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A Novel Cell Surface Polysaccharide in *Pseudomonas putida* WCS358, Which Shares Characteristics with *Escherichia coli* K Antigens, Is Not Involved in Root Colonization

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Previously we have shown that flagella and the O-specific polysaccharide of lipopolysaccharide play a role in colonization of the potato root by plant growth-promoting *Pseudomonas* strains WCS374 and WCS358. In this paper, we describe a novel cell surface-exposed structure in *Pseudomonas putida* WCS358 examined with a specific monoclonal antibody. This cell surface structure appeared to be a polysaccharide, which was accessible to the monoclonal antibody at the outer cell surface. Further study revealed that it does not contain 2-keto-3-deoxyoctonate, heptose, or lipid A, indicating that it is not a second type of lipopolysaccharide. Instead, the polysaccharide shared some characteristics with K antigen described for *Escherichia coli*. From a series of 49 different soil bacteria tested, only one other potato plant growth-promoting *Pseudomonas* strain reacted positively with the monoclonal antibody. Mutant cells lacking the novel antigen were efficiently isolated by an enrichment method involving magnetic antibodies. Mutant strains defective in the novel antigen contained normal lipopolysaccharide. One of these mutants was affected in neither its ability to adhere to sterile potato root pieces nor its ability to colonize potato roots. We conclude that the bacterial cell surface of *P. putida* WCS358 contains at least two different polysaccharide structures. These are the O-specific polysaccharide of lipopolysaccharide, which is relevant for potato root colonization, and the novel polysaccharide, which is not involved in adhesion to or colonization of the potato root.

The potential of *Pseudomonas* bacteria to act as biocontrol agents has been described for many important crops, e.g., wheat (33), sugar beets (31), and potatoes (14, 21). These biocontrol agents offer a promising alternative to chemical pesticides, some of which are a threat to the environment. A variety of mechanisms can be involved in microbial plant protection, but efficient colonization of the rhizosphere is always essential, no matter whether the mechanism is delivery of biopesticides, physical exclusion of deleterious microbes, or competition for nutrients in the rhizosphere (23). Poor colonization of the rhizosphere often is the limiting factor in the application of biocontrol microbes. In order to improve this crucial step, it is necessary to increase our knowledge of the molecular mechanisms of root colonization.

Structures on the bacterial surface are likely candidates to play a role in colonization, since they may mediate interaction with the plant root. In previous studies we showed that flagella as well as the O-specific polysaccharide of lipopolysaccharide (LPS) are relevant for efficient colonization of potato roots (9, 10). Flagella are involved in the colonization of the deeper root parts of the potato plant root (9). However, a role of flagella in root colonization was not found in other microbe-plant systems (16, 29). Mutants lacking the O-specific polysaccharide of LPS are impaired in colonization of the deeper root parts of the potato root system, suggesting that this structure also plays a role in efficient root colonization (10). De Mot and Vanderleyden showed that a bacterial surface protein is involved in the adhesion of bacterial cells to wheat roots (6). Furthermore, bacterial cells that are agglutinated by root agglutinins appeared to perform better in root colonization than nonagglutinable cells (1), suggesting that the interaction with plant root components plays a role in root colonization.

During our search for cell surface molecules, we detected a novel polysaccharide at the cell surface of a root-colonizing *Pseudomonas putida* strain, WCS358. Characterization of the polysaccharide revealed that it lacks characteristics of LPS but shares some characteristics with K antigens. We accordingly studied whether it was involved in the interaction with the plant root.

MATERIALS AND METHODS

Bacteria and growth conditions. *P. putida* WCS358 and its mutant, LWP358-43b, which lacks the O-specific polysaccharide, have been described elsewhere (8, 14). For colonization experiments, a Tn*5*-marked derivative of WCS358 was used (11). Cells were grown in King's B medium (20) or standard succinate medium (24) at 28°C for 16 h under vigorous agitation. Tn*5* mutants were induced according to the method described by Simon et al. (30). In order to test the specificity of the monoclonal antibody, the following species were used: *Pseudomonas fluorescens* and *P. putida* (43 strains), *Erwinia carotovora* (2 strains), *Xanthomonas campestris* (1 strain), *Agrobacterium rhizogenes* (1 strain), and *Rhizobium leguminosarum* bv. viciae (2 strains).

Monoclonal and polyclonal antibodies. Spleen cells of a BALB/c mouse, immunized with washed cells of strain WCS358, were fused to Sp2/0 myeloma cells. Three weeks later, hybridoma culture supernatants were screened for antibody production. A preselection of hybridomas was made with an enzyme-linked immunosorbent assay (ELISA) with cells of strain WCS358 as the antigen. After subcloning of the positive hybridomas, three cell lines were obtained that produced monoclonal antibodies which recognized cells of the wild-type strain, WCS358, but not cells of its mutant, LWP358-43b, which lacks the O-specific polysaccharide. Ascites was prepared from one of the three monoclonal anti-

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bodies, MC13.6. The lipid A-specific monoclonal antibody 177 was kindly provided by T. R. De Kievit (5).

A rabbit was immunized intradermally with 10^9 cells of strain WCS358 emulsified in Freund's incomplete adjuvant. Three booster injections were given at 2-week intervals. Serum was obtained 2 weeks after the last injection and stored at −20°C. The resulting polyclonal antiserum responds predominantly with the LPS of strain WCS358.

Immunological techniques. For the immunological colony blot assay, colonies were streaked on King's B medium plates and after 16 h of growth were blotted to nitrocellulose. Cells were detected with monoclonal antibody MC13.6 and alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.). The alkaline phosphatase was visualized with nitrotetrazolium blue and 5-bromo-4-chloro-3-indolylphosphate as the substrate (13).

