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Mutational Changes in Physicochemical Cell Surface Properties of Plant-Growth-Stimulating *Pseudomonas* spp. Do Not Influence the Attachment Properties of the Cells

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Bacteriophage-resistant mutant strains of the root-colonizing *Pseudomonas* strains WCS358 and WCS374 lack the O-antigenic side chain of the lipopolysaccharide, as was shown by the loss of the typical lipopolysaccharide ladder pattern after analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These strains differed from their parent strains in cell surface hydrophobicity and in cell surface charge. The observed variation in these physicochemical characteristics could be explained by the differences in sugar composition. The mutant strains had no altered properties of adherence to sterile potato roots compared with their parental strains, nor were differences observed in the firm adhesion to hydrophilic, lipophilic, negatively charged, or positively charged artificial surfaces. These results show that neither physicochemical cell surface properties nor the presence of the O-antigenic side chain plays a major role in the firm adhesion of these bacterial cells to solid surfaces, including potato roots.

The potential of *Pseudomonas* spp. to act as biocontrol agents in agriculture has been widely recognized (4, 7, 14, 23). A presumed prerequisite for its successful application is extensive colonization of plant surfaces, e.g., of roots or leaves (2, 7, 17). A better understanding of the factors involved in colonization of the plant surface will finally help us to improve the performance of plant-beneficial *Pseudomonas* strains in the field. However, up till now very little is known about the molecular aspects of this colonization process. One of the early steps presumably involves the binding between the bacterial cell and the plant surface.

Firm binding of *Pseudomonas* cells to radish roots and bean roots has been described (1, 11), but the molecular mechanism of this binding process is largely unknown. According to the literature, adhesion of bacteria to eucaryotic cells can either be a very specific process which involves receptor-ligand interactions (5, 12) or it can be quite nonspecific in that it can be explained in terms of hydrophobicity and the electrical charge of the bacterial cell surface (22, 26). Our laboratory has been interested for some time in the mechanism of colonization of potato roots by certain fluorescent *Pseudomonas* spp. It was shown that the *Pseudomonas* strains *P. putida* WCS358 and *P. fluorescens* WCS374, which efficiently colonize the root system, possess lipopolysaccharides (LPSs) with long O-antigenic polysaccharide chains (6). These polymers are presumed to protrude from the outer membrane into the medium (15, 16, 19). It can be expected that mutations causing a loss of these polysaccharide chains will change the cell surface characteristics, including hydrophobicity and electrical charge. We therefore decided to construct mutants without the O-antigenic side chain to use them to test whether these physicochemical cell surface properties are involved in the firm adherence of the bacteria to various surfaces. The results show that mutant strains lacking the O-antigenic side chain indeed differ in their cell surface hydrophobicity and cell surface charge. Differences in the sugar composition between the parent and the mutant LPSs accounted fairly well for the observed differences in physicochemical properties. These strains were used to study the relevance of the physicochemical cell surface properties for the firm adhesion to defined artificial surfaces and to sterile potato roots.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Relevant characteristics of *P. putida* WCS358 and *P. fluorescens* WCS374 are described elsewhere (6, 8, 10). Unless otherwise indicated, cells were grown in King B medium (13) at 28°C for 16 h under vigorous aeration. To measure the adhesion to Sephadex beads, bacterial cells were radioactively labeled by growth for 16 h in King B medium supplemented with 10 μCi of [^{35}S]methionine per ml (specific activity, 1,151 Ci/mmol). Prior to use, the cells were washed three times in phosphate buffer (10 mM sodium phosphate, pH 7.2) to remove extracellular [^{35}S]methionine. For the determination of adhesion to potato roots, strains WCS358 and WCS374 and their respective LPS-mutant strains LWP358-43b and LWP374-30b (see below) were marked with transposon Tn*5*, which contains a kanamycin resistance marker, by the method described by Simon et al. (24). To avoid choosing a single mutant whose fitness is accidentally impaired by the Tn*5* insertion, approximately 50 Tn*5*-containing derivatives were mixed and grown for three successive cycles in King B medium supplemented with final concentrations of kanamycin and nalidixic acid of 25 and 20 μg/ml, respectively. These Tn*5*-containing populations did not differ from their parental strains in growth rate in either King B medium or in minimal salts medium (27) supplemented with 1% glucose.

