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Colonization of Soybean Roots by *Pseudomonas* and *Serratia* Species: Relationship to Bacterial Motility, Chemotaxis, and Generation Time

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ABSTRACT

Scher, F. M., Kloepper, J. W., Singleton, C., Zaleska, I., and Laliberte, M. Colonization of soybean roots by *Pseudomonas* and *Serratia* species: Relationship to bacterial motility, chemotaxis, and generation time. Phytopathology 78:1055-1059.

Thirty-two bacterial strains representing *Pseudomonas putida*, *P. fluorescens*, and *Serratia* spp. were isolated from soil or water. All strains colonized soybean roots in laboratory, greenhouse, and field assays when applied as seed inoculants. Colony-forming units (cfu) ranged from log 1.9 to 6.1 cfu/g of root. All strains colonized soybean seeds at values ranging from log 1.4 to 7.0 cfu per seed. Mean generation times in culture media were not significantly different among the three bacterial types and did not correlate to root or seed colonization levels. *P. putida* and *P. fluorescens* exhibited significantly ($P = 0.05$) greater motility and chemotaxis toward soybean exudates in soft agar (0.2%) and capillary assays than did *Serratia* spp. There was no significant positive correlation between motility or chemotaxis and root or seed colonization by the bacteria, with one exception: chemotaxis of *P. fluorescens* on exudate agar significantly correlated to root colonization, but only in the laboratory assay, where overhead watering was eliminated. In the same assay, *Serratia* spp. showed a significant negative correlation between motility, chemotaxis, and root colonization. Motility was not required for root colonization. A Tn*5* nonmotile mutant of *P. putida* RW3 (RW3-) colonized roots and was distributed along them as well as the motile parent. Thus, use of motility, chemotaxis, or laboratory generation time assays will not likely improve the isolation and identification of superior root-colonizing bacteria.

Establishment of plant growth-promoting rhizobacteria and disease-suppressing bacteria on seeds and root systems is well recognized as a critical step toward their effectiveness (19). Nevertheless, the key characteristics of a bacterial root colonizer are not clearly understood. *Pseudomonas* spp. are a widely studied group of beneficial rhizobacteria (5,7,9,11,14,17,20) that possesses several characteristics that would appear to aid in colonization, including motility, chemotactic response (2,15,18), and fast growth rate (4). However, the relative importance of these characteristics for root colonization is not known.

Scher et al (15) showed that *Pseudomonas* spp. exhibited chemotaxis up to 2 cm in soil toward soybean seed exudates and subsequently colonized seeds. However, the relationship of chemotaxis to root colonization was not investigated. For a *Rhizobium* spp., chemotaxis provided a competitive advantage over nonmotile strains (1), but infection and nodulation of clover by motile and nonmotile *Rhizobium trifolii* were the same (13).

Howie et al (8) showed that three nonmotile mutants of *P. fluorescens*, suppressive to take-all of wheat, colonized roots and suppressed take-all as well as the wild type. Motility was not required for root colonization of wheat by *P. fluorescens* in their system. However, De Weger et al (6) reported that four Tn*5*-induced mutants of *P. fluorescens* without flagella were impaired in their ability to colonize potato roots. They indicated that motility was required for colonization of potato by *P. fluorescens*.

The purpose of this study was to determine the importance of bacterial motility, chemotaxis, and generation time in establishing bacterial colonization of soybean roots. Thirty-two strains representing *P. fluorescens*, *P. putida*, and *Serratia* spp., as well as one nonflagellated mutant of *P. putida*, were employed.

MATERIALS AND METHODS

Bacterial strains. Thirty-two bacterial strains were isolated from soil or water and were characterized taxonomically according to standard microbiological techniques (4). Seven strains were classified as *P. putida*, seven as *P. fluorescens*, and 18 as *Serratia* spp. Strains were isolated from corn or soybean seeds buried in soil collected from eastern Canada and held at 10–14 C, or from stream water with no preselection to seed colonization. Strain RW3 (*P. putida*) was isolated from corn seeds buried in James Bay soil. The Tn5 mutant of RW3 (RW3-) was described in a previous report (15). Spontaneous rifampicin-resistant mutants of all strains were isolated on *Pseudomonas* agar F (PAF) (Difco, Detroit, MI) containing 200 μg of rifampicin per milliliter and were used throughout this study.