For Western blot (immunoblot) analysis, the electrophoresed patterns were transferred to Immobilon-P sheets (Millipore, Bedford, Mass.) and incubated with monoclonal antibody or with the polyclonal antiserum, followed by incubation with alkaline phosphatase-conjugated anti-mouse or anti-rabbit immunoglobulin G, respectively. Detection of bound antibodies was performed as described above. In order to obtain wide separation patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, samples (60 μl) were applied to horizontal slots approximately 7 cm in width. After blotting of the electrophoresed patterns to an Immobilon-P sheet, the sheet was cut in two: one part was developed with the polyclonal rabbit antiserum, and the other part was developed with monoclonal antibody MC13.6.

For bacterial agglutination assays, cells of the strain to be tested from a 16-h-old culture were washed and resuspended to an A_{620} of 1.0 in 10 mM phosphate buffer (pH 7.2). A dilution series of monoclonal antibody MC13.6 (50 μl) was applied to U-shaped wells of microtiter plates (Greiner, Solingen, Germany), followed by addition of 50 μl of the cell suspension. After 16 h of incubation at 4°C, the titer (i.e., the highest dilution which causes agglutination of the cells) was determined.

For the analysis of column fractions, an ELISA was used. Twenty-microliter aliquots of the column fractions were used to coat the wells of microtiter plates (Greiner), followed by an incubation with either monoclonal antibody MC13.6 or a polyclonal antiserum. For detection of bound antibodies, wells were incubated with peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Sigma), respectively, followed by addition of the substrate *o*-phenylenediamine-dihydrochloride (0.2 mg/ml)–H_2O_2 (0.015%). The reaction was stopped by addition of 1 N H_2SO_4, after which the A_{490} in the wells was measured with a Bio-Rad microplate reader (model 3550).

SDS-PAGE. Polyacrylamide gels were prepared as described previously (22). For analysis of complete cells, approximately 10^8 CFU was solubilized by incubation for 15 min at 95°C in the standard sample mixture as described previously (22) and incubated for 15 min at 95°C. Cell envelopes were isolated by differential centrifugation after ultrasonic disruption (22). Complete cells or cell envelopes were treated with proteinase K (Sigma) and subsequently used to analyze cell wall polysaccharides (7). After electrophoresis, gels were blotted to Immobilon-P membranes (Millipore) or subjected to the silver stain procedure described by Tsai and Frasch (32).

Mild hydrolysis of LPS on Western blots. Western blots containing LPS fractions were boiled in 10% acetic acid for 2.5 h. During this treatment, the O-specific polysaccharide was hydrolyzed from the lipid A part, leaving the latter bound to the blot (4). Subsequently, the blot was washed three times in phosphate-buffered saline (PBS; 10 mM phosphate buffer [pH 7.2], 0.155 M NaCl) to neutralize the blot. Bound molecules on the blot were detected with monoclonal antibody 177 or MC13.6 or the polyclonal antiserum (described above).

Isolation of the antigen recognized by MC13.6. LPS was isolated according to the method described by Darveau and Hancock (3). The resulting material, which also contained the antigen recognized by MC13.6, was fractionated on a Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.7 by 50 cm) at room temperature as described by Peterson and McGroarty (25). Approximately 25 mg of LPS in column buffer (0.25% deoxycholate, 0.2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0]) was applied to the column, and 4-ml fractions were collected at a flow rate of 8 ml/h. After a first analysis of the column fractions by an ELISA and by SDS-PAGE and Western blotting, fractions were selected for pooling in groups of six. To remove detergent and buffer, the pooled fractions were extensively dialyzed against buffer lacking deoxycholate at 28°C and subsequently dialyzed against demineralized water at 4°C. The dialyzed fractions were lyophilized and suspended in water in 1/40 of the original volume and used for sugar analyses.

Sugar analyses. Neutral sugar was determined according to the method of Dubois et al. (12) with glucose as the standard. Heptose was assayed according to the method of Wright and Reber (34) with manno-heptulose (Sigma) as the reference. 2-Keto-3-deoxyoctonate (KDO) was determined by the thiobarbituric acid method (19) with commercial KDO (Sigma) as the standard. When required in the latter procedure, the normal hydrolysis step for the fractions (8 min at 100°C in 0.25 N H_2SO_4) was modified according to the method of Carof et al. (2) (24 h at room temperature in 25% hydrofluoric acid).

The polysaccharide in pooled fractions 31 to 38 and 39 to 45 (described later) was hydrolyzed. Neutral sugars in the polysaccharide were determined after alditol acetate conversion to derivatives by gas-liquid chromatography on ECNSS-M [poly(ethylene succinate-methylcyanoethyl siloxane)-M] at 170°C. The carbazol reagent was used for the detection of hexuronic acid, and for the detection of amino sugars, gas-liquid chromatography and the Morgan Elson reaction were used (7).

Isolation of the MC13.6 antigen from the culture supernatant. The cells were removed by centrifugation from a 16-h-old culture in King's B medium, and the resulting cell pellet was analyzed by Western blotting as the total cell fraction. The supernatant was passed through a cellulose nitrate filter (0.45-μm-pore size; Sartorius, Frankfurt, Germany), and 3 volumes of ice-cold (−20°C) 96% ethanol was added to the resulting filtrate. The mixture was left at 4°C for 16 h, after which the precipitated material was recovered by centrifugation. The pellet was dissolved in demineralized water, treated with ribonuclease and deoxyribonuclease, and lyophilized. The material was analyzed by Western blotting or by immunoelectrophoresis, after which the center trough was filled with 2% Cetavlon (hexadecyltrimethylammonium bromide) (18). Precipitation arcs were judged 20 h later. The relative distribution of the polysaccharide between the extracellular material and the total cells was estimated by evaluation of the intensity of the reactions on the blot and the relative amounts applied to the blot.