Siderophore production by mutant and parent strains was compared by fluorescence of the supernatant of an iron-limited culture under UV irradiation (366 nm). The motility of parent and mutant strains was tested on King B medium solidified with 0.3% agar (9).

Bacteriophage techniques. Phages were isolated from var-

ious soil or ditch water samples taken from the areas around Leiden and Baarn in The Netherlands. A 50-ml volume of King B medium supplemented with either 10 g of soil or 10 ml of water was inoculated with 1 ml of a stationary-phase culture of one of the *Pseudomonas* strains and was cultured overnight at 28°C under vigorous aeration. Subsequently, the bacteria were killed by adding a few drops of chloroform to the culture. Cell and soil debris was removed by centrifugation, and the supernatant fluid was mixed with the host strain and plated in a top layer containing King B medium solidified with 0.6% agar. For obtaining smooth bacterial layers with strain WCS358, it was necessary to supplement the top layer with 2 mM $CaCl_2$.

To obtain pure phage suspensions, individual plaques were plated again with their host strain through two successive cycles. High-titer stocks were obtained from bacterial layers (0.6% agar) showing confluent lysis. Titers of 10^9 to 10^{11} PFU/ml were obtained for the smaller- and the larger-plaque-forming phages, respectively. The phage stocks were stored in King B medium containing 0.5% chloroform at 4°C. Spontaneous phage-resistant mutants were isolated with a frequency of 10^5 to 10^6 from confluent lysis plates. To obtain pure mutant strains, single colonies were picked and purified twice.

Isolation and analysis of cell envelopes and LPS. After disruption of the cells, cell envelopes were isolated by differential centrifugation (18). LPS was isolated as described by Darveau and Hancock (3). Cell envelope proteins and LPSs were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described elsewhere (6, 8). Neutral sugars and amino sugars of the LPSs were quantitatively determined by gas-liquid chromatography and by amino acid analysis, respectively. Experimental details of these analyses and of the colorimetric methods used for the quantitative determination of heptose and 2-keto-3-deoxyoctonate (KDO) have been described previously (6).

Measurement of bacterial hydrophobicity and electrophoretic mobility. Hydrophobicity of the cell surface was determined by measuring the contact angle of a water drop on a homogeneous bacterial cell layer collected by filtration on a 0.45-μm (pore size) micropore filter (Sartorius), as described by van Loosdrecht et al. (25). As a measure of the electrical charge of the bacterial cell surface, the electrophoretic mobility of the cells was determined (26) by laser Doppler velocimetry with a Zeta Sizer (Malvern Instruments, Malvern, England). Cells were suspended in 75 mM phosphate-buffered saline containing the following (per liter of deionized water): 0.21 g of KH_2PO_4, 0.89 g of K_2HPO_4, and 3.69 g of NaCl.

Adhesion to Sephadex beads. An amount of 0.1-g Sephadex beads (Sephadex G-25, Sephadex LH-20, CM-Sephadex C-25, or DEAE-Sephadex A-25; all from Pharmacia, Uppsala, Sweden) was allowed to swell in glass tubes at 60°C for 20 h in phosphate buffer. After the swollen beads were washed three times with phosphate buffer, they were mixed with 0.5 ml of a ^{35}S-labeled cell suspension of 1×10^9 CFU/ml, unless otherwise indicated, and were incubated routinely for 1 h on a rotary shaker at 250 rpm. For time course studies, incubation periods ranged from 2 to 120 min. After the incubation period, the beads were allowed to settle and the supernatant fluid was discarded. The beads were washed four times by being mixed (extension 1500, Vibrofix VF1 Electronic) in 5 ml of phosphate buffer, after which the bacteria still attached were considered to be firmly bound. Finally the beads were transferred to scintillation vials, and 8 ml of scintillation fluid (Quickszint 212; Zinsser Analytic, Göttingen, Federal Republic of Germany) was added. The radioactivity associated with the beads, determined by using the ^{35}S channel of a type 1214 Rackbeta liquid scintillation counter (LKB Instruments, Inc., Rockville, Md.) was used to calculate the number of firmly bound cells.

