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Characterization of Root Surface and Endorhizosphere Pseudomonads in Relation to Their Colonization of Roots

RON VAN PEER,[1]* HELMA L. M. PUNTE,[1] LETTY A. DE WEGER,[2] AND BOB SCHIPPERS[1]

Willie Commelin Scholten Phytopathological Laboratory, Department of Plant Pathology, State University of Utrecht, Javalaan 20, 3742 CP Baarn,[1] and Department of Plant Molecular Biology, Leiden University, 2311 VJ Leiden,[2] The Netherlands

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An extensive colonization of the endorhizosphere by fluorescent pseudomonads was observed in tomato plants grown on artificial substrates. These studies reveal that a significantly higher percentage of pseudomonads obtained from the endorhizosphere (30%) reduced plant growth than those obtained from the root surface (4%). Lipopolysaccharide patterns, cell envelope protein patterns, and other biochemical characteristics indicated that *Pseudomonas* isolates obtained from the endorhizosphere are distinct from *Pseudomonas* isolates obtained from the root surface. Isolates from the endorhizosphere especially were able to recolonize the endorhizosphere of both sterile and nonsterile tomato roots. The ability of the endorhizosphere isolates to colonize the endorhizosphere significantly correlated with their agglutination by tomato root agglutinin but did not correlate with chemotaxis to seed exudates of tomato. No correlation between colonization of the endorhizosphere and agglutination by root agglutinin could be demonstrated for the root surface isolates. We propose that agglutination of specific *Pseudomonas* strains by root agglutinin is of importance in the initial phase of adherence of bacteria to the root surface.

Previous studies demonstrated the presence of pseudomonads both on the root surface and in the endorhizosphere of tomatoes (18). Pseudomonads were predominant in the endorhizosphere of tomatoes grown on artificial substrates such as rockwool or nutrient film cultures, compared with tomatoes grown in soil. Bacteria producing phytotoxic metabolites (particularly gaseous components) in the endorhizosphere of hydroponically grown plants are deleterious to plant growth, because their metabolites are entrapped in the root tissue and do not easily diffuse, thereby exhibiting more dramatic effects on the plant physiology than metabolites produced on the root surface (B. Schippers, P. A. H. M. Bakker, A. W. Baker, and R. van Peer, Proc. Brighton Crop Prot. Conf., p. 611–614, 1988). The present study was initiated to elucidate the question of whether populations of pseudomonads inhabiting the endorhizosphere are different from those inhabiting the root surface. Chemotaxonomic characteristics such as lipopolysaccharide patterns and cell envelope protein patterns were used in addition to other biochemical characteristics. The observed differences in the colonization of the endorhizosphere between *Pseudomonas* spp. isolated from either the root surface or the endorhizosphere are related to specific properties, such as their interactions with the plant root agglutinins, adherence to the plant root, and chemotaxis.

MATERIALS AND METHODS

Samplings. Pseudomonads were isolated and selected from roots of glasshouse tomatoes (*Lycopersicon esculentum* Mill cv. Moneymaker or cv. Turbo) grown on rockwool, on a nutrient film culture, or in a hydroponic system (18). A sample (0.3 g) of root pieces was shaken vigorously for 1 min in 5 ml of 0.1 M $MgSO_4$ and dilution plated on modified King's medium B (KB) (10) to enumerate root surface-inhabiting pseudomonads. Root pieces were surface sterilized with H_2O_2 (10%; 15 s), rinsed three times with $MgSO_4$ (0.1 M), ground with a mortar and pestle, and then homogenized and dilution plated to enumerate the endorhizosphere-inhabiting pseudomonads.

Colonies representing different morphological types were randomly selected and purified. A total of 213 pseudomonads were collected in this way.

Bacterial culture conditions. A loop of bacteria from a 24-h-old culture was suspended in 0.1 M $MgSO_4$. A total of 500 μl of this suspension was added to KB agar plates and incubated for 24 h at 27°C. These cultures were suspended in sterile distilled water (SDW) and were used for inoculation of plants (10^8 cells ml^{-1}). For isolation of lipolysaccharides (LPS), a stationary-phase culture was diluted 100-fold in KB and cultivated for 24 h at 28°C.

Screening of pseudomonads for effects on plant growth. For the screening of bacterial effects on plant growth, plastic containers (200 ml) were used. They were filled with a nutrient solution [0.6 g of Nutriflora-T (Windmill Holland B.V.) $liter^{-1}$ and 10 g of $Ca(NO_3)_2$ $liter^{-1}$]. Each container was covered with a petri dish lid with seven holes, each of which held a 1-ml pipette tip filled with sterilized vermiculite. Tomato seeds were sterilized by rinsing with 1% sodium hypochlorite for 1 min. After being rinsed with SDW, the seeds were germinated on sterile filter paper in a petri dish. One germinated seed was placed on the vermiculite in each pipette tip and covered with a thin layer of vermiculite.

Seedlings were bacterized with 0.5 ml of a bacterial suspension (10^8 cells ml^{-1}) from cultures grown for 24 h at 27°C. After 14 days of growth in a growth cabinet at 21°C, fresh weights of plants were recorded for four replicates of each treatment. Isolates with statistically significant effects on seedling growth and other randomly chosen isolates up to a total of either 50 root surface isolates or 50 endorhizosphere isolates were tested twice for their effects on growth of 5-week-old tomato plants grown in a hydroponic system

* Corresponding author.