Root-colonization assay. Bacterial strains were tested for their capacity to colonize soybean (*Glycine max* L. 'Maple Arrow') roots under laboratory, greenhouse, and field conditions. Soybean seeds were soaked in aqueous suspensions of rifampicin-resistant bacterial strains for 5 min before planting. Bacteria grown for 24 hr in tryptic soy broth (TSB) (Difco) at 150 rpm and 23 C were rinsed once with 0.1 M $MgSO_4$ (by centrifugation at 2,000 *g*) and then resuspended in 0.1 M $MgSO_4$ to a density of log 7.0 colony-forming units (cfu) per milliliter. Seed inoculation resulted in approximately log 4 cfu per seed. Control seeds were soaked in 0.1 M $MgSO_4$.

For the laboratory root-colonization assay (bag assay), a variation on our earlier test tube assay (*16*) was designed to eliminate the need for watering plants (and inadvertently spreading bacteria from seeds to roots) and to facilitate root sampling. Polyethylene tubing (5.5 cm × 25 cm) was heat-sealed at one end to form a long, narrow bag. Fifty g of a 1:1 (v:v) field soil-perlite mixture (at 20% moisture) was placed into each bag. Field soil, collected from the Allelix research center near Caledon, Ontario, was a clay loam with 3% organic matter, pH 7.0, total exchange capacity (M.E.) 14, and with the following nutrient levels in ppm: nitrate nitrogen 4, phosphorous 1, potassium 2, calcium 70, magnesium 16, sodium 0.5, boron 0.4, iron 550, manganese 1,320, copper 2, and zinc 7. Once an inoculated or control seed had been pressed 2 cm into the soil in the bag, the bag was heat-sealed at the top. Bags were held upright in test tube racks in growth chambers (16 hr of light) at 25 C for 1 wk. They were then slit open, and roots were excised at the crown, weighed, and washed by vortexing for 20 sec in 9 ml of 0.1 M $MgSO_4$. The wash was plated onto PAF

TABLE 1. Root colonization of soybean by three bacterial types across three assays: bag, pot, and field

Bacterial type and strain[a]	Log cfu/g of root		
	Bag	Pot	Field
Pseudomonas putida			
RW3	3.7	5.7	3.7
17-29	4.0	5.3	5.4
17-93	2.4	3.9	3.5
W24	4.6	5.6	2.5
W31	4.9	5.2	4.0
W40	2.8	5.5	1.9
W43	2.7	4.4	4.0
Mean	3.6	5.1	3.6
P. fluorescens			
1-206	4.4	5.6	5.1
1-226	4.4	5.5	4.6
17-2	4.3	5.0	5.1
17-62	4.2	4.7	3.4
17-76	4.2	4.8	4.5
W12	4.4	5.9	3.9
W30	5.7	6.1	4.4
Mean	4.5	5.4	4.4
Serratia spp.			
1-102	3.8	4.9	4.8
1-189	3.6	5.0	5.0
1-224	4.1	5.0	4.1
2-16	2.5	5.1	5.1
2-18	3.9	4.8	5.2
2-67	2.1	4.9	3.0
2-68	3.5	5.3	3.6
W6	3.5	5.8	4.8
W9	5.1	4.4	4.4
W29	4.5	5.9	4.6
W32	4.1	4.8	3.2
W34	4.3	4.4	4.6
W35	4.4	5.1	4.8
W36	4.1	4.9	4.5
Mean	3.9	5.0	4.4
F Value	NS[b]	NS	NS

[a] Strains with identifying numbers prefixed by 1-, 2-, or 17- were isolated from corn or soybean seeds buried in soil collected from eastern Canada and held at 10–14 C. Strains prefixed by W were isolated from stream water with no preselection to seed colonization. Strain RW3 was isolated from corn seed buried in soil collected from James Bay, Canada.

[b] NS indicates that *F* value is nonsignificant (for comparison among means of species).

