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Relationship between Rapid, Firm Adhesion and Long-Term Colonization of Roots by Bacteria

DOUGLAS W. JAMES, JR.,* TREVOR V. SUSLOW, AND KATHERINE E. STEINBACK
Advanced Genetic Sciences, Inc., Oakland, California 94608

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For rhizobacteria to exert physiological effects on plant growth, the bacteria must first effectively colonize the root surface. To examine the relationship between long-term colonization of root systems and adherence to roots in the short term, a binding assay was developed. Adherence was determined by incubating roots of intact radish seedlings with bacteria, washing and homogenizing the roots, and dilution plating the resulting homogenate. Irreversible binding of bacteria was rapid, reaching half-maximum by 5 min. All of the rhizosphere bacteria tested showed similar, concentration-dependent binding (ranging from 10^4 to 10^8 CFU/ml), as well as long-term colonization of radish roots under sterile conditions. *Escherichia coli*, which is not a root colonizer, showed about 10-fold less binding, but still demonstrated concentration-dependent binding and rapid kinetics of adherence at high concentrations (10^6 to 10^8 CFU/ml). The bacteria tested were very different with respect to source or habitat and plant response, yet they showed similar concentration-dependent binding. There was no correlation between the relative hydrophobicities of the cell surfaces of strains and the adherence of the strains to roots. Binding of *Pseudomonas fluorescens* E6-22 was promoted by divalent cations (Ca^{2+} and Mg^{2+}) at concentrations of 5 to 10 mM, whereas monovalent cations (Na^+ and K^+) had little effect; electrostatic phenomena may partially explain adherence in the short term, an important prelude to long-term colonization of root surfaces.

Colonization of plant root surfaces by rhizobacteria must occur before subsequent effects of the bacteria on promotion or limitation of plant growth can take place (25). A strategy to identify bacteria as bacterization or biological control agents must select not only for bacteria that are capable of antagonism against plant pathogens, but also for the ability to effectively colonize roots (27). The screening of various soil and rhizosphere bacteria for the ability to colonize plant roots involves introducing inoculant onto seeds or seed pieces (i.e., tubers or bulbs) and, at intervals in plant development to maturity, sampling the root system for bacterial presence and number (11, 15, 24, 28, 32). Because plant development requires considerable time, an assay can take weeks or months. Therefore, a rapid assay which predicts colonization on roots would be a valuable tool in studying the factors and events involved in the colonization process, as well as the genetics of plant-microbe interactions (27). Bacterial attachment is an early event in the colonization of various surfaces by bacteria (16) and is amenable to the development of such an assay.

Most of the details pertaining to the mechanism of bacterial attachment to surfaces are unclear. However, bacterial adherence is known to be a time-dependent phenomenon (for a review see reference 6). Marshall et al. (17) have described adherence of marine bacteria to glass surfaces as a two-phase process; first, there is an instantaneous, reversible adsorption phase which is interpreted to be a balance between the repulsion and attraction energies of the electrical double layer and van der Waals forces, respectively, and second, there is a time-dependent irreversible adherence phase, which is presumably mediated by the adhesive properties of extracellular polymers. The reversible, instantaneous phase is characterized experimentally by evaluating the persistent adherence of bacterial cells to a surface after washing with water, buffer, or dilute salt solutions. Bacteria remaining on the surface are considered to be firmly attached. Irreversible adherence of *Pseudomonas tolaasii* to barley roots has been reported to be optimal in the presence of the divalent cations Ca^{2+}, Mg^{2+}, and Sr^{2+} (20). This effect probably involves an electrostatic interaction, as divalent cations are known to facilitate adhesion of two surfaces which each possess a net negative charge (5, 29). A role for hydrophobic interactions in the adherence of bacteria to surfaces has also been suggested. In experiments with a marine pseudomonad, attachment was greatest to hydrophobic plastics (7). The surfaces of many plant tissues are hydrophobic due to the presence of waxes. The adherence of fungal spores to bean hypocotyls can be reduced if the root surfaces are first treated with surfactant or if the surface wax is removed with chloroform (33).