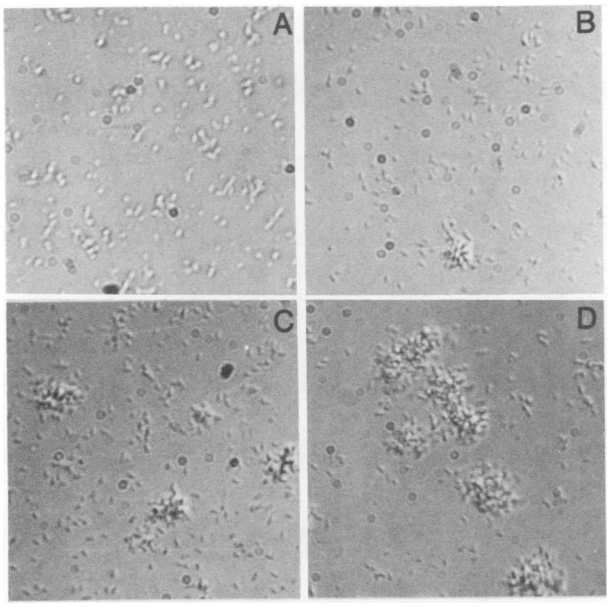

FIG. 1. Classes of agglutination of *Pseudomonas* cells by root agglutinin of tomato plants. (A) Class 0; (B) class 1; (C) class 2; (D) class 3. See text for definition of classes.

(18). For each treatment, 10 plants were bacterized by the addition of a bacterial suspension to the nutrient solution (final concentration, 10^6 cells ml^{-1}).

Antibiotic-resistant mutants. Spontaneous rifampin-resistant mutants of some isolates were obtained by streaking wild types of the isolates on KB agar plates supplemented with 150 mg of rifampin (KBrif) $liter^{-1}$. Colonies that grew on these plates were purified and cultured repeatedly on KB with increasing concentrations of rifampin, up to 500 mg $liter^{-1}$. These mutants were compared with their wild types regarding their fluorescence, growth rates, production of C_2H_4 or HCN, and in vitro antagonism with some reference strains (WCS417 Rif^r, WCS358, and WCS374). None of these mutants differed from their parental strains.

Characterization of pseudomonads. (i) LPS and cell envelope protein patterns. Cell envelopes were obtained by differential centrifugation after disruption of the cells by ultrasonic treatment (12). Cell envelope samples containing approximately 20 μg of protein were solubilized in standard sample mixture and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (12). Cells were stained with fast green. For the analysis of LPS, the samples were first subjected to treatment with proteinase K (17), after which 10-fold dilutions were applied to each lane. Gels were then stained with silver reagent (17).

(ii) Biochemical characterization. Representative isolates from every group with reproducible stimulating, inhibiting, or neutral effects on plant growth were chosen for further experimentation. A total of 26 isolates were identified biochemically by the methods described by Schaad (13) and Stanier et al. (16). Results were scored positive or negative, and corresponding characteristics were expressed as clusters.

Root colonization. Strains were tested for their potential to colonize the root surface or endorhizosphere of sterile tomato roots. Roots of sterile seedlings were soaked for 5 min in an aqueous suspension of bacterial strains (10^8 cells ml^{-1}) or a mixture of 100 isolates selected from either the root surface or the endorhizosphere. These isolates were mixed in a ratio of 1:1 (final concentration, 10^6 cells of each

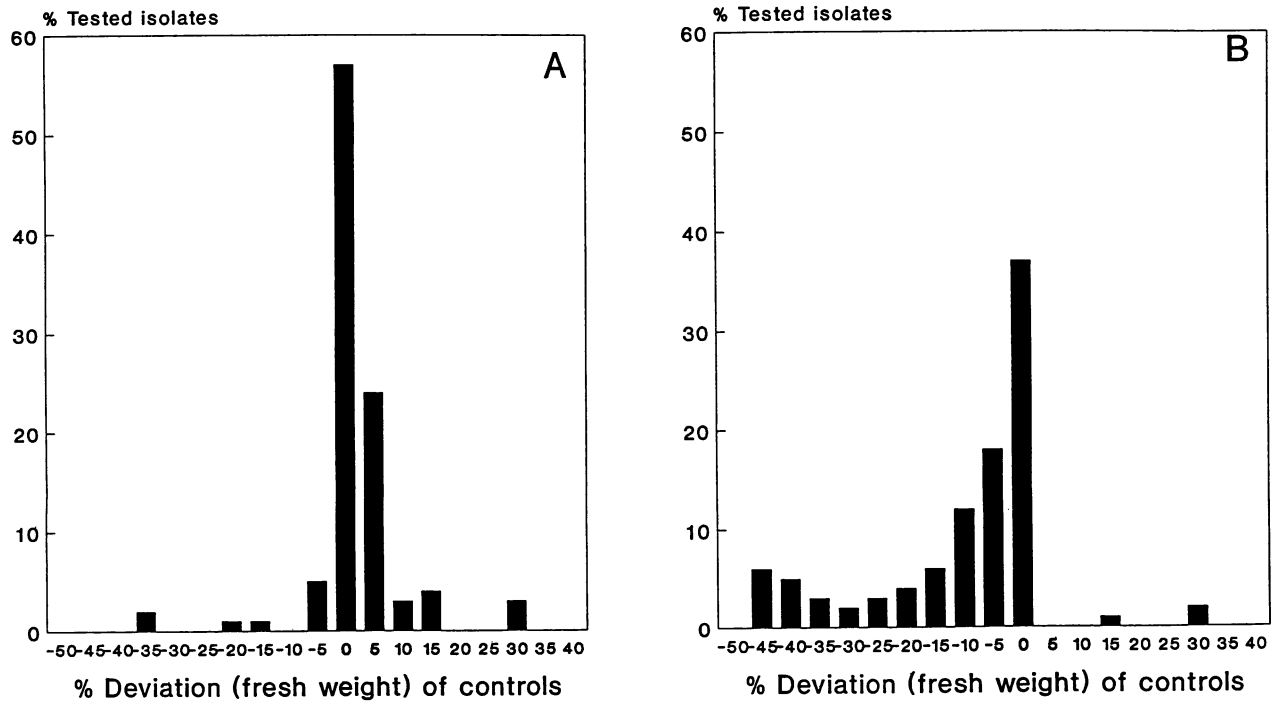

FIG. 2. Effects of pseudomonads selected from the root surface (A) and from the endorhizosphere (B) on tomato seedlings. Controls were not inoculated with pseudomonads.

