectable flagellin in complete cells or in the culture supernatant of these strains. Therefore, these three nonmotile mutants appear impaired in the synthesis of wild-type flagellin.

Role of motility in root colonization. Previous studies had shown that there is chemotactic attraction of rhizobacteria by root exudate (5, 12), root mucilage (19), and seed exudate (26). Furthermore, *P. aeruginosa* cells move through moist soil a distance of 2 cm in 24 h (11). Scher et al. (26) showed that *Pseudomonas putida* could move 2 cm in 48 h to imbibing soybean seeds in raw soil. These studies indicate that chemotactic movement of bacteria in the rhizosphere can take place and suggest that motility can play a role in the colonization of roots.

The root-colonizing abilities of the nonmotile mutants and the wild-type strain WCS374 were compared in a greenhouse root colonization assay. In this assay the soil was not irrigated from the top, but soil humidity was provided from below (Fig. 1). Each of the four nonmotile mutants had a reduced ability to colonize growing roots (Table 1). Before drawing the conclusion that motility plays an essential role in efficient root colonization, we have to consider the possibility that the mutations leading to the nonmotile phenotype have pleiotropic effects. The *chvB* mutation in *Agrobacterium tumefaciens*, which causes defective attachment to plant tissues, also appears to affect the formation of flagella (8). Similarly, the nonmotile phenotype in *E. coli* was found among mutants lacking the LPS sugar heptose (1). However, no evidence has been found for pleiotropic effects in our mutants to date since they have wild-type properties with

respect to composition of the LPSs and proteins in the cell envelope, production of siderophores, and growth rate in King B medium or minimal medium. Moreover, the Tn*5* insertions in the four nonmotile mutants are independent, and it is not likely that the lack of motility in each of these mutants is due to different pleiotropic mutations which by themselves affect the root-colonizing ability of the strain. The results of this study therefore strongly suggest that flagella of *P. fluorescens* WCS374 play an essential role in the colonization of potato roots.

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