In this study we examined rapid, firm adherence of bacteria to roots with the ultimate objective of developing a short, routine method for assessing the colonization potential of bacteria on roots. Our results indicate that under sterile conditions, bacteria which proliferate significantly on radish roots also adhere to roots in the short term. The effects of various factors, including time, bacterial concentration, bacterial surface charge and hydrophobicity, pH, and cations, are discussed in the context of possible adherence mechanisms.

MATERIALS AND METHODS

Bacterial strains and plant material. The bacterial strains used in this study are listed in Table 1. All of the bacteria except *Escherichia coli* were cultured at 28°C in King's medium B broth or agar (10); *E. coli* cultures were grown at 37°C in Luria broth or agar (19) supplemented with 0.5% (wt/vol) NaCl and 0.1% (wt/vol) glucose. Radish seeds (*Raphanus sativus* L. cv. Comet; Ferry Morse, Mountain View, Calif.) were surface sterilized by immersion in 70% (vol/vol) ethanol for 1 min, followed by treatment in 1.6%

* Corresponding author.

TABLE 1. Serial washing of inoculated radish roots[a]

Strain	Total no. of cells (log CFU)				No. of cells on two roots (log CFU)[b]
	Wash 1	Wash 2	Wash 3	Wash 4	
P. fluorescens E6-22	8.98 ± 0.15[c]	4.31 ± 0.07	3.49 ± 0.09	2.85 ± 0.15	3.84 ± 0.20[c]
Pseudomonas sp. strain WD-13	8.86 ± 0.15	4.69 ± 0.11	3.41 ± 0.24	<2[d]	3.67 ± 0.30
Flavobacterium sp. strain MtCa-7	8.82 ± 0.25	4.33 ± 0.26	3.17 ± 0.12	2.78 ± 0.14	4.17 ± 0.12
S. marcesens QMB 1466	8.01 ± 0.11	3.43 ± 0.23	<2	<2	3.54 ± 0.08
E. coli AB1157	8.02 ± 0.13	3.19 ± 0.28	<2	<2	3.55 ± 0.23

[a] After standard incubation in bacterial suspensions adjusted to densities of approximately 10^9 CFU/ml, two radish seedlings were successively washed in 9-ml volumes of washing buffer (20 mM phosphate containing 0.1% [wt/vol] peptone); each step included brief sonication followed by blending with a Vortex mixer. Number of CFU was determined in wash solutions and for excised roots by dilution plating (see text). Washes were performed in triplicate.

[b] Determined from root grinds.

[c] Mean ± standard error.

[d] The approximate detection limit was 10^2 CFU.

(vol/vol) hypochlorite for 10 min. After being thoroughly rinsed in sterile distilled water, the seeds were germinated and grown in the dark for 4 days on moistened filter paper in petri dishes that were 7 cm deep.

Adherence assay. Bacteria in late log phase were harvested from the culture broth by centrifugation at 2,000 × *g* for 10 min. The resulting bacterial pellets were suspended in 20 mM sodium phosphate buffer (pH 7.0). Cell densities were adjusted turbidimetrically by dilution in buffer to concentrations ranging from 10^4 to 10^9 CFU/ml. The adherence assay was initiated by transfering two 4-day-old radish seedlings (length, 2 to 3 cm; fresh weight, 35 to 50 mg) to a test tube (13 by 100 mm) containing 1 ml of bacterial suspension. After 20 min of incubation at 23°C, the seedlings were removed from the suspension and rinsed by blending in a Vortex mixer for 10 s in 10 ml of fresh incubation buffer, and those cells remaining were considered to be firmly bound (Table 1). The bacterial population densities adhering to the root surfaces were determined by homogenizing the excised roots in buffer, dilution plating the homogenates onto an appropriate agar medium by using a Spiral-plater (Spiral Systems, Cincinnati, Ohio), and counting the bacterial colonies on the plates after 2 days of growth. The cell densities of the initial bacterial suspensions were also determined.