Adhesion to sterile potato roots. Sterile potato plant roots of the potato cultivar Bintje were maintained on medium described by Murashige and Skooge (20) (pH 5.8) supplemented with 2% sucrose and solidified with 0.8% agar. The culture vessels (type GA7; Magenta Corp., Chicago, Ill.) were placed in a growth chamber at 28°C with a day length of 14 h. For the cultivation of sterile potato roots, eight plantlets were placed on a metal grid and were cultivated on 100 ml of liquid Murashige-Skooge medium. After 10 days of growth, root tips of 3 cm were cut off. Three of these pieces were incubated with 1.0 ml of a bacterial cell suspension in phosphate buffer (5×10^8 CFU/ml, unless otherwise indicated) under agitation at 100 rpm. For time course studies, incubation times varied from 1 to 120 min; bacteria and roots were routinely incubated for 1 h at room temperature, after which the root pieces were transferred to 10.0 ml of phosphate buffer and were washed four times by vortexing (extension 1500, Vibrofix VF1 Electronic) for 10 s in 10.0 ml of phosphate buffer. The bacteria still attached to the root surface after this treatment were considered to be firmly bound to the root surface. Their number was determined by homogenizing the root pieces by means of a type 10-T homogenizer (Ystral, Dottingen, Federal Republic of Germany). The viability of the bacterial population was not affected by this procedure. Bacterial cell numbers in the washes and in the homogenates were determined by dilution plating on King B medium supplemented with kanamycin (100 μg/ml) and chloramphenicol (10 μg/ml). The bacteria were grown at 28°C, and the colonies were scored after 2 days.

RESULTS

Isolation of phages and phage-resistant mutants. Seventeen phages were isolated which lysed *P. putida* WCS358, giving rise to either very small plaques (less than 1 mm in diameter) or somewhat larger ones (approximately 1 mm in diameter). By using resistance to one of the latter plaque-type phages, phage HK58-5, one phage-resistant mutant (LWP358-5c) was selected, which upon analysis by SDS-polyacrylamide gel electrophoresis showed a shorter ladder pattern for its LPS compared with its parent strain WCS358 (Fig. 1, lanes 1 and 2), indicating that the average O-antigenic side chain was reduced in length. Neither of the phages enabled us to select mutant strains which completely lacked the ladder pattern. Therefore a new phage, HK58-43, was isolated by using mutant strain LWP358-5c as the host strain to select from the population of LWP358-5c cells a mutant strain, LWP358-43b, which was resistant to phage HK58-43. Strain LWP358-43b had lost the LPS ladder pattern completely, as shown after SDS-polyacrylamide gel electrophoresis (Fig. 1, lane 3).

All six isolated phages which recognize strain *P. fluorescens* WCS374 caused very large plaques (approximately 1 cm in diameter) on their host strain, WCS374. Forty spontaneous phage-resistant mutant strains were isolated, the majority of which lacked the LPS ladder pattern which was observed for the wild-type strain WCS374 (Fig. 1, lane 4). One of these mutant strains, LWP374-30b (Fig. 1, lane 5), selected by plating host cells of strain WCS374 with phage HK74-30, was chosen for further study. On an SDS-poly-

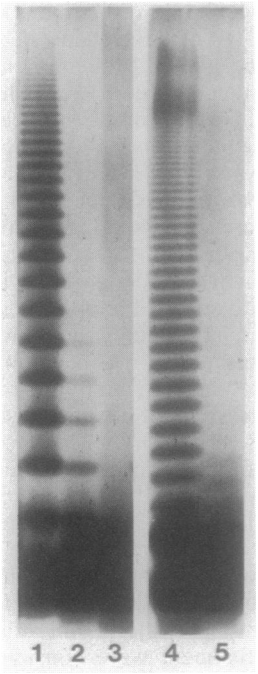

FIG. 1. Silver-stained patterns of proteinase K-treated cell envelopes obtained after SDS-polyacrylamide gel electrophoresis. Lanes: 1, WCS358; 2, LWP358-5c; 3, LWP358-43b; 4, WCS374; 5, LWP374-30b.

acrylamide gel the LPS pattern of strain LWP374-30b showed a heavy spot near the front of the gel and three tiny bands, which move to a slightly shifted position in the gel when compared with the corresponding bands of the wild-type strain WCS374 (Fig. 1, lanes 4 and 5). This may have been caused by changes in the inner core of the LPS of mutant strain LWP374-30b.