TABLE 2. Soybean seed colonization by strains of three bacterial species

Bacterial type and strain[a]	Log cfu per seed
Pseudomonas putida	
RW3	1.7
17-29	6.5
17-93	5.6
W24	2.5
W31	1.4
W40	4.6
W43	4.4
Mean	3.8
P. fluorescens	
1-206	3.6
1-226	3.8
17-2	6.8
17-62	7.0
17-76	6.0
W12	5.5
W30	4.9
Mean	5.4
Serratia spp.	
1-102	3.2
1-189	5.0
1-224	5.4
2-16	3.6
2-18	6.2
2-67	4.5
2-68	5.3
W6	6.6
W9	3.1
W29	4.0
W32	4.3
W34	4.4
W35	4.4
W36	4.8
Mean	4.6
F value	NS[b]

[a] Strains with identifying numbers prefixed by 1-, 2-, or 17- were isolated from corn or soybean seeds buried in soil collected from eastern Canada and held at 10–14 C. Strains prefixed by W were isolated from stream water with no preselection to seed colonization. Strain RW3 was isolated from corn seed buried in soil collected from James Bay, Canada.

[b] NS indicates that *F* value is nonsignificant (for comparison among means of species).

containing 200 μg of rifampicin, 50 μg of cycloheximide, and 60 μg of benlate per milliliter (PAF-RCB) with a spiral plater (Spiral Systems, Bethesda, MD). Plates were incubated for 48 hr at 30 C, and colony count determinations were made by a Spiral Systems laser counter. Mean cfu/g of root values were determined by averaging log population densities (12) of six replicates per treatment.

In the greenhouse root-colonization assay (pot assay), bacterial strains were tested on soybeans grown in the field soil-perlite mix in 10-cm-diameter plastic pots (one per pot) at 25 C (±5 C). Seeds were treated as for the bag assay, and plants were watered overhead daily. At 1 wk, plants were harvested, and assessment of colonization was performed as above.

Additionally, strains were assessed for colonization under field conditions. The field was located on the Allelix research center, from which soil for the bag and pot assays was taken. The same inoculation/harvest procedures were followed, except that plants were harvested at 2 wk instead of 1 wk (the same developmental stage as the other assays). Field temperatures ranged from 14 to 25 C during the 2 wk.

Seed-colonization assay. A modification of a previously described assay (10) was used to assess seed-colonization capacity of the bacterial strains. Bacteria were grown for 24 hr in TSB at 150 rpm and 23 C, centrifuged (2,000 *g*), washed in 0.1 M $MgSO_4$, and adjusted to log 9.0 cfu/ml in 0.1 M $MgSO_4$ by optical density. Thirty ml of the bacterial suspension was added to 600 g of soil, and soybean seeds were placed in eight portions of soil (one seed per portion) in 25 × 100 mm petri dishes. Spermosphere populations after 4 days at 14 C were determined by spiral plating seed dilutions onto PAF-RCB.

Generation times. In vitro assessment of doubling time was made by determining optical densities (at 780 nm) of TSB liquid culture (50 ml in 250-ml Erlenmeyer side-armed flasks), shaking (150 rpm) at 25 C. Doubling time during log phase of growth was considered generation time.

Chemotaxis. In vitro chemotaxis was assessed by two previously described assays (15). First, bacteria from 24-hr PAF cultures were spotted onto soft (0.2%) agar containing soybean-seed exudate (5%). Seed exudate was collected as previously described (15). After 24-hr incubation at 23 C, bacterial swarms were evident because of bacterial attraction to and movement toward a bacterial-induced nutrient gradient. Diameters of swarms were determined and used to compare chemotactic capacities of the strains. In the second assay, the number of bacteria entering 1-μl capillaries containing seed exudate or phosphate buffer (0.01 M,

TABLE 3. Chemotactic swarm diameters for strains of three bacterial species on soft (0.2%) agar containing soybean seed exudate