Colonization test. Bacterial densities (CFU) were determined by dilution plating the various homogenates and suspensions described below. Surface-sterilized radish seeds were inoculated with bacteria by incubating the seeds for 20 min in bacterial suspensions adjusted turbidimetrically to densities of approximately 10^8 CFU/ml. The initial density of cells on the seeds after incubation with bacteria was determined by homogenizing five replicate seeds in buffer (about 10^6 CFU of bacteria per seed). The seeds were removed from the bacterial suspensions without rinsing and planted in sterile, screw-capped, 50-ml plastic centrifuge tubes that were filled to a depth of 5 cm with sterilized potting mixture that was premoistened with half-strength Hoagland solution (9). The tubes were incubated in a controlled growth chamber under a cycle which consisted of 12 h of light and 12 h of darkness at 22 and 19°C, respectively. Light was supplied by warm-white fluorescent tubes and incandescent bulbs at an intensity of approximately 1,200 microeinsteins s^{-1} m^{-2}. After 25 days the plants were removed from the tubes, and the roots were washed in 100 ml of washing buffer (6.25 g of KH_2PO_4, 8.75 g of K_2HPO_4, 1 g of Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 1 liter of distilled water; pH adjusted to 7.0) with shaking at 200 rpm for 30 min. Eight replicates for each bacterial treatment were run. Colonization was expressed as the fold change in CFU detected on the roots plus root washings divided by the CFU in the initial seed homogenate.

Hydrophobicity assay. The relative hydrophobicities of the surfaces of various bacterial strains were determined by observing the change in absorbance of cell suspensions after partitioning with *n*-hexadecane (22). Log phase bacterial cells were pelleted and suspended twice in buffer at pH 7.2 (16.95 g of K_2HPO_4, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_2O$, distilled water to 1 liter). Test tubes containing 1 ml of suspended bacteria and up to 200 μl of *n*-hexadecane were agitated for 2 min with a Thermolyne Maxi Mix mixer (Sybron, Dubuque, Iowa). The aqueous and organic phases were allowed to separate for 15 min, and the absorbance of the aqueous phase was measured at 400 nm by using a Perkin-Elmer model Lambda 1 spectrophotometer.

RESULTS

Adherence of bacteria to radish roots. A time course to determine the adherence of several strains of bacteria to radish roots was conducted to establish conditions for binding (Fig. 1). With the rhizobacteria *Pseudomonas* sp. strain WD-13 and *Pseudomonas fluorescens* E6-22, the number of cells bound increased rapidly in the first few minutes, reached half-maximum by 5 min, and markedly leveled off by 10 min. The adherence of *E. coli* stabilized at a lower concentration (10 to 20 CFU/root), although half-maximum binding occurred with the same time course as half-maximum binding of the other bacteria. A difference in the concentration dependence of binding was also observed (Fig. 2). All of the bacteria tested, including strains of *Flavobacterium*, *Agrobacterium*, and *Serratia*, strain WD-13, the *E. coli* strain, and the three fluorescent pseudomonads (strains E6-22, E6-24b, and NZ130), demonstrated linear, concentration-dependent binding behavior. As the concentration of bacteria in the suspension increased (10^4 to 10^8 CFU/ml), a linear increase in the number of bacteria adhering to roots was observed. However, for the *E. coli* strain, no concentration-dependent adherence was observed at low concentrations (10^4 and 10^5 CFU/ml), which may reflect values near the limit of detection in this study. At higher concentrations (10^6 to 10^8 CFU/ml), *E. coli* showed linear, concentration-dependent adherence. Throughout the concentration range tested, the level of adherence of *E. coli* was about 10% of the level observed for the other bacteria tested. In no case was saturation of binding sites on the roots observed.

To assess how firmly the various bacteria became attached to the roots during the 20-min incubation period, a serial washing experiment was conducted. Table 1 shows that

most of the bacteria were removed in the first wash (ca. 10^8 CFU), whereas in the second through fourth washes only 10^4, 10^3, and 10^2 CFU were removed. Thus, since the first wash was effective in removing bacteria from the roots and multiple wash steps would have been impractical, we used a single washing step as our criterion of firm adherence. Indeed, bacteria did become irreversibly attached to the roots, as indicated by the numbers of bacteria detected in the root grinds after four washes.