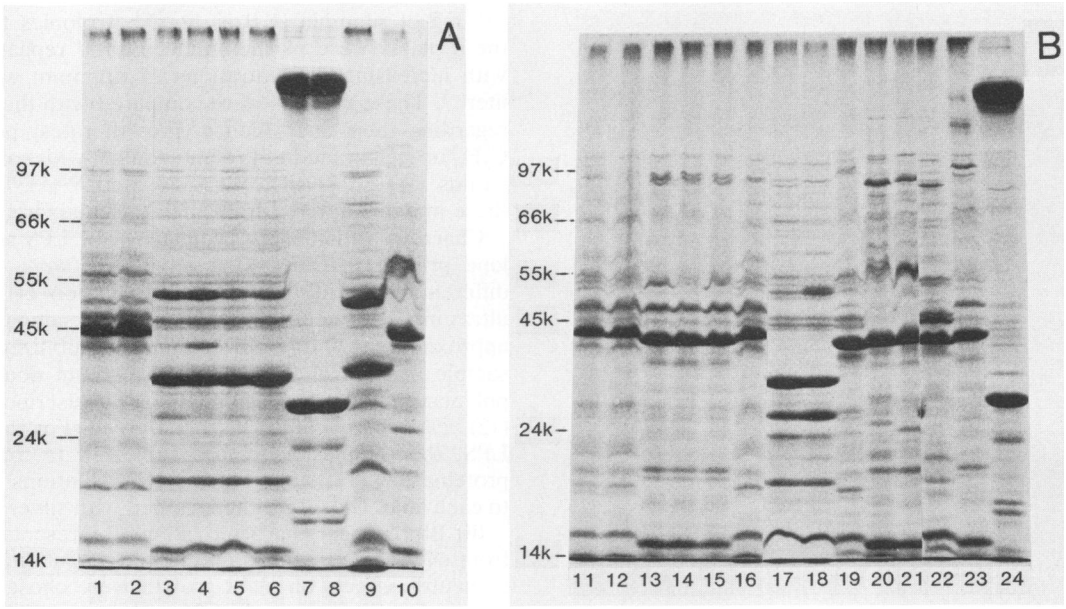

FIG. 3. Cell envelope protein patterns of *Pseudomonas* isolates obtained from the root surface (A) and the endorhizosphere (B). Lanes: 1, RS94; 2, RS194; 3, RS117; 4, RS112; 5, RS111; 6, RS8; 7, RS17; 8, RS12; 9, RS19; 10, RS3; 11, En4; 12, En413; 13, En25; 14, En27; 15, En23; 16, En415; 17, En7; 18, En401; 19, En3; 20, En89; 21, En59; 22, En114; 23, En416; 24, En105. All isolates were run on the same gel.

isolate ml^{-1}). Roots were then rinsed three times in SDW and incubated for different lengths of time in a moist petri dish. The total root length of five seedlings was measured. Roots were macerated in 3 ml of 0.1 M $MgSO_4$ with or without root surface disinfection (10% H_2O_2; 15 s) and dilution plated on KB. Contamination of roots was checked by dilution plating of nonbacterized treatment samples. Plates were incubated for 48 h at 27°C. Mean CFU centimeter of $root^{-1}$ were determined by averaging log population densities of six replicates per treatment.

In addition, rifampin-resistant (Rif^r) mutants of isolates RS48, RS17, En25, and En415 were tested for their root-colonizing capacity in a nonsterile nutrient film system (13). Tomato plants were bacterized with 10 ml of a bacterial

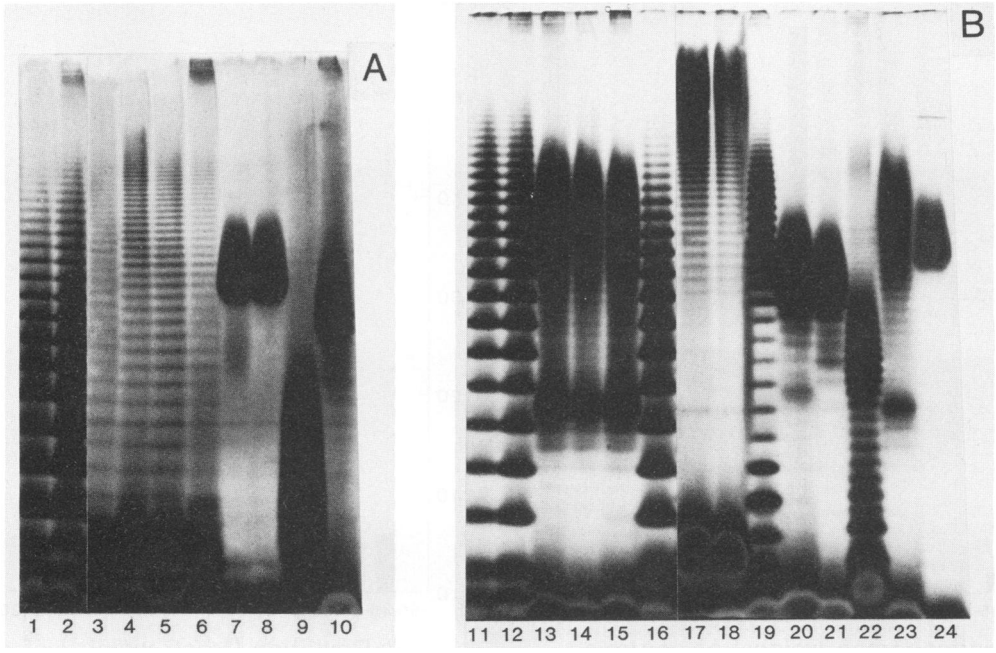

FIG. 4. Silver-strained LPS patterns of proteinase K-treated cell envelopes after gel electrophoresis of *Pseudomonas* isolates obtained from the root surface (A) or from the endorhizosphore (B). Lane numbers correspond with those defined in the legend to Fig. 3. All isolates were run on the same gel.