The mutant strains LWP358-5c, LWP358-43b, and LWP374-30b did not differ from their parent strains in growth rate, siderophore production, cell envelope protein pattern, or motility.

Chemical analysis of LPS. The sugar composition of the LPS preparations of parent strains WCS358 and WCS374 and mutant strains LWP358-5c, LWP358-43b, and LWP374-30b were comparatively analyzed (Table 1). The LPSs of mutant strains LWP358-5c and LWP358-43b contained substantially less glucose than the LPS of the parent strain WCS358. Quinovosamine, which is a constituent of the O-antigenic side chain (6), was strongly reduced in the LPS of strain LWP358-5c and was completely absent from the LPS of strain LWP358-43b. Other sugars were present in approximately similar relative amounts in mutant and parent strain. The LPS of mutant strain LWP374-30b lacked fucose and contained considerably less glucose than the LPS of its parent strain WCS374. Levels of other sugars, most likely constituents of the core of the LPS, were often considerably increased for the rough LPS compared with the LPS of the parent strain WCS374.

TABLE 2. Electrophoretic mobilities and contact angles of water for parent strains WCS358 and WCS374 and their mutant strains

Strain	Contact angle (°)[a]	Electrophoretic mobility (10^{-8} m/V · s)[b]
WCS358	40	−2.2
LWP358-5c	23	−2.4
LWP358-43b	25	−2.5
WCS374	16	−0.5
LWP374-30b	23	−2.6

[a] Averaged standard deviation, 1.5°.
[b] Average standard deviation, 0.15 × 10^{-8} m/V · s.

Hydrophobicity and electrophoretic mobility. The contact angle of water on a layer of WCS358 cells was significantly higher than on a layer of WCS374 cells (Table 2), indicating that the cell surface hydrophobicity of strain WCS358 was higher than that of strain WCS374. The electrophoretic mobility of these two wild-type strains differed from each other in such a way that strain WCS358 had a higher electrokinetic mobility than strain WCS374. A decrease in the length of the O-antigenic side chain (LWP358-5c) or a complete lack of this polysaccharide chain (LWP358-43b) in strain WCS358 resulted in a decrease in the cell surface hydrophobicity and a slight increase in the electrokinetic mobility of the cell surface. The mutant strain LWP374-30b, lacking the O-antigenic side chain of strain WCS374, showed an increase in the cell surface hydrophobicity as well as in the electrokinetic mobility (Table 2).

Adhesion properties of parent and mutant strains to Sephadex beads. Sephadex beads with defined artificial surfaces (G-25, hydrophilic; LH-20, lipophilic; CM, negatively charged; DEAE, positively charged) were used to study the adhesion characteristics of the strains. By using radioactively labeled bacteria, it was shown that less than 1% of the added bacteria (5×10^8 CFU) remained associated with the hydrophilic (G-25), lipophilic (LH-20), or negatively charged (CM) Sephadex beads, with no significant differences in adhesion between the wild-type and the mutant strains (data

TABLE 1. Comparative analysis of the LPS of strains WCS358 and WCS374 and the respective LPS-mutant strains[a]

Strain	Composition of LPS (% by wt)									
	KDO	Heptose	Glucose	Fucose	Rhamnose	Quinovosamine	Glucosamine	Glucosamine phosphate	Galactosamine	Alanine
WCS358	3.1	2.0	9.6	0	0	15–18	5.9	1.7	2.0	0.8
LWP358-5c	2.9	2.8	4.0	0	0	1.2–1.5	4.8	1.1	1.8	1.3
LWP358-43b	3.1	1.6	4.7	0	0	0	5.8	1.6	2.0	1.2
WCS374	1.4	3.2	9.5	1.9	0.6	0	1.7	0.2	0	0.7
LWP374-30b	5.0	4.2	1.6	0	2.2	0	5.8	1.1	0	0.8