Colonization experiments. Table 2 compares the abilities of various bacteria (chosen from representatives known to elicit different plant growth responses) to colonize the surfaces of radish roots under sterile conditions. Colonization was assessed by comparing the bacterial populations associated with the roots after 25 days with the number initially inoculated onto the seeds. All of the rhizosphere bacteria showed changes in the numbers of CFU on the root systems; these changes ranged from an increase of approximately 70-fold for the *Serratia* strain to a 330-fold increase for strain WD-13. The plant growth-promoting pseudomonads (strains E6-22, E6-24b, and NZ130) showed increases of approximately 40- to 100-fold. In contrast, the population of *E. coli*, which is not characteristically associated with roots, only increased fourfold. In the absence of competition, all rhizosphere bacteria proved to be colonizers, whereas *E. coli* did not significantly colonize.

Adherence of *P. fluorescens* E6-22. Several factors that determined optimum adherence of strain E6-22 to radish roots were studied. Maximum adherence of strain E6-22 was observed at neutral pH by using sodium phosphate, Tris, and PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] buffers (data not shown). Adherence of strain E6-22 was determined to be a cation-dependent phenomenon. As shown in Fig. 3, addition of either $MgCl_2$ or $CaCl_2$ resulted in dramatic

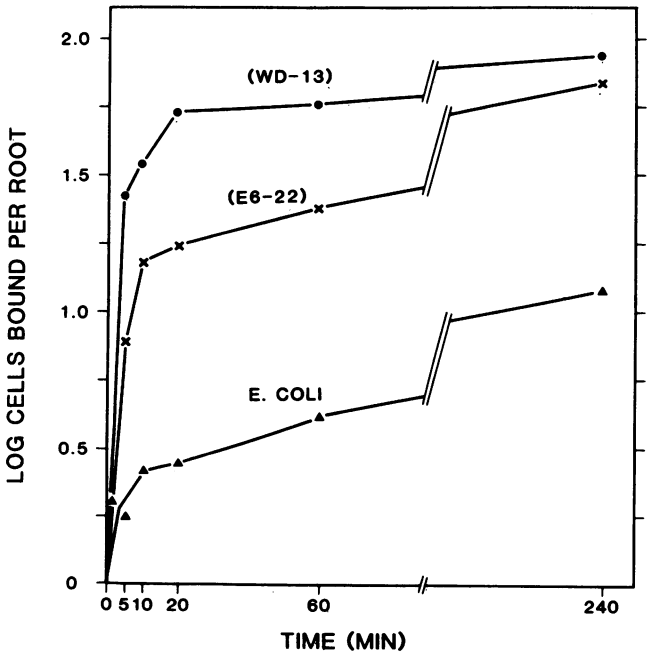

FIG. 1. Time course of bacterial adherence to radish roots. The assay was carried out as described in the text. The bacteria were adjusted to concentrations of approximately 10^5 CFU/ml and were incubated with radish roots for different times. Symbols: ●, strain WD-13; ×, strain E6-22; ▲, *E. coli*.