suspension (10^8 CFU ml^{-1}). Root pieces (0.3 g) were sampled 2 weeks after bacterization to determine colonization of the rhizosphere by the isolates tested.

Adherence to the root surface. Roots of sterile tomato seedlings were immersed in bacterial suspensions (10^8 cells ml^{-1}) for 30 min. Preliminary results demonstrated that incubation for a longer period of time did not increase the number of adhering cells. Adherence of cells was quantitated by the method described by Anderson et al. (3). Intact roots were sequentially transferred under sterile conditions through four washes in 10 ml of water and then homogenized. Cells present on the root surface after four washes were considered to be firmly adhered. After each transfer, the tube was agitated for 60 s by a Vortex. The washed cells and homogenates were diluted and plated (100 μl) onto KB or KBrif. Each treatment was replicated six times, with two roots per replicate.

Chemotaxis. In vitro chemotaxis towards seed exudates of tomato was assessed by using a modification of the method of Adler (1). A total of 10 surface-sterilized tomato seeds were placed in 50 ml of SDW in 100-ml Erlenmeyer flasks and incubated on a rotary shaker (50 rpm) for 5 days at 21°C. The liquid containing the seed exudates was lyophilized and resuspended in 10 ml of SDW. Six capillary tubes (sterilized in 70% ethanol) containing seed exudate or phosphate buffer (0.01 M; pH 7.0) were sealed at one end and placed in a cuvette with an aqueous suspension of the individual isolates (10^8 CFU ml^{-1}). The number of bacterial cells entering the capillary tubes after 30 min was determined by plating their contents onto KB. Chemotaxis was expressed as the ratio CFU entering the capillary tube with seed exudate/CFU entering the tube without seed exudate.

Agglutination. Tomato seeds were either grown on vermiculite and harvested after 21 days or grown on hydroponic culture and harvested after 42 days. Root agglutinin was obtained by the method of Anderson (2). Roots were carefully removed from either the vermiculite or the nutrient solution. Roots grown in vermiculite were swirled by hand for 15 min in 200 ml of SDW in a 1-liter beaker, whereas the sterile nutrient solution was used to obtain agglutinins from the roots grown under sterile conditions. Roots were then allowed to drain for 10 min. The root wash was filtered (Whatman no. 1 filter paper) and centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was mixed with 1.5 g (dry weight) of prehydrated CM-Sephadex C-50 (Pharmacia Fine Chemicals) and gently stirred with a magnetic stirrer for 15 min. The slurry was filtered and treated similarly with 1.5 g (dry weight) of prehydrated DEAE-Sephadex A-50 (Pharmacia Fine Chemicals). The filtrate was lyophilized and suspended in 3 ml of SDW. Ethanol (9 ml; 96%) was added, the filtrate was then held overnight at 4°C, and the precipitate was collected by centrifugation. SDW was added to the precipitate (1 ml g [fresh weight] of $root^{-1}$) and allowed to stand for 30 min at 4°C. Insoluble substances were removed by centrifugation at 10,000 × *g* for 15 min at 4°C. Root agglutinins were also collected from 21-day-old sterile tomato plants. Agglutination was examined microscopically. The activity was ranked from 0 to 3 on the following scale: 0, no agglutination; 1, weak agglutination of only two or three cells; 2, clear agglutination of several clusters of three or more cells; and 3, complete agglutination of the majority of cells (Fig. 1).

RESULTS

Effects on plant growth. In the initial screening tests, 4% of the 101 tested *Pseudomonas* isolates obtained from the root surface significantly reduced plant weight, whereas 7% demonstrated significant plant growth stimulation. In contrast, 30% of the 112 *Pseudomonas* isolates obtained from the endorhizosphere reduced plant growth and only 2% stimulated plant growth (Fig. 2). In the second screening, only 2% of the root surface isolates and 18% of the endorhizosphere isolates showed reduced plant growth. For both populations, 2% of the isolates stimulated plant growth.

TABLE 1. Clusters of *Pseudomonas* isolates that were obtained from the root surface or the endorhizosphere based on LPS and cell envelope protein patterns or biochemical characteristics

Cluster no.	Clusters of isolates based on:	
	LPS and CEP[a] patterns	Biochemical characteristics
1	RS94, RS194, En4, En403, En415	RS94, RS194, En3, En403, En114
2	RS8, RS111, RS112, RS117	RS8, RS111, RS112, RS117, RS48[b]
3	En401, En7	En401, En7, En91[b]
4	En23, En25, En27	En23, En25, En27 (En4)
5	RS12, RS17	RS19, En416
6	En89, En59	
Nonclustering isolates[c]	RS3, RS19, En105, En114, En416, En3	RS3, RS12, RS17, En89, En105, En415, En59

[a] CEP, Cell envelope protein.
[b] LPS and cell envelope protein patterns were not determined.
[c] Each isolate demonstrated unique LPS and cell envelope protein patterns or biochemical properties.