Mutant enrichment with immunomagnetic particles. A collection of Tn*5* mutants of strain WCS358 were washed once in PBS and subsequently diluted in incubation buffer (PBS supplemented with 0.3 M NaCl and 0.5% Tween 80) to a final concentration of 10^6 CFU/ml. Monoclonal antibody MC13.6 was added to 1.0 ml of this suspension, which was then incubated at room temperature under continuous gentle mixing. After 1 h, the suspension was washed once and resuspended in fresh incubation buffer. Thirty-five microliters of a suspension of magnetic particles coupled to goat anti-mouse immunoglobulin G antibodies (BioMag 4340; Biolab, Limal, Belgium) was washed twice in PBS, resuspended in incubation buffer, and added to the suspension of cells loaded with antibody. This suspension was gently mixed at room temperature, and after 1 h, a samarium-cobalt magnet was positioned at the outside surface of the tube. After 15 min, the immunomagnetic complexes were concentrated at one side of the tube. The remaining suspension, enriched for cells that were not recognized by the monoclonal antibody, was pipetted off and used for a second enrichment cycle. Small samples (100 μl) from the starting suspension and from the suspension remaining after each enrichment cycle were plated on King's B medium to obtain colonies that were used for further screening in immunological colony blot assays. The remaining suspension was used as an inoculum for liquid King's B medium, and after 16 h of growth, the culture was used for the third and final enrichment cycle.

Plant assays. Sterile potato plantlets were cultivated as described previously (11). For the determination of the adhesion of the bacterial cells to sterile potato roots, three 3-cm-diameter pieces of sterile roots were incubated in a suspension of bacteria (10^7 CFU/ml) in 10 mM phosphate buffer (pH 7.2). After 10 and 60 min, the pieces were taken out of the suspension and washed in phosphate buffer by vigorous vortexing (IKA-Vibrofix; VF1 Electronic, Staufen, Germany) for 1 min. Subsequently, the root pieces were rinsed quickly in fresh phosphate buffer, put in an Eppendorf tube with glass beads, and shaken for 20 min on a Vortex Genie 2 (Scientific Industries, Bohemia, N.Y.) with a plastic adaptor for 50 Eppendorf tubes. The number of *Pseudomonas* cells released from the root pieces was determined by plating with a Spiral plater, model CU (Spiral System Instruments, Inc., Bethesda, Md.). The mean values and the standard deviations of the log-transformed data of three replicas were determined.

Colonization of potato roots was tested in a greenhouse assay as described previously (15). Bacterial suspensions were prepared from cells grown for 48 h at 27°C on King's B agar plates, washed twice, and resuspended in sterile distilled water (10^9 CFU/ml). Ten potato stem cuttings with roots approximately 1 cm in length were dipped into the bacterial suspension of one of the Tn*5*-marked *Pseudomonas* strains and planted in petri dishes containing clay soil. After 7 days of growth in the greenhouse, the root samples were taken from different depths (0 to 2, 2 to 4, and 4 to 6 cm), and the number of kanamycin-resistant bacteria on the roots was determined by dilution plating of the root suspensions on modified King's B medium (14) supplemented with streptomycin (200 μg/ml) and kanamycin (200 μg/ml). The data from 10 replicas were log transformed, followed by calculation of the mean and the standard deviation. Two independent experiments gave similar results.

RESULTS

Preliminary characterization of a novel cell surface polysaccharide. Three monoclonal antibodies were isolated that reacted with wild-type cells of *P. putida* WCS358 but not with cells of its mutant, LWP358-43b, which lacked the O-specific polysaccharide. In an ELISA, they reacted with whole cells, cell envelopes, and the LPS fraction of the wild-type strain, WCS358. Furthermore, 40× diluted ascites fluid of these monoclonal antibodies agglutinated cells of WCS358, indicating that the antigen is accessible at the outer surface of the cells. None of these reactions was observed when the mutant LWP358-43b was used instead of the wild-type strain, WCS358. Since all three monoclonal antibodies showed similar

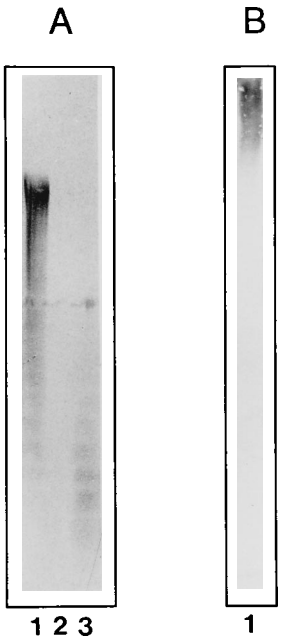

FIG. 1. Western blot of polyacrylamide gels containing SDS (A) and lacking SDS (B), developed with MC13.6, showing proteinase K-treated cells of WCS358 (lanes 1) and of the mutant strains PCL5801 (lane 2) and PCL5802 (lane 3).

reaction patterns, one of the monoclonal antibodies, MC13.6, was chosen for the rest of this study.

Although the reactions described are indicative of a reaction with the LPS of strain WCS358, Western blot analysis of whole cells or cell envelopes of strain WCS358 did not reveal the expected LPS ladder, but the antibodies instead reacted with an antigen that appeared on Western blots as a broad region with a faint ladder pattern (Fig. 1A). The broad region was observed when the sample was applied to a small slot and may contain high-molecular-weight complexes of the antigen. A similar broad region (but higher in the gel) was also detected in polyacrylamide gels lacking SDS (Fig. 1B). When proteinase K-treated cells were applied to a 7-cm-broad trough, a clear ladder pattern was observed (Fig. 2). However, the phase of this ladder pattern was different from that of the LPS ladder, as can be observed in Fig. 2. Here the broad lane has been cut into two. In the left part, the normal LPS ladder pattern was visualized by the polyclonal antiserum, whereas the right part revealed the ladder pattern of the novel antigen recognized by monoclonal antibody MC13.6.