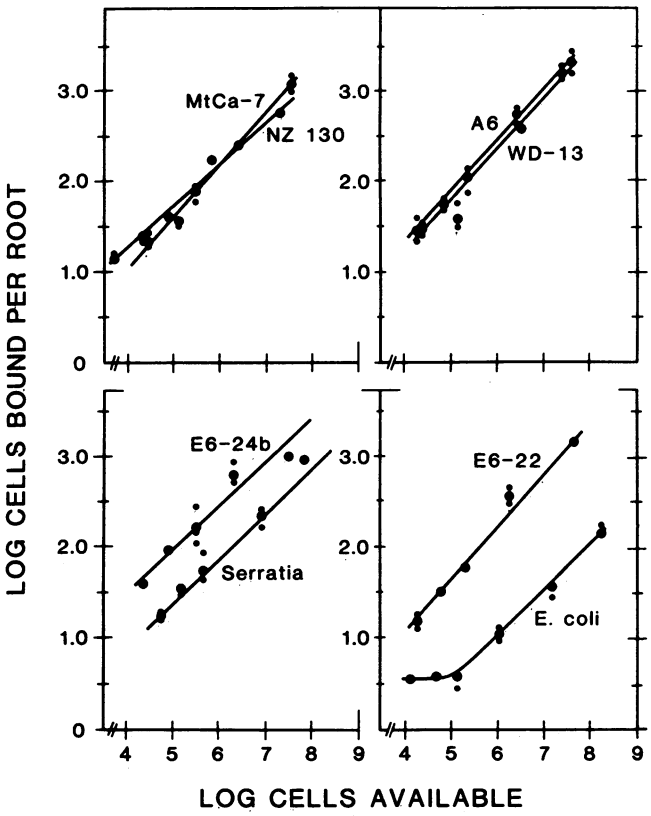

FIG. 2. Effect of bacterial concentration on adherence to radish roots. The bacteria were adjusted to different concentrations (*X*-axis), and the number of cells adhering to the roots *Y*-axis was determined as described in the text. The averages of three experiments are plotted.

increases in binding. At the optimum concentration of divalent cations (5 mM), the number of bacteria adhering to radish roots was almost threefold higher than the level of binding observed in the absence of cations. The divalent cation-stimulated adherence was reversed to basal levels of binding if the divalent cation was omitted from the rinsing buffer (data not shown). To test whether endogenous divalent cations biased the determination of concentration optima, bacterial cells were treated either with chelators [50 mM EDTA for Mg^{2+}, 50 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid for Ca^{2+}] or with 50 mM NaCl for 60 min (to displace divalent cations) and then exhaustively washed with buffer. These treatments did not alter the observed optimum divalent cation concentrations for bacterial adherence. The effects of monovalent cations on binding were also studied (Fig. 4). Addition of either KCl or NaCl at concentrations less than 20 mM had no significant effect on the adherence of strain E6-22 to radish roots, whereas concentrations greater than 50 mM were inhibitory. This inhibition could have been the result of monovalent cations (KCl or NaCl) displacing tightly bound divalent cations required for maximal bacterial adherence. Data shown in Fig. 4 support this interpretation, because a slight stimulation of bacterial adherence by NaCl was observed after displacement of endogenous divalent cations in prior washing steps.

Relative hydrophobicities of the bacteria. The relative hydrophobicities of several bacterial strains were determined based on their tendency to partition into *n*-

TABLE 2. Comparison of the colonization abilities of the bacteria used

Strain	Origin	General plant response[a]	No. of cells per seed initially (log CFU)	No. of cells per root system after 25 days (log CFU)	Increase in colonization (fold)[b]
P. fluorescens					
E6-22	Celery roots	PGPR	6.36 ± 0.03[c]	8.10 ± 0.15[c]	72
E6-24b	Subculture of strain E6	PGPR	6.46 ± 0.08	7.97 ± 0.09	36
NZ130	Radish roots	PGPR	6.35 ± 0.01	8.26 ± 0.11	104
Flavobacterium sp. strain MtCa-7	Sugar beet roots	DRB	6.35 ± 0.03	>8	~45
Pseudomonas sp. strain WD-13	Sugar beet roots	DRB	5.67 ± 0.01	8.10 ± 0.13	334
Agrobacterium tumefaciens A6	Plant tumors	Pathogen	5.96 ± 0.03	7.76 ± 0.07	67
S. marcesens QMB 1466	ATCC 990	Neutral	6.13 ± 0.02	>8	~74
E. coli AB1157[d]	Humans	Neutral	5.26 ± 0.04	5.71 ± 0.15	4

[a] PGPR, Plant growth-promoting rhizobacteria; DRB, deletrious rhizobacteria.
[b] The procedure used is described in the text.
[c] Mean ± standard error.
[d] Genotype: *ara gal his lac leu mtl pro rps sup thi tsx* (29).

hexadecane. The eight strains tested demonstrated a wide range of hydrophobicity (Fig. 5). Both strain WD-13 and strain E6-24b were strongly hydrophobic. Strain E6-22 and the *Serratia* strain were moderately hydrophobic, strain NZ130 and the *Agrobacterium* strain were slightly hydrophobic, and the *Flavobacterium* and *E. coli* strains were relatively hydrophilic. No differences in hydrophobicity were observed when the bacteria were grown in King medium B, Luria medium, or NAG medium (14). Stationary phase *E. coli* cells were more hydrophobic than log phase cells (data not shown), as reported by Rosenberg et al. (22).