Strain characterization. (i) LPS and cell envelope protein patterns. LPS and cell envelope protein patterns of root surface and endorhizosphere *Pseudomonas* isolates were very diverse (Fig. 3 and 4). Of the 24 isolates, 6 demonstrated unique LPS and cell envelope protein patterns (Table 1). Six clusters consisting of two or more isolates with identical or very similar patterns were distinguished (Table 1). In most cases, both LPS and cell envelope proein patterns did not differ between individual isolates of a cluster. Exceptions were isolate En3 (from the endohizosphere) of which the LPS pattern but not the cell envelope protein pattern was clearly different from that of isolates En89 and En59 and isolates RS12 and RS17 (from the root surface) which had the same LPS patterns as but different cell envelope protein patterns from En89 and En59. On the basis of LPS and cell envelope protein patterns, isolates obtained from the root surface differed from the *Pseudomonas* isolates obtained from the endorhizosphere, except for isolates RS94 and RS194; their patterns were identical to those of isolates En4, En403, and En415 (Table 1).

(ii) Biochemical characterization. Biochemical characteristics of *Pseudomonas* isolates are summarized in Table 2. Of the 26 isolates, 7 exhibited unique characteristics that did not correspond with each other and that were not closely related to one of the clusters of other strains (Table 1). Five different clusters of isolates with closely related biochemical characteristics could be distinguished (Table 1). Except for isolates RS94 and RS194, the biochemical characteristics of which were identical to those of isolates En105 and En114, identical types were not found between isolates obtained from the root surface and those obtained from the endorhizosphere. Isolates En105 and RS12 also have very similar characteristics.

Root colonization. Colonization of the total rhizosphere (root surface and endorhizosphere) and of the endorhizo-

TABLE 2. Biochemical characterization of *Pseudomonas* isolates obtained from the root surface or the endorhizosphere

Parameter	Result or characteristic for isolate:																								
	RS94	RS194	RS117	RS112	RS111	RS8	RS17	RS12	RS19	RS3	RS48	En4	En403	En23	En25	En27	En415	En7	En401	En3	En89	En59	En114	En416	En105	En91
Effects on plant growth[a]	0	0	0	0	+	−	0	0	0	−	0	0	0	0	0	0	−	−	+	−	−	−	−	−	0	0
Fluorescence	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Oxidase	±	±	−	±	−	−	+	+	±	−	±	+	+	±	+	±	+	−	+	+	−	−	−	±	+	±
Pectolytic activity	−	−	−	−	−	−	−	−	−	+	−	+	−	−	±	−	−	−	−	−	±	+	−	−	−	±
Arginine dihydrolase	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Levan production	−	−	+	+	+	+	+	−	−	−	+	−	−	+	+	+	−	−	−	−	−	−	−	+	+	+
Production of poly-β-hydroxybutyrate	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−
Growth at:																										
4°C	+	±	+	+	±	+	±	−	−	+	±	−	±	±	±	+	−	+	+	−	+	±	−	±	−	−
27°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37°C	±	±	−	−	−	−	−	−	+	+	−	+	+	−	−	−	+	−	−	+	±	−	+	−	−	−
40°C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Gelatine hydrolysis	−	−	+	+	+	+	±	+	+	+	+	−	−	+	+	+	−	+	+	−	+	−	−	+	+	+
Hypersensitive reaction in tobacco by[b]:																										
Cells	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Supernatant	+	+	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+	−	+	+	+
Denitrification	−	−	+	+	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Soft rot in potato	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Growth on various C sources																										
Mannitol	−	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	−	+	+	+
Inositol	−	−	+	+	+	+	−	−	+	−	+	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+
Trehalose	−	−	+	+	+	+	−	−	+	−	+	+	−	+	+	+	+	+	+	−	+	−	−	+	−	+
Sucrose	−	−	+	+	+	+	+	−	+	+	+	−	−	+	+	+	−	−	−	−	−	−	−	+	−	+
Cellobiose	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−
Sorbitol	−	−	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+
Calcium lactate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	−	+	+	+	+	+	+	−	−	+	+	+	−	+	+	+	+	+	+	−	+	+	−	+	−	+
L-Rhamnose	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Tartrate	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−
Erythritol	−	−	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+

[a] See Materials and Methods. Effects are distinguished by analysis of variance as inhibitory (−), promoting (+), or neutral (0).
[b] Cells, Leaves injected with viable cells; Supernatant, leaves injected with the equivalent of cell-free culture supernatant.

sphere of sterile tomato roots by individually tested isolates is presented in Table 3. After 2 h, significant differences in root colonization were observed between many isolates. However, after 6 h, only a few isolates significantly differed in colonization of the total rhizosphere. In contrast, after 6 h of incubation, many differences were still obvious with respect to the colonization of the endorhizosphere (Table 3). The mean numbers of CFU centimeter of root^{-1} obtained from the endorhizosphere after inoculation with root surface or endorhizosphere isolates were significantly different. The same tendency was found when a mixture of 100 root surface isolates or 100 endorhizosphere isolates was used (Fig. 5). This suggests that colonization of the endorhizosphere mainly occurs within a specific group of pseudomonads.

Results from experiments performed under gnotobiotic conditions cannot simply be applied to nonsterile conditions, since properties such as antagonism and rhizosphere competence, for example, are not measured in those experiments. Four isolates (RS48 Rif^r, RS17 Rif^r, En25 Rif^r, and En415 Rif^r) differing in their root colonization (Table 3) and agglutination patterns (Table 4) were tested in a nonsterile nutrient film system for their capacity to colonize the endorhizosphere. All four isolates colonized the root surface, but only the isolates obtained from the endorhizosphere could be enumerated from both the root surface and the endorhizosphere. In this system, the two isolates obtained from the root surface could not be detected in the endorhizosphere (Table 4).