Fractions obtained by the method of Darveau and Hancock (3) for the isolation of LPS contained the polysaccharide antigen. Rivera et al. showed the occurrence of at least two types of chemically and antigenically distinct LPS molecules in different strains of *Pseudomonas aeruginosa* (27). In order to investigate whether our novel antigen represents a second type of LPS, it was separated from the known LPS. To this end, the fraction obtained by the method of Darveau and Hancock was applied to a Sephadex G-200 gel filtration column. The column fractions were assayed with an ELISA by using MC13.6 as well as the polyclonal antiserum. The elution profile obtained with MC13.6 showed a peak after the void volume (from fraction 30 on) that slowly decreased in the next fractions (up to fraction 64) (Fig. 3). The elution profile as detected by the polyclonal antiserum showed a small first peak and a broad plateau. The first peak may represent the new antigen, which also reacts with the polyclonal antiserum as well as the presence of some contaminating LPS material in these fractions (see also the silver-stained gel and the Western blot developed with polyclonal antiserum [Fig. 3]). When the fractions were subjected to SDS-PAGE and subsequently silver stained, the broad region of unresolved material typical for the antigen was observed in fractions 32 through approximately 57. This material runs lower in the gel in the subsequent lanes, suggesting that the molecules which cause this smear decrease in size. Also, the LPS molecules were resolved in molecules with decreasing sizes, starting from fraction 49, as can be observed on the Western blot developed with polyclonal antiserum. The antigen was faintly detected with the polyclonal antiserum. The Western blot developed with MC13.6 (Fig. 3) clearly revealed that the antigen was present in fractions 32 through approximately 57.

In order to study the nature of the new antigen, several sugar analyses were performed with pooled, dialyzed column fractions (Fig. 4). Neutral sugar was found in relatively high quantities in the fractions containing the novel antigen, whereas the fractions containing LPS molecules showed lower quantities. In the fractions containing the LPS molecules (fractions 49 to 97), the amounts of the LPS-specific sugars heptose and KDO increased with the decreasing molecule sizes of the LPS. In the fractions containing large LPS molecules (fractions 49 to 57) and in the fractions containing only the new antigen (fractions 31 to 48), KDO and heptose were not detected. It has been reported that in some LPS species, KDO can be substituted and thereby can become less responsive to the thiobarbiturate assay (2). Stronger hydrolysis conditions will increase the amount of reactive KDO in these species (2, 27). We therefore subjected the fractions containing the antigen to more rigorous hydrolysis conditions with hydrofluoric acid, but no response in the thiobarbituric assay was observed.

Analysis of the monosaccharide constituents in the new antigen revealed glucose as the only neutral sugar present. Amino sugars and hexuronic acids were not detected.

A typical constituent of LPSs is the lipid A part. To study whether the new antigen contains lipid A, the pooled column fractions were analyzed on a Western blot with the lipid A-

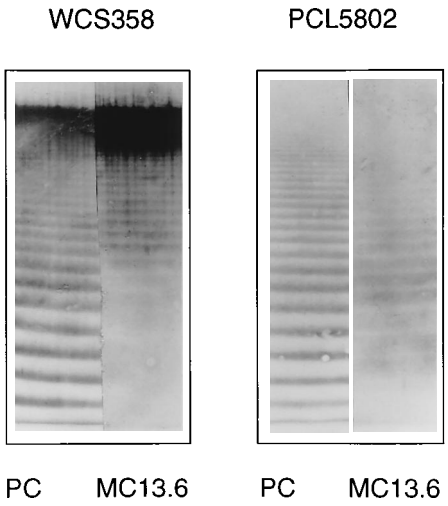

FIG. 2. Western blot analysis of proteinase K-treated cells of wild-type strain WCS358 and mutant strain PCL5802. The samples were applied to SDS-polyacrylamide gels in broad lanes, electrophoretically separated, and blotted to Immobilon-P sheets. Each sheet was cut into two parts; one part was developed with the polyclonal antiserum (PC), and the other part was developed with the monoclonal antibody MC13.6. The polyclonal antiserum has predominantly O specificity, and it therefore predominantly stains LPS.

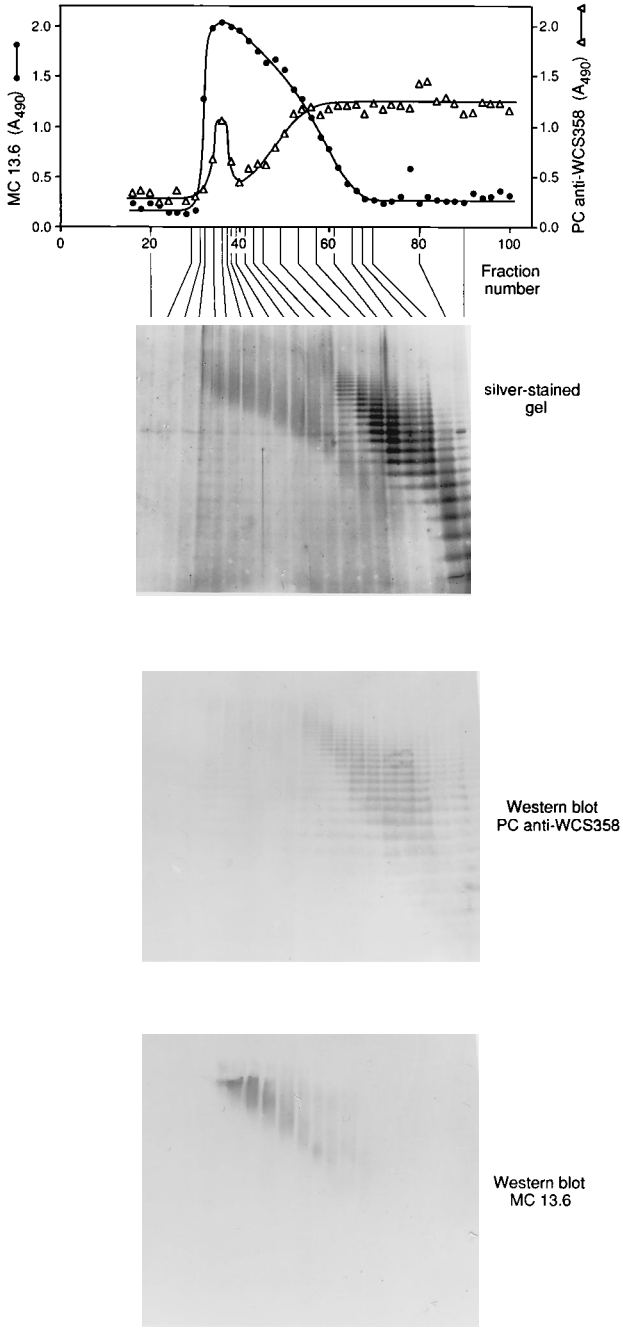

FIG. 3. Fractionation of the polysaccharide fraction of *P. putida* WCS358 obtained by the method of Darveau and Hancock on Sephadex G-200. The fractions were analyzed with an ELISA for reaction with either the anti-WCS358 polyclonal antiserum (PC) (△) or the monoclonal antibody MC13.6 (●). The fractions were analyzed on SDS-polyacrylamide gels that were silver stained (upper gel) or blotted to Immobilon-P sheets and developed with the polyclonal antiserum (middle gel) or the monoclonal antibody MC13.6 (bottom gel). This fractionation was performed twice with similar results.