[a] Data on the LPS of the parent strains WCS358 and WCS374 have been published previously (6). LPS from mutant strains LWP358-5c, LWP358-43b, and LWP374-30b and their corresponding parent strains purified contained less than 1% contaminating nucleic acid and protein, except for the LPS preparation of strain LWP358-5c, in which a protein contamination of 5% was determined. The values given represent the percentages (wt/wt) of at least two determinations (KDO, heptose, and neutral sugars) or a single determination (amino sugars). The amount of quinovosamine was estimated from the peak integral (6).

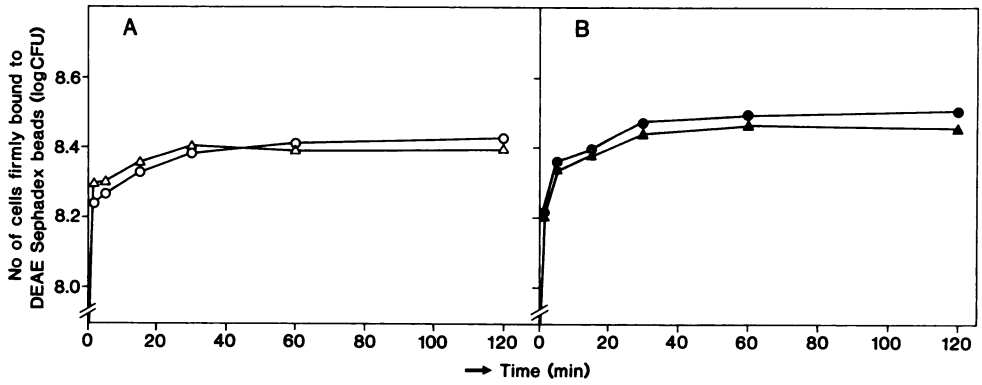

FIG. 2. Number of cells firmly bound to DEAE-Sephadex beads after various incubation times in 0.5 ml of a bacterial suspension (10^9 CFU/ml) of (A) strain WCS374 (○) and its LPS-mutant strain LWP374-30b (△) or (B) strain WCS358 (●) and its LPS-mutant strain LWP358-43b (▲). The results shown are mean values of the logarithm of the number of firmly bound cells determined in two replicates. Standard deviations were within the size of the symbol.

not shown). In contrast, the positively charged DEAE-Sephadex beads showed a high affinity for these bacterial cells, since 60 to 70% of the added cells became firmly attached to this material. The number of cells firmly bound to DEAE-Sephadex beads was monitored over time (Fig. 2). No significant differences between the parent strains and their corresponding LPS-mutant strains were observed. Incubation of the beads at cell concentrations ranging from 1×10^7 to 5×10^9 CFU/ml also did not result in significant differences between the parent strains and their LPS-mutant strains (data not shown).

Adhesion properties of parent and mutant strains to sterile potato roots. The abilities of strains WCS358 and WCS374 and their respective LPS-mutant strains LWP358-43b and LWP374-30b to adhere to sterile potato roots were studied. After incubating approximately 5×10^8 CFU with three 3-cm pieces of sterile potato roots for 1 h, 10^7 to 10^8 CFU were released from the roots during the first rinse. In the subsequent rinses this number gradually decreased from 10^6 to 10^5 CFU. After four rinses, 10^5 to 10^6 CFU were still bound to the root segments. Figure 3 shows the number of bacteria firmly bound to the root segments after various incubation times. No significant differences were observed for the wild-type strains and their LPS-mutant strains LWP358-43b and LWP374-30b. Incubation of the root segments with different cell concentrations ranging from 1×10^7 to 5×10^8 CFU/ml did not result in differences in the number of firmly bound cells between parent and LPS-mutant strains (data not shown).