Adherence to the root surface. After the first wash, most of the cells were removed from the root surface; thereafter, numbers in the washes slowly decreased with the wash steps (Table 5). In particular, strain En7, and to some lesser extent

TABLE 3. Colonization of the rhizosphere of sterile tomato roots by *Pseudomonas* isolates selected from the root surface and from the endorhizosphere

Pseudomonas isolates from:	Log CFU cm of root^{-1} from:					
	Total rhizosphere			Endorhizosphere		
	0.5 h	2 h	6 h	0.5 h	2 h	6 h
Root surface						
RS94	5.5	6.2	6.3	3.3	4.9	4.2
RS194	4.9	5.5	5.8	1.9	2.6	3.0
RS117	4.3	5.8	5.9	1.5	3.7	3.6
RS112	5.0	5.5	5.8	2.3	2.4	2.4
RS111	5.4	5.7	5.8	2.3	3.0	3.2
RS8	5.4	6.0	6.3	1.9	2.8	3.1
RS17	4.8	5.6	5.3	1.1	1.3	1.5
RS12	5.0	5.8	5.9	2.2	2.9	3.1
RS19	5.2	5.4	5.7	1.8	3.0	3.1
RS3	5.5	5.9	6.4	2.9	3.6	4.0
RS48	4.1	5.5	6.0	2.4	2.8	2.9
Mean	5.0	5.7	5.9	2.1[a]	3.0[a]	3.1[a]
Endorhizosphere						
En4	4.7	5.8	6.0	3.0	4.4	5.0
En403	5.0	5.4	5.6	2.2	3.1	3.3
En23	4.4	4.9	5.7	2.9	3.6	3.9
En25	5.3	5.9	5.9	2.8	3.5	3.5
En27	5.2	6.0	5.8	2.6	3.4	3.2
En415	4.9	5.8	6.3	3.0	3.9	4.9
En7	5.8	6.0	6.0	5.0	4.2	4.6
En401	4.7	5.6	6.1	4.0	4.9	5.2
En3	5.3	5.8	5.9	2.8	3.3	3.4
En89	5.1	5.5	5.7	3.0	3.6	3.8
En59	4.8	5.4	5.8	3.6	4.1	4.3
En114	4.4	5.8	6.4	2.8	3.6	4.1
En416	5.4	6.3	6.5	2.8	3.3	3.6
En105	4.7	5.6	5.9	2.4	3.2	3.5
En91	5.2	5.5	6.4	2.2	3.4	3.8
Mean	5.0	5.7	6.0	3.0[a]	3.7[a]	4.0[a]

[a] Differences in colonization (mean values) of the endorhizosphere between isolates obtained from the root surface and the endorhizosphere are significantly different ($P \leq 0.05$) after 0.5, 2, and 6 h of incubation (Wilcoxon test).

strain En415, adhered strongly to the root surface. No prominent differences were observed between the isolates from the root surface and those from the endorhizosphere.

Chemotaxis. Endorhizosphere pseudomonads exhibited significantly higher capillary chemotaxis towards tomato seed exudates than root surface pseudomonads (Table 6).

Agglutination. A total of 100 randomly chosen *Pseudomonas* isolates from both the root surface and the endorhizosphere were tested for their agglutinative responses to tomato agglutinins present in root washes. A statistically significantly higher percentage of endorhizosphere isolates demonstrated strong agglutinative responses (classes 2 and 3) than root surface isolates (Table 7, Expt I). Similar results were found for experiments with a selected group of 26 isolates (Table 7, Expt II). The agglutinative responses were independent of the age of the bacterial culture or the plant and demonstrated the same tendencies when root agglutinin was obtained from sterile roots (R. van Peer, unpublished results).

DISCUSSION

Compared with root surface isolates, a significantly higher percentage of *Pseudomonas* isolates obtained from the endorhizosphere inhibited the growth of tomato seedlings (Fig. 2) and 5-week-old plants as well. These observations suggest that endorhizosphere-inhabiting pseudomonads, compared with root surface-inhabiting pseudomonads, possibly exhibit different characteristics that are responsible for their effects on plant growth. Growth inhibition by some isolates was still measurable 2 months after bacterization (van Peer, unpublished results). When LPS patterns, cell envelope protein patterns, and biochemical characteristics were compared, it was demonstrated that except for strains RS94 and RS194 no resemblances were found between isolates obtained from the root surface and those obtained from the endorhizosphere (Table 1). Note that strains RS94 and RS194 are the only *Pseudomonas putida* strains from the root surface. Therefore, we conclude that in general, the endorhizosphere isolates are distinct from root surface isolates. On the basis of similar LPS patterns, cell envelope protein patterns, and/or biochemical characteristics, 14 of 26 isolates could be arranged in six clusters of LPS-cell envelope protein patterns and five clusters based on biochemical differences (Table 1). Some isolates (e.g., RS12 and RS17) demonstrated identical LPS and cell envelope protein patterns despite differences in biochemical characteristics. Consequently, care should be taken to use LPS and cell envelope protein patterns only for strain identification. On the basis of resemblances of LPS, cell envelope protein patterns, and biochemical characteristics, it is tempting to conclude that isolates present in the same cluster represent different species or biovars. Doing so, we distinguished at least *P. putida* and *Pseudomonas fluorescens* (with three biovars), represented by two or more isolates. The 12 remaining isolates belong to other species or biovars, represented by single isolates (Table 8). This demonstrated once more that certain (dominant) species or biovars are present on the root surface while others inhabit the endorhizosphere.