DISCUSSION

Our results suggest that bacterial strains which colonize radish roots also adhere to root surfaces in the short term. However, this observation is subject to limitations which diminish the value of the adherence assay as a means of identifying bacteria with colonization potential. Both rapid adherence and colonization assays were conducted under sterile conditions in the absence of competition from other microbes. Therefore, these tests only determined the potential ability of a given bacterial strain to adhere or proliferate or both at the rhizoplane. Another indication that conclusions from a sterile colonization test may not be extended to greenhouse or field tests comes from our observation that two of the bacterial strains, strain WD-13 and the *Serratia* strain (both good colonizers under sterile conditions), were not effective colonizers in nonsterilized field soil. Furthermore, rapid adherence is only one of several events in the colonization process, which may also include taxis toward or along the root (for a review see reference 2) and long-term adhesion to the surface mediated by the production of extracellular polysaccharides, such as cellulose (18), or structures, such as fimbriae (12). Conditions promoting growth of bacteria on the root surface are also important to successful colonization and proliferation of bacteria over the root system (31). Therefore, in addition to the limitations described above, the complexity of the colonization process in nature restricts the ability of our adherence assay to predict whether a given bacterium will colonize a root surface.

The rapid adherence of bacteria to radish roots probably does not involve specific recognition events. This concept is supported by the results of time course and concentration dependence experiments (Fig. 1 and 2). All of the bacteria tested demonstrated linear, concentration-dependent adherence. The level of adherence of *E. coli* was 10-fold less than the level of adherence of the other bacteria tested but was still linear and concentration dependent at the higher concentrations. A similar, linear concentration dependence has been reported for adherence of several strains of *E. coli* to human tissue culture cells (8). Thus, the adherence assay

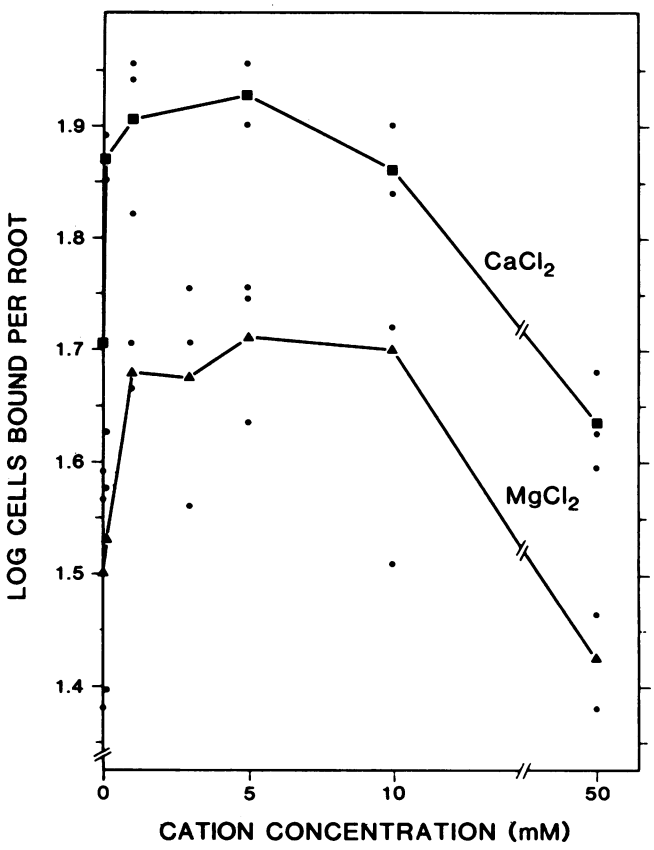

FIG. 3. Effect of divalent cation concentration on adherence of *P. fluorescens* E6-22 to radish roots. PIPES buffer (5 mM; pH 7.5) was used to wash and adjust the cells to a concentration of 3×10^5 CFU/ml. Different final concentrations of $CaCl_2$ or $MgCl_2$ were included in the assay and wash buffers. Symbols: ■, $CaCl_2$, ▲, $MgCl_2$. The averages of three replicates are plotted.

does not distinguish the rhizosphere bacteria from one another, but readily discriminates *E. coli* from rhizosphere bacteria. This observed difference could be due to a number of factors, including topographic surface features unique to rhizosphere bacteria or some combination of physical forces, such as electrostatic or hydrophobic interactions.