DISCUSSION

Bacteria adhere to a variety of solid surfaces, including plant roots. Bacterial adhesion may be either based on specific receptor-ligand interactions (5, 12) or governed by nonspecific interactions between the bacterial surface and the adhesion surface (11, 22, 26). In the latter case, the physicochemical properties of the cell surface, i.e., cell surface hydrophobicity and cell surface charge, are believed to be of prime importance. This report focused on the question whether these physicochemical cell surface characteristics of the root-colonizing strains *P. putida* WCS358 and *P. fluorescens* WCS374 are important for the firm adhesion to potato plant roots and to a number of well-defined solid surfaces. To test this notion, we wanted to obtain mutant strains with cell surface properties different from those of the parent strains. Since capsular polysaccharide was not detected in cells of strains WCS358 and WCS374 (L. A. de Weger and J. W. H. de Voogt, unpublished results), it seemed reasonable to predict that LPS,

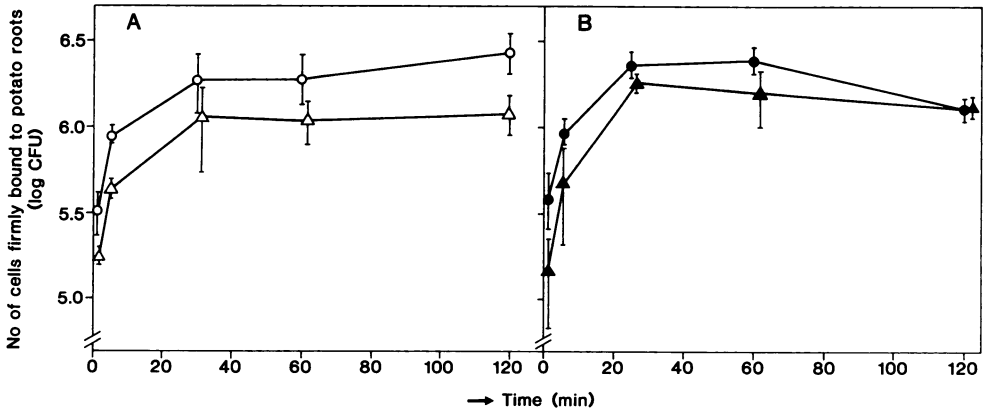

FIG. 3. Number of cells firmly bound to three 3-cm-long segments of sterile potato roots after incubation for various periods of time in 1.0 ml of a bacterial suspension (5×10^8 CFU/ml) of (A) strain WCS374 (○) and its LPS-mutant strain LWP374-30b (△) or (B) strain WCS358 (●) and its LPS-mutant strain LWP358-43b (▲). The results shown are mean values and standard deviations (bars) of the logarithm of the number of bound cells determined in three replicates.

with its long O-antigenic side chains (6), is a major determinant of the physicochemical surface characteristics of the cell. Therefore, mutants lacking the O-antigenic side chain were isolated (Fig. 1) and were characterized both chemically (Table 1) and with respect to their physicochemical surface properties (Table 2).

After being selected with specific phages, mutants were obtained from which the O-antigenic side chain is shorter (LWP358-5c) or absent (LWP358-43b and LWP374-30b) (Fig. 1). Chemical analysis of the sugar composition of the wild-type and mutant strains (Table 1) indicates that the O-antigenic side chain of strain WCS358 consists of quinovosamine and a small amount of glucose, whereas the O-antigenic side chain of strain WCS374 consists of glucose and some fucose. Since glucose is still present in all mutant strains, this sugar is most likely also a constituent of the core of both *Pseudomonas* strains. Similar levels of the constituents of the lipid A (e.g., glucosamine and glucosamine phosphate) and the inner core (e.g., KDO and heptose) were detected in the LPSs of the wild-type strain WCS358 and the derived LPS mutants (Table 1), suggesting that the lipid A and the core contribute to a high degree to the weight of the wild-type LPS. This presumably indicates that in the wild-type strain relatively few LPS molecules with long O-antigenic side chains are present and that, therefore, the dominant LPS molecules are those with a short O-antigenic side chain or lacking the O-antigenic side chain. The presence of a minority of the LPS molecules with long O-antigenic side chains has also been described for wild-type *P. aeruginosa* cells (21). In contrast, in mutant strain LWP374-30b most components of the lipid A (e.g., glucosamine and glucosamine phosphate) and the core (e.g., KDO and rhamnose) are considerably increased, indicating that the O-antigenic side chain forms a substantial part of the weight of the LPS of strain WCS374. Therefore, it is likely that strain WCS374 contains predominantly LPS molecules with long O-antigenic side chains.