specific monoclonal antibody 177 (5). A lipid A spot was detected at the front of the gel in the lanes containing the LPS molecules (pooled fractions 49 to 97), while such a spot was absent in the fractions containing the new antigen (pooled fractions 31 to 48) (data not shown). Since the monoclonal antibody can only respond to the unmasked lipid A epitope (4),

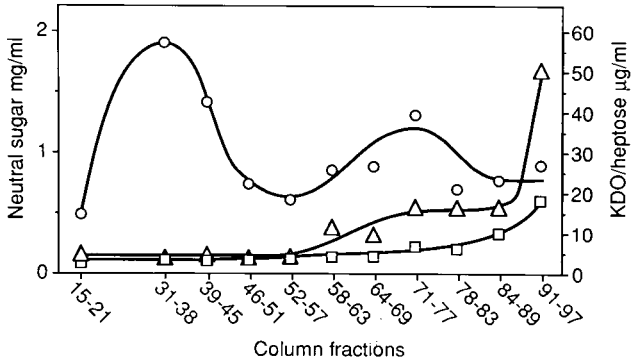

FIG. 4. Sugar analysis of pooled column fractions obtained after the gel filtration shown in Fig. 3. The fractions were analyzed for the presence of neutral sugar (○), KDO (□), and heptose (△).

we subsequently removed the core saccharides and oligosaccharides from the fractions in order to test whether lipid A was present in the fractions containing the new antigen. Therefore, an acid hydrolysis was performed with the Western blot. This hydrolysis resulted in partial hydrolysis of the O-specific polysaccharides, as judged from control blots that were developed with the polyclonal antibody and the monoclonal antibody (data not shown). When the hydrolyzed blot was developed with the lipid A-specific monoclonal antibody, ladder patterns were observed in the lanes containing the LPS molecules, while no reaction was observed in the lanes containing the new antigen (Fig. 5).

Analysis by Western blots of material that was precipitated from the supernatant of a stationary-phase culture showed that the antigenic structure was also present in the medium of the cells. According to these Western blots, it was estimated that up to 40% of the material present in total cells could be found in the culture supernatant. By immunoelectrophoresis, this

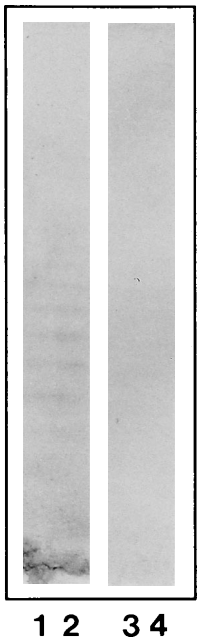

FIG. 5. Western blot containing pooled column fractions 31 to 38 (lane 4), 39 to 45 (lane 3), 71 to 77 (lane 2), and 78 to 83 (lane 1). The blot was subjected to a mild hydrolysis followed by immunodetection with monoclonal antibody 177.

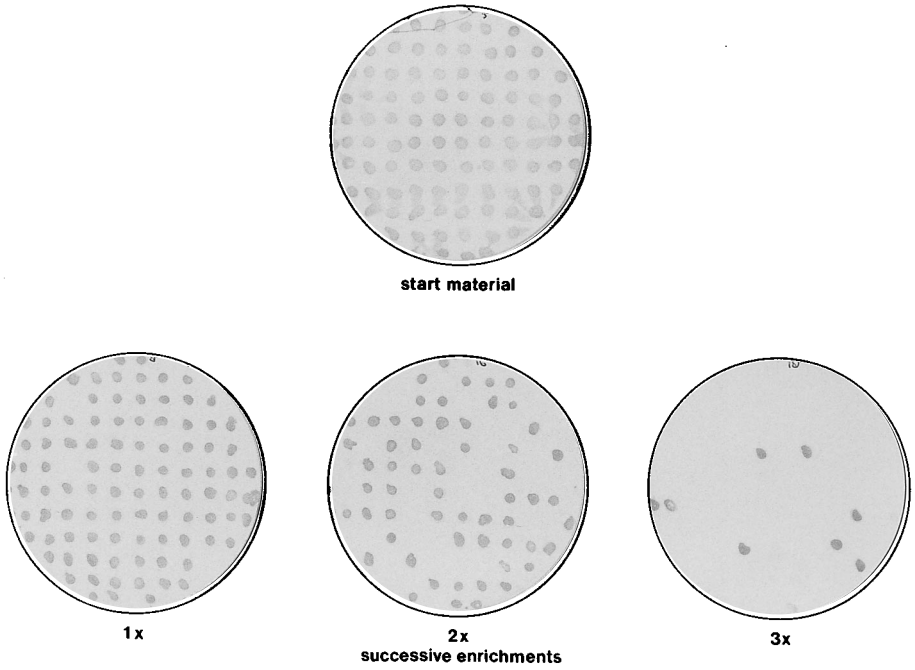

FIG. 6. Colony blots containing mutants of strain WCS358 developed with the monoclonal antibody MC13.6. Each colony blot contains 100 colonies derived from the starting suspension (upper blot) or from the suspensions that have passed subsequent enrichment cycles (three blots at the bottom).

material moved to the positive pole and formed precipitation arcs with the quaternary ammonium salt Cetavlon, which is also observed for K antigens of *Escherichia coli* (18). These results show that the polysaccharide is negatively charged.