Saturation of adherence to the roots would be expected if specific, high-affinity adherence sites were present on either the root or bacterial cell surfaces. This was not the case over a concentration range of 10^5 CFU/ml. Although the bacteria were isolated from widely different sources and differ with respect to influence on plant growth and ability to colonize, they nevertheless rapidly adhered to radish roots in the same time- and concentration-dependent fashion. This is a further indication that specific recognition may not be involved in rapid adherence.

Nonspecific interactions have been implicated in other bacteria-plant systems. Pueppke (21) noted that adherence of several rhizobia to roots was independent of plant species and the ability of *Rhizobium* strains to infect and nodulate. Leben and Whitmoyer (13) found that with representatives of seven bacterial genera (including *E. coli*, *Serratia marcescens*, *Pseudomonas lachrymans*, and several other plant-pathogenic bacteria) the number of bacteria that adhered to leaves was proportional to the number applied regardless of whether the plants were known hosts. These authors also observed that a constant proportion of bacteria were removed from leaves during each successive step of a serial washing procedure and concluded that a nonselective mechanism may be involved.

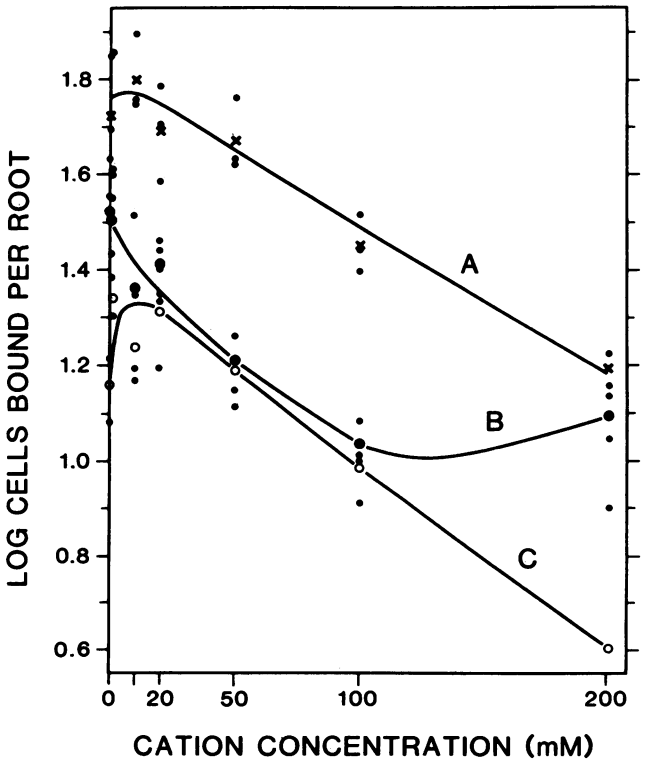

FIG. 4. Effect of monovalent cation concentration on adherence of *P. fluorescens* E6-22 to radish roots. The conditions were as described in the legend to Fig. 3. Cells were adjusted to a concentration of 1.6×10^5 CFU/ml. Different final concentrations of KCl or NaCl were included in the assay and wash buffers. Symbols: ×, KCl (line A); ●, NaCl (line B); ○, NaCl but with endogenous divalent cations exchanged from the bacteria with two 50 mM NaCl washes, followed by two washes with plain buffer (line C). The averages of three replicates are plotted.