In general, isolates obtained from the endorhizosphere (re)colonized the endorhizosphere in higher numbers than isolates obtained from the root surface (Table 3). Endorhizosphere colonization occurs inter- and intracellularly, sometimes as far as the endodermis (R. van Peer, W. L. M. Punte, and B. Schippers, unpublished data). Since no correlation was demonstrated between endorhizosphere colonization of the individual isolates and inhibitory effects on plant growth, it is suggested that it is not the presence of pseudomonads as such that is responsible for the growth reduction of plants. Presumably, growth reduction is mediated by the production of certain metabolites. Metabolites have a more significant effect on the physiology of the plant and thereby possibly on plant growth when they are produced and accumulated in the endorhizosphere than when they are produced on the root surface. Detailed electron microscopic studies demonstrated histochemical differences between the root surface and the endorhizosphere (8, 9). These observations suggest that components that accumulate in the endorhizosphere are different from those that accumulate on the root surface; these components may consequently favor the colonization of certain pseudomonads in these areas. It is possible that not all isolates from the endorhizosphere are endorhizosphere colonizers. Some isolates may have established themselves in the endorhizosphere because the presence of wounds or dead cells in the root surface provided a substrate for these bacteria. They may also have entered the endorhizosphere by aggregation with endorhizosphere colonizers. Similarly, the root surface carries some endorhizosphere colonizers. However, this last group composes only a minor proportion of the isolates obtained from the root surface (Fig. 5). Strains RS3, RS94, and RS117 may be

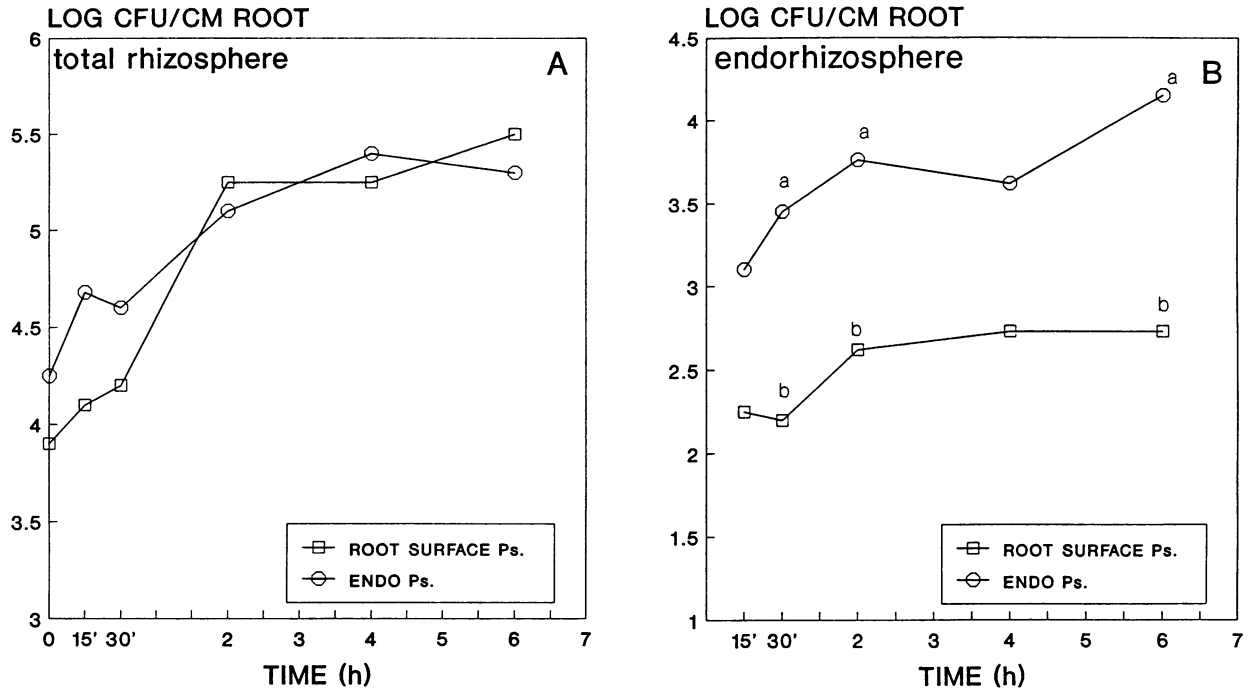

FIG. 5. Recolonization of the total rhizosphere (root surface and endorhizosphere) (A) and the endorhizosphere only (B) of sterile tomato roots by a mixture of 100 isolates obtained from either the root surface or the endorhizosphere. Ps, Pseudomonads.

designated endorhizosphere colonizers. However, strains En3, En25, En27, En91, and En416 are probably root surface colonizers because their colonization of the endorhizosphere (expressed as a percentage of colonization of the total rhizosphere) is comparable to that of isolates obtained from the root surface.

The correlation coefficient for chemotactic response and endorhizosphere colonization was low but was higher for isolates obtained from the endorhizosphere (0.4) than for isolates obtained from the root surface (0.1). This suggests only a minor role for chemotaxis as a factor in endorhizosphere colonization. Unlike Anderson et al. (3), we found no correlation between total rhizosphere colonization and agglutination with root agglutinin. It is possible that differences in root surface colonization are masked because cells of a strain that strongly agglutinates do aggregate. When dilution plated, these aggregates are represented only by a single colony and their numbers are underestimated. In contrast, we demonstrated a significant correlation between agglutination of isolates obtained from the endorhizosphere with root agglutinin of sterile tomato roots and colonization of the endorhizosphere ($r = 0.7$). Such a correlation was not found for isolates obtained from the root surface. Adherence of pseudomonads to the root surface was significantly correlated with their agglutinative response as well (Table 5). Agglutination of bacteria has been suggested to play a role in the adherence and colonization of root surfaces (2, 3) but never in the colonization of the endorhizosphere. Apparently, the ability of pseudomonads to agglutinate with root agglutinin is beneficial for pseudomonads with the capacity to colonize the endorhizosphere. Strongly agglutinating pseudomonads will firmly adhere to the root surface and are not easily removed (Table 5). We suppose that agglutination

TABLE 4. Colonization of the rhizosphere of tomato plants grown in a nutrient film culture by rifampin-resistant mutants of *Pseudomonas* isolates RS48, RS17, En25, and En415 that were obtained from either the root surface or the endorhizosphere

Strain (Rif^r) from:	Agglutination class[a]	CFU g (fresh wt) of root^{-1} from:	
		Root surface	Endorhizosphere
Root surface			
RS48	0	5.1	
RS17	1	5.9	
Endorhizosphere			
En25	2	5.1	3.0
En415	3	5.4	3.5

[a] For definition of classes, see text.