Specificity of monoclonal antibody MC13.6. To test the specificity of the antibodies, a series of 49 different soil bacteria (e.g., several *Pseudomonas* species, *Erwinia* spp., *A. rhizogenes*, and *Rhizobium* spp.) were tested in an ELISA. Only one strain, a plant growth-promoting *Pseudomonas* strain, A1, isolated in the United States (21), was recognized. This strain has cell envelope protein and LPS patterns similar to those of strain WCS358 (7, 8). When proteinase K-treated cell envelopes were analyzed by Western blotting, a broad region similar to the one observed for strain WCS358 (Fig. 1) was detected (data not shown).

Isolation and characterization of mutants defective in the novel polysaccharide structure. Since the novel polysaccharide antigen is accessible at the outer surface of the cell and is also present in another potato plant growth-promoting *Pseudomonas* strain, we decided to investigate whether the structure is involved in the interaction with the plant root. Therefore, we isolated mutants defective in this novel polysaccharide. A method in which magnetic antibodies are used to enrich a cell suspension for mutant cells lacking the antigen was developed. Initial experiments showed that it is relevant to increase the salt concentration of the incubation buffer by addition of 0.3 M NaCl to the original PBS in order to reduce nonspecific binding of cells to the magnetic beads. In a model experiment with cells of strain WCS358 and its mutant, LWP358-43b, which lacks the O-specific polysaccharide (which is not recognized by MC13.6), in a fixed ratio, it was found that after each enrichment cycle (see Materials and Methods), the ratio of mutant to wild type increased by a factor of 10. The method was therefore used to isolate Tn*5* mutants of strain WCS358 that do not react with MC13.6. After each enrichment cycle, 200 to 300 colonies obtained from the enriched suspensions were tested in an immunological colony blot for reactivity with MC13.6 (Fig. 6). The 300 colonies tested from the suspension at the start of the experiment all reacted with MC13.6, suggesting that the percentage of MC13.6-negative mutants in the starting suspension was less than approximately 0.3%. After the enrichment method described above was used, this percentage increased from 3% after the first cycle to 23% in the second cycle. After the third cycle, 93% of the cells in the remaining suspension were mutant cells that did not react with MC13.6 at all or showed a significantly decreased reaction (Fig. 6). This finding shows that this method is very powerful for the enrichment of cell surface mutants.

Among the mutants isolated above, some strains showed a weak response with MC13.6 in an immunological colony blot, e.g., mutant strain PCL5802, while other strains did not react with MC13.6 at all (e.g., mutant strain PCL5801). The two mutants mentioned above were characterized in more detail. Neither the generation times in minimal medium nor the cell envelope protein or LPS pattern obtained by SDS-PAGE of these mutants differed from those of the wild-type strain. The latter observation was especially interesting, since strain LWP358-43b, the mutant lacking the O-specific polysaccharide, did not react with monoclonal antibody MC13.6 (see Materials and Methods). Furthermore, 10 other, different MC13.6-unreactive mutant strains showed LPS patterns similar to those of the wild-type strain (data not shown).

In contrast to wild-type strain WCS358, cells of mutant strains PCL5801 and PCL5802 were not agglutinated by MC13.6. Western blot analysis of whole cells of the wild-type and mutant strains revealed different reaction patterns among these strains (Fig. 1A and 2). When the polyclonal rabbit antiserum was used to develop the blots, both mutants as well as wild-type strain WCS358 showed the regular LPS ladder pattern (Fig. 2), whereas the patterns obtained with MC13.6 were different for the mutants and the wild type. Cells of mutant strain PCL5802, which showed a faint reaction with

MC13.6 in the colony blot, showed another ladder pattern which was shifted in comparison with that of the wild type (Fig. 2). Cells of mutant strain PCL5801, the mutant that did not show any reaction with MC13.6 in colony blots, also did not show a reaction in the Western blot analysis with MC13.6 (Fig. 1A, lane 2). These results show that mutant PCL5801 lacks the polysaccharide antigen, whereas the polysaccharide of mutant PCL5802 seems to be smaller but still contains the characteristic epitope.

Adhesion to and colonization of potato roots by mutant and wild-type cells. The abilities of the mutant strains PCL5801 and PCL5802 and the wild-type strain to adhere to sterile potato plant roots were compared. The number of cells that adhered to the sterile potato root pieces after incubation for 10 min in bacterial suspensions of strain WCS358 and mutant strains PCL5801 and PCL5802 were similar (mean values and standard deviations of the log-transformed determinations were 4.2 ± 0.2, 4.3 ± 0.1, and 4.3 ± 0.1, respectively). Comparable results were obtained after a 1-h incubation.

The root-colonizing abilities of mutant strain PCL5801 and the wild-type strain were compared by analysis of the roots of plants inoculated with either one of the strains. Segments of the roots were taken from three different depths. From segments taken from the upper part of the root (0 to 2 cm in depth), approximately 10^8 CFU/g of root were reisolated, and from segments taken from the lower part (4 to 6 cm in depth), 10^4 CFU/g of root were found. However, no significant differences were found between the plants inoculated with the wild-type strain and those inoculated with mutant strain PCL5801 (mean values and standard deviations of the log-transformed determinations of root samples taken from 10 individual plants at depths of 4 to 6 cm were 4.7 ± 0.8 and 4.3 ± 0.4, respectively).

DISCUSSION

A new antigenic structure on the cell surface of *P. putida* WCS358, a plant growth-promoting, potato root-colonizing strain, was discovered with a monoclonal antibody. This antibody agglutinates the cells at a very low titer. Apparently the monoclonal antibody can bind to the antigen of two adjacent cells. Therefore, at high concentrations of monoclonal antibody, agglutination of the cells can occur. This result indicates that the molecule is accessible at the cell surface. From a series of 49 different potato rhizosphere bacteria, only one strain appeared to contain a similar epitope, namely, another plant growth-promoting *Pseudomonas* strain, A1. This strain, isolated in the United States (also from potato roots) (21), had been shown to possess cell envelope protein and LPS patterns by SDS-PAGE similar to those of strain WCS358 (7, 8). The presence of this antigenic structure in another plant-beneficial strain and its location at the outer surface of the cell increased our interest in this polysaccharide.