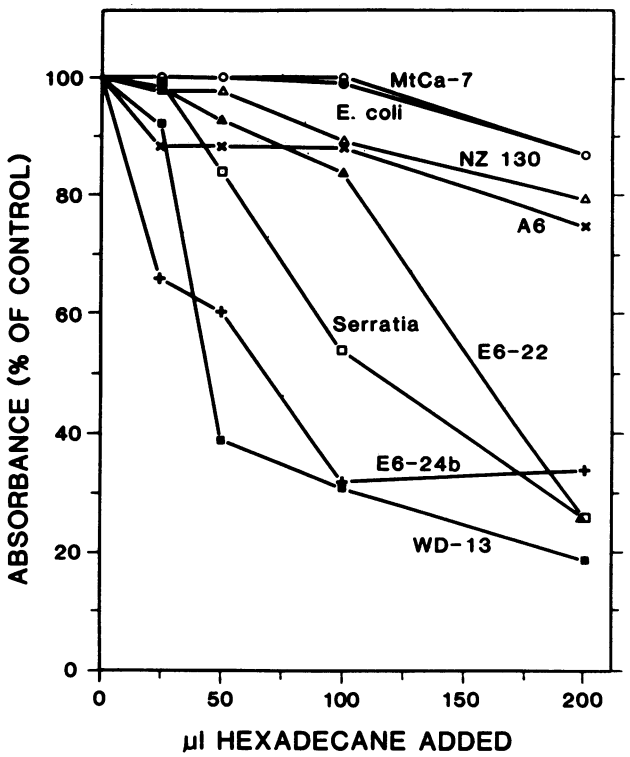

FIG. 5. Relative hydrophobicities of various bacteria. The assay was carried out as described in the text.

In contrast to the proposal of nonselective association, specific mechanisms have been suggested, including lectin-mediated adsorption of *Rhizobium trifolii* to clover (3, 4) and agglutination of fluorescent pseudomonads by leachates from roots and shoots of bean (1). In the more recent of the *Rhizobium* studies, Dazzo et al. described a sequence beginning with rapid random attachment of clumps of bacteria, followed by specific reversible interactions between bacterial and plant surfaces (phase 1 attachment) and later production of extracellular microfibrils for firm adhesion (phase 2 adhesion). The criteria of these authors for specific attachment were that the bacteria could be dislodged from the clover roots with 2-deoxy-D-glucose, a hapten for the specific lectin trifoliin A. Also, the bacteria did not attach to control alfalfa seedling roots or clover cell wall fragments, which are both devoid of trifoliin A. In the report on pseudomonads it was noted that bacteria in agglutinated form adhere to surfaces more readily than free bacteria. The authors suggested that specific agglutination may function to promote colonization in the phylloplane or rhizoplane. Although we did not direct our study toward lectins and agglutinating activity, the similar time- and concentration-dependent adherence behaviors of the various bacteria which we tested do not indicate that there is a selective recognition mechanism which governs rapid bacterial adherence. However, the possibility of specific interactions in longer-term adherence events remains an open question.

Hydrophobic properties of cell surfaces have been discussed in explaining the physical basis of bacterial adherence (23). However, in the present study no correlation

between bacterial adherence properties and bacterial surface hydrophobicity was found. Furthermore, there was no obvious relationship between hydrophobicity and long-term colonization potential, ruling out any major role of hydrophobic interactions in the colonization process.

Binding of *P. fluorescens* E6-22 to radish roots is stimulated by 5 to 10 mM Mg^{2+} or Ca^{2+}. Similar cation effects have been reported for adherence of *P. tolaasii* to barley roots (20), adherence of *Pseudomonas aeruginosa* to steel (26), and adherence of marine pseudomonads to glass (17). These divalent cation effects are thought to indicate involvement of electrostatic forces in the adherence of bacteria to surfaces (17). Cations are believed to mask the repulsion of electrical double layers, thus allowing two surfaces having net negative charges to approach and in some cases to adhere to one another. This spontaneous, cation-mediated process could not involve de novo synthesis of extracellular adhesive polymers, as has been suggested for mechanisms of long-term binding. Rather, the surface properties already present at the bacterial and root surfaces govern the cation-stimulated binding, as has been suggested by Stanley (26). In view of the results presented in this paper and the observations of other workers, we suggest that the surface charge properties of bacterial and plant cells play a role in rapid, firm adhesion and that this binding is strongly promoted by cation-mediated events involving physical masking of negative charges at the cell surface. Although short-term binding properties cannot be taken as an indicator of long-term colonization potential, it is clear that the surface properties of very different strains of bacteria possess similar physical properties which are essential for effective adherence in the short term.

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