The long polysaccharide chains protrude from the bacterial cell surface (15, 16, 19) and thus are likely to be important for the physicochemical cell surface characteristics like hydrophobicity and charge. The data on the hydrophobicity of the two wild-type strains are consistent with this notion. The major constituents of the O antigen of strains WCS358 and WCS374 are quinovosamine and glucose, respectively. Quinovosamine can be assumed to be more hydrophobic than glucose because of the presence of an NH_2 and an H group, where glucose has OH groups. This difference explains the higher water contact angle, i.e., hydrophobicity, of strain WCS358 compared with that of strain WCS374. When the O-antigenic side chain is not present, the core oligosaccharide of the LPS will determine the physicochemical surface characteristics. Since the core oligosaccharides of these *Pseudomonas* species have many components in common (e.g., KDO, heptose, glucose, and alanine), it was not surprising to find that both the contact angles and the electrophoretic mobilities of the O-antigen-lacking mutants are similar (Table 2). The influence of the sugar composition of the LPS on the physicochemical surface characteristics may be inferred by comparing the mutant strains with their parental strains. Loss of the hydrophilic O-antigenic side chain in strain WCS374 results in an increase in the hydrophobicity of the cell surface. The increase in the cell surface charge of the mutant LWP374-30b may be explained by exposure of the negative charges of the inner-core constituents, e.g., KDO and phosphate groups, which in the wild type are masked by the neutral O-antigenic side chain. Elimination of the quinovosamine-containing O-antigenic side chain of strain WCS358 renders the surface more hydrophilic, because the sugars in the core are more hydrophilic than quinovosamine. The influence of the O-antigenic side chain of strain WCS358 on the cell surface charge is relatively small, which is consistent with the previously discussed conclusion that in the wild-type strain, LPS molecules lacking the side chain or with a short side chain may dominate. Loss of the O-antigen structure hardly influences the electrophoretic mobility, presumably since the negative charges of the core components are already relatively well exposed in the wild-type strain.

The considerable differences in hydrophobicity between strains WCS358 and LWP358-43b and in hydrophobicity as well as in cell surface charge between strains WCS374 and LWP374-30b make these strains ideal tools for studying the influence of physicochemical cell surface characteristics on adhesion properties. The adhesion of all strains to hydrophilic, lipophilic, or negatively charged Sephadex beads was extremely low (less than 1% of the cells), while the cells adhered to a greater extent (60 to 70%) to the positively charged DEAE-Sephadex beads. No significant difference in the kinetics of the firm adhesion to DEAE-Sephadex was observed between the wild-type strain and the LPS mutant (Fig. 2). In the studies of the firm adhesion of bacterial cells to sterile potato plant roots, a low percentage of the cells adhered to the root segments (less than 2%). Again, no significant differences between the wild types and their derived LPS-mutant strains in the number of adhered cells were found (Fig. 3). These results show that neither these differences in the cell surface charge and hydrophobicity between these wild-type and mutant strains nor the presence of the O-antigenic side chain of the LPS is relevant for the firm adhesion to the artificial surfaces and potato plant roots.

In conclusion, the O-antigenic side chain of the LPS of *Pseudomonas* strains WCS358 and WCS374 does influence physicochemical properties of the cells, like hydrophobicity and cell surface charge, but this structure does not seem to contribute substantially to firm adhesion to artificial surfaces or sterile potato plant roots. Furthermore, the results show that the variations in physicochemical properties of these *Pseudomonas* strains are not of prime importance for the adhesion phenomena studied. Therefore, the adhesion process may be governed by an alternative mechanism, involving specific receptor-ligand type interactions.

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