Preliminary characterization revealed that the antigenic structure is a polysaccharide. Although it appeared to be present in the LPS fraction isolated according to the procedure described by Darveau and Hancock (3), analysis by SDS-PAGE revealed that the antigenic structure gives a ladder pattern different from the LPS of this strain (Fig. 2) or it forms a broad band in the gel (Fig. 1A). For several *P. aeruginosa* strains, it has been shown that two types of LPS can be present in a single strain (25, 27, 28). In order to test whether the new polysaccharide at the cell surface of strain WCS358 is a second type of LPS, we separated the two polysaccharides on a gel filtration column and analyzed their composition. Analysis of the fractions containing the new antigen indicated that it is not a second type of LPS since (i) it lacks lipid A (Fig. 5) and (ii) it lacks the LPS-specific sugars heptose and KDO. The latter sugars were also not detected in the high-molecular-weight fractions of LPS. This is probably due to the fact that heptose and KDO are only present in the core of the LPS molecule and that when the O-specific polysaccharide is large, the relative amounts of heptose and KDO are too small to be detected. It has been reported that the LPSs of various bacterial species (e.g., of the genera *Bordetella*, *Vibrio*, and *Aeromonas*) can contain KDO that is less responsive in the thiobarbiturate test because of substitutions in the molecule (2). More severe hydrolysis conditions can make this KDO more reactive (2). However, our novel polysaccharide did not show any reaction in the thiobarbiturate assay after a more rigorous hydrolysis. Analysis of the monosaccharides in the polymer revealed the presence of glucose. No other sugar could be detected.

In conclusion, the absence of KDO, heptose, and lipid A makes it unlikely that the novel polysaccharide is a second LPS. Instead, this polysaccharide shares characteristics with certain group II capsular polysaccharides (K antigens) of *E. coli* (17): it is an additional, negatively charged surface polysaccharide antigen which is not only cell associated but also secreted into the medium. Colorimetric determinations with the polysaccharide precipitated from the culture supernatant revealed the absence of acidic sugars and the presence of phosphate (unpublished data), which explains its acidic character. K antigens which are structurally similar to the *E. coli* K antigens were discovered in two other rhizosphere microorganisms, i.e., *Rhizobium fredii* and *Rhizobium meliloti* (26). Similar to our antigen, these anionic K antigens appeared to run in polyacrylamide gels without detergents (Fig. 1B). This is in contrast to LPS, which forms aggregates that cannot enter the gel without detergent.

Approximately 40% of the polysaccharide was excreted into the medium, leaving the remainder bound to the cells. However, this material was not enough to provide a visible capsule with an India ink stain (unpublished data). Still, the material might offer some sort of protection to the cells, especially in the rhizosphere. Therefore, the role of this polysaccharide in colonization of the potato root was further studied. Mutants lacking the antigen were isolated. For the isolation of these mutants, a novel enrichment method utilizing immunomagnetic particles was developed. Model experiments with WCS358 and its mutant, LWP358-43b, in a fixed ratio showed that each enrichment cycle results in an increase in the ratio of mutant to wild-type cells by a factor of 10. With this enrichment method, mutants lacking the antigenic determinant were found with a frequency of approximately 10^{-4} to 10^{-3}. This high mutation frequency may be due to spontaneous loss of the antigen, since these mutants were also found with a high frequency after selection with a specific phage (unpublished data), thus not needing to be coupled to the Tn*5* insertion. Mutant strain PCL5801 responds to the antibody neither in a colony blot nor on Western blots, indicating that the antigen is lacking. Mutant strain PCL5802 showed a faint reaction on a colony blot but was not agglutinated by the antibody. In this mutant, the ladder pattern was shifted to the lower part of the gel (Fig. 1 and 2), indicating that the antigen is still present in the mutant. However, the polysaccharide may be smaller and therefore less accessible to the antibody.

Mutant strain PCL5801, which completely lacks the antigenic determinant of the polysaccharide, was compared with the wild type for its ability to adhere to and to colonize potato roots. It appeared to be unaffected in both of these characteristics, indicating that the novel polysaccharide is not involved in attachment to and colonization of the plant root. Mutant strain

LWP358-43b, lacking the O-specific polysaccharide of LPS, has previously been shown to be impaired in the efficient colonization of the deeper root parts of the potato root system (10). In this study, it became apparent that mutant LWP358-43b not only lacks the O-specific polysaccharide but also lacks the novel polysaccharide. We have now shown that the latter polysaccharide does not affect the colonization ability of the cells. This result justifies our former conclusion (10) that the lack of the O-specific polysaccharide is responsible for the reduced colonization ability of this mutant. In conclusion, at least two types of polysaccharides are present at the cell surface of the plant growth-promoting strain WCS358: one of the polysaccharides (the LPS) is relevant for root colonization, whereas the newly described polysaccharide is not involved in this process.