TABLE 5. Adherence and agglutination of several *Pseudomonas* strains to sterile tomato roots after serial washings[a]

Pseudomonas strain	Log CFU				No. of cells on roots (CFU cm^{-1})	Agglutination class[b]
	Wash 1	Wash 2	Wash 3	Wash 4		
RS48	7.5 (0.3)	5.4 (0.4)	3.9 (0.2)	2.4 (0.4)	3.6 (0.3)	0
RS8	6.9 (0.5)	5.1 (0.2)	4.4 (0.3)	3.1 (0.4)	3.5 (0.3)	0
RS17	7.5 (0.1)	4.1 (0.3)	3.0 (0.3)	1.9 (0.1)	3.9 (0.2)	1
RS112	7.3 (0.3)	4.6 (0.2)	3.4 (0.2)	2.1 (0.3)	4.0 (0.1)	1
RS3	7.6 (0.4)	5.1 (0.2)	3.8 (0.1)	2.7 (0.3)	4.0 (0.1)	3
En3	7.1 (0.3)	5.0 (0.1)	3.3 (0.2)	2.4 (0.1)	3.9 (0.3)	1
En25	7.5 (0.2)	4.8 (0.2)	3.6 (0.2)	1.6 (0.2)	3.9 (0.2)	2
En91	7.1 (0.1)	4.7 (0.3)	3.2 (0.1)	2.3 (0.1)	3.7 (0.2)	2
En415	7.9 (0.2)	5.6 (0.1)	4.0 (0.4)	2.4 (0.2)	4.3 (0.3)	3
En7	7.3 (0.4)	4.8 (0.3)	3.1 (0.1)	2.1 (0.2)	4.9 (0.4)	3

[a] For each volume, the standard error is given in parenthesis.
[b] For definition of classes, see text.

TABLE 6. Chemotactic responses of *Pseudomonas* isolates obtained from the root surface or the endorhizosphere toward tomato seed exudate

Isolate	Chemotactic response[a]
RS94	1.4
RS194	1.1
RS117	1.1
RS112	2.3
RS111	1.3
RS8	1.2
RS17	1.3
RS12	1.1
RS19	1.8
RS3	1.0
RS48	1.3
Mean	1.4
En4	1.9
En403	1.3
En23	1.3
En25	1.4
En27	1.4
En415	(17.8)
En7	2.3
En401	1.3
En3	2.2
En89	1.8
En59	1.5
En114	2.0
En416	2.4
En105	1.9
En91	2.3
Mean	1.8

[a] Expressed as the quotient of the log of CFU in capillary tubes containing seed exudate and the log of CFU in capillary tubes containing phosphate buffer. Mean values for isolates obtained from the root surface and the endorhizosphere are significantly different ($P \leq 0.05$) by the Wilcoxon test. The value for isolate En415 is excluded.

of bacteria with root agglutinin is of significant importance in the initial phase of adherence of the bacterium to the root cell.

The observation that four rifampin-resistant mutants (RS48 Rif^r, RS17 Rif^r, En25 Rif^r, and En415 Rif^r) differed in their abilities to colonize nonsterile tomato roots, which correlated with agglutinability (Table 4), supports the hypothesis that agglutination indeed is important in colonization of the endorhizosphere. On the other hand, immobilization of bacteria through agglutination with plant agglutinin has also been demonstrated (11, 14, 15). Notable was the observation that differences in the colonization of the rhizosphere of nonsterile roots between strain En415 and strain En25 were less pronounced than differences in the colonization of the rhizosphere of sterile roots (Tables 3 and 4). Further investigations are required to explain such a difference.

TABLE 7. Percentage of *Pseudomonas* isolates from the root surface and from the endorhizosphere demonstrating agglutinative responses with root agglutinin of tomatoes[a]

Agglutination class[b]	% of agglutinative isolates from:			
	Root surface		Endorhizosphere	
	Expt I	Expt II	Expt I	Expt II
0	54	62	32	27
1	23	21	22	27
2	21	17	32	29
3	2	0	14	17

[a] Experiment I and experiment II were performed with 100 randomly chosen isolates and 26 selected isolates, respectively.
[b] For definition of classes, see text.

TABLE 8. Distinction of species and biovars of pseudomonads on the basis LPS and cell envelope protein patterns and biochemical characteristics[a]

Isolate(s)	Species and biovar
RS94, RS194, En403	*P. putida*
RS8, RS111, RS112, RS117	*P. fluorescens* biovar II
RS12	Unclassified
RS17	Unclassified
RS19	*P. fluorsecens* biovar I
RS3	*P. fluorescens* biovar IV
En401, En7	*P. fluorescens* biovar V
En23, En25, En27	*P. fluorescens* biovar I
En416	*P. fluorescens* biovar I
En105	Unclassified
En114	*P. putida*
En415	*P. putida*
En3	*P. putida*
En4	*P. putida*
En89	*P. fluorescens* biovar III
En59	*P. putida*

[a] Isolates were obtained from the root surface or the endorhizosphere of tomato plants grown in hydroponic cultures.

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