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REFERENCES

1. **Anderson, A. J., P. Habibzadegah-Tari, and C. S. Tepper.** 1988. Molecular studies on the role of a root surface agglutinin in adherence and colonization by *Pseudomonas putida*. Appl. Environ. Microbiol. **54:**375–380.
2. **Carof, M., S. Lebbar, and L. Szabo.** 1987. Do endotoxins devoid of 3-deoxy-d-manno-2-octulosonic acid exist? Biochem. Biophys. Res. Commun. **143:** 845–847.
3. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155:**831–838.
4. **De Kievit, T. R. (University of Guelph).** 1994. Personal communication.
5. **De Kievit, T. R., and J. S. Lam.** 1993. Immunochemistry and biochemistry of the core-lipid A region of *Pseudomonas aeruginosa* lipopolysaccharide, abstr. 82, p. 126. *In* Pseudomonas 1993. Fourth International Symposium on Pseudomonas: Biotechnology and Molecular Biology.
6. **De Mot, R., and J. Vanderleyden.** 1991. Purification of a root-adhesive outer membrane protein of root-colonizing *Pseudomonas fluorescens*. FEMS Microbiol. Lett. **81:**323–328.
7. **de Weger, L. A., B. Jann, K. Jann, and B. Lugtenberg.** 1987. Lipopolysaccharides of *Pseudomonas* spp. that stimulate plant growth: composition and use for strain identification. J. Bacteriol. **169:**1441–1446.
8. **de Weger, L. A., R. van Boxtel, B. van der Burg, R. A. Gruters, F. P. Geels, B. Schippers, and B. Lugtenberg.** 1986. Siderophores and the outer membrane proteins of antagonistic plant-growth-stimulating, root-colonizing *Pseudomonas* spp. J. Bacteriol. **165:**585–594.
9. **de Weger, L. A., C. I. M. van der Vlugt, A. H. M. Wijfjes, P. A. H. M. Bakker, B. Schippers, and B. Lugtenberg.** 1987. Flagella of a plant growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. J. Bacteriol. **169:**2769–2773.
10. **de Weger, L. A., M. C. M. van Loosdrecht, P. A. H. M. Bakker, B. Schippers, and B. Lugtenberg.** 1989. *Pseudomonas* spp. with mutational changes in the O-antigenic side chain of their lipopolysaccharide are affected in their ability to colonize potato roots. NATO ASI (Adv. Sci. Inst.) Ser. Ser. H Cell Biol. **36:**197–202.
11. **de Weger, L. A., M. C. M. van Loosdrecht, H. E. Klaassen, and B. Lugtenberg.** 1989. Mutational changes in physicochemical cell surface properties of plant growth-stimulating *Pseudomonas* spp. do not influence the attachment properties of the cells. J. Bacteriol. **171:**2756–2761.
12. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
13. **Ey, P. L., and L. K. Ashman.** 1986. The use of alkaline phosphatase conjugated antimouse immunoglobulin with immunoblots for determining the specificity of monoclonal antibodies to protein mixtures. Methods Enzymol. **121:**497–509.
14. **Geels, F. P., and B. Schippers.** 1983. Selection of antagonistic fluorescent *Pseudomonas* spp. and their root colonization and persistence following treatment of seed potatoes. Phytopathol. Z. **108:**193–206.
15. **Glandorf, D. C. M., L. G. L. Peters, I. van der Sluis, P. A. H. M. Bakker, and B. Schippers.** 1993. Crop specificity of rhizosphere pseudomonads and the involvement of root agglutinins. Soil Biol. Biochem. **25:**981–989.
16. **Howie, W. J., R. J. Cook, and D. M. Weller.** 1987. Effects of soil matric potential and cell motility on wheat root colonization by fluorescent pseudomonads suppressive to take all. Phytopathology **77:**286–292.
17. **Jann, B., and K. Jann.** 1990. Structure and biosynthesis of the capsular antigens of *Escherichia coli*. Curr. Top. Microbiol. Immunol. **150:**19–42.
18. **Jann, K.** 1985. Isolation and characterization of capsular polysaccharides (K antigens) from *Escherichia coli*, p. 375–379. *In* M. Sussman (ed.), The virulence of *Escherichia coli*. Academic Press, London.
19. **Keleti, G., and W. H. Lederer.** 1974. Handbook of micromethods for the biological sciences, p. 74–75. Van Nostrand Reinhold Co., Inc., New York.
20. **King, E. O., K. Ward, and D. E. Raney.** Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. **44:**301–307.
21. **Kloepper, J. W., J. Leong, M. Teintze, and M. N. Schroth.** 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature (London) **286:**885–886.
22. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen,** 1975. Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. FEBS Lett. **58:**254–258.
23. **Lugtenberg, B. J. J., L. A. de Weger, and J. W. Bennett.** 1991. Microbial stimulation of plant growth protection from disease. Curr. Biotechnol. **2:** 457–465.
24. **Meyer, J. M., and M. A. Abdallah.** 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. J. Gen. Microbiol. **107:**319–328.
25. **Peterson, A. A., and E. J. McGroarty.** 1985. High-molecular-weight components in lipopolysaccharides of *Salmonella typhimurium*, *Salmonella minnesota*, and *Escherichia coli*. J. Bacteriol. **162:**738–745.
26. **Reuhs, B. L., R. W. Carlson, and J. S. Kim.** 1993. *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-D-manno-2-octulosonic acid-containing polysaccharides that are structurally analogous to group II K antigens (capsular polysaccharides) found in *Escherichia coli*. J. Bacteriol. **175:**3570–3580.
27. **Rivera, M., L. E. Bryan, R. E. W. Hancock, and E. McGroarty.** 1988. Heterogeneity of lipopolysaccharides from *Pseudomonas aeruginosa*: analysis of lipopolysaccharide chain length. J. Bacteriol. **170:**512–521.
28. **Rivera, M., and E. J. McGroarty.** 1989. Analysis of a common-antigen lipopolysaccharide from *Pseudomonas aeruginosa*. J. Bacteriol. **171:**2244–2248.
29. **Scher, F. M., J. W. Kloepper, C. Singleton, I. Zaleska, and M. Laliberte.** 1988. Colonization of soybean roots by *Pseudomonas* and *Serratia* species: relationship to bacterial motility, chemotaxis and generation time. Phytopathology **78:**1055–1059.
30. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilisation system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.
31. **Suslow, T. V., and M. N. Schroth.** 1982. Rhizobacteria of sugar beets: effects of seed application and root colonization on yield. Phytopathology **72:**199–203.
32. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
33. **Weller, D. M., W. J. Howie, and R. J. Cook.** 1988. Relationship between in vitro inhibition of *Gaeumannomyces graminis* var. *tritici* and suppression of take-all of wheat by fluorescent pseudomonads. Phytopathology **78:**1094–1100.
34. **Wright, B., and P. A. Reber.** 1972. Procedure for determining heptose and hexose in lipopolysaccharides—a modification of the cysteine-sulfuric method. Anal. Biochem. **49:**307–319.