Bacterial type and strain[a]	Swarm diameter (mm)
Pseudomonas putida	
RW3	23
17-29	26
17-93	7
W24	41
W31	42
W40	35
W43	37
Mean	30
P. fluorescens	
1-206	33
1-226	37
17-2	41
17-62	45
17-76	13
W12	35
W30	70
Mean	39
Serratia spp.	
1-102	12
1-189	36
1-224	12
2-16	21
2-18	9
2-67	29
2-68	13
W6	31
W9	15
W29	20
W32	6
W34	7
W35	4
W36	6
Mean	16
LSD (0.05)[b]	12

[a] Strains with identifying numbers prefixed by 1-, 2-, or 17- were isolated from corn or soybean seeds buried in soil collected from eastern Canada and held at 10–14 C. Strains prefixed by W were isolated from stream water with no preselection to seed colonization. Strain RW3 was isolated from corn seed buried in soil collected from James Bay, Canada.

[b] Least significant difference value for comparison among means of species.

TABLE 4. Number of bacteria entering capillaries containing phosphate buffer (control) or soybean exudate

Bacterial type and strain[a]	Log cfu/capillary	
	Control	Exudate
Pseudomonas putida		
RW3	3.2	4.0
17-29	4.0	5.0
17-93	3.4	4.1
W24	3.4	4.3
W31	3.8	4.4
W40	4.5	3.8
W43	2.9	3.8
Mean	3.6	4.2
P. fluorescens		
1-206	3.0	3.9
1-226	3.0	4.4
17-2	3.0	4.5
17-62	2.8	4.1
17-76	1.6	3.4
W12	2.9	4.3
W30	3.4	4.3
Mean	2.8	4.1
Serratia spp.		
1-102	2.0	4.1
1-189	0.5	2.3
1-224	...	...
2-16	2.5	3.7
2-18	1.4	1.9
2-67	1.9	3.9
2-68	2.2	3.8
W6	1.4	1.9
W9	1.4	2.4
W29	...	1.9
W32	2.4	2.2
W34	1.3	0.5
W35	...	...
W36	...	1.5
Mean	1.2	2.1
LSD (0.05)[b]	0.9	1.2

[a] Strains with identifying numbers prefixed by 1-, 2-, or 17- were isolated from corn or soybean seeds buried in soil collected from eastern Canada and held at 10–14 C. Strains prefixed by W were isolated from stream water with no preselection to seed colonization. Strain RW3 was isolated from corn seed buried in soil collected from James Bay, Canada.

[b] Least significant difference value for comparison among means of species.

pH 7) was determined by plating the contents of capillaries onto PAF plus 100 μg of rifampicin per milliliter. Higher numbers of bacteria entering capillaries containing exudate than those containing buffer was considered an indication of chemotactic attraction to the exudate. The number of bacteria entering capillaries with buffer only was considered a measure of baseline motility.

Correlation of root and seed colonization to bacterial characteristics. Regression analysis was performed to compare bacterial characteristics to root colonization. Individual strain values for generation time, seed colonization, swarm diameter, capillary motility, and chemotaxis were compared to root colonization values of the same strains within a bacterial type. Resultant r values indicated the positive, neutral, or negative correlation of each characteristic to root colonization within each bacterial type.

Comparative root and seed colonization of RW3 and a nonmotile mutant of RW3. A Tn*5*-induced nonmotile (nonflagellated) insertional mutant of RW3 (RW3-) was employed to determine the necessity of motility for root colonization in two experiments. In the first, colonization by both strains on soybean seeds and whole root systems was followed for 7 days by using the bag assay. The seed and emerging radical were sampled together for the first 3 days; thereafter, roots were removed and sampled separately. In the second experiment, roots were sampled at 7 days only and were segmented at 4-cm intervals. Each segment was weighed and plated separately to determine bacterial population densities at several locations along the root system.

Statistical analysis. Mean values for root and seed colonization, generation time, capillary motility and chemotaxis, and swarm diameter on soft exudate agar for each bacterial type were analyzed by analysis of variance. Mean values for each bacterial type, rather than for individual strains, were used to compare responses by the three bacterial types. If a significant F value occurred, means were separated by the least significant difference value. All experiments were repeated at least once. Significance, when indicated, is at the $P = 0.05$ level.

RESULTS

Root colonization. All strains tested in the bag, pot, and field assays colonized roots to $\geq$log 2.5 cfu/g of root in at least two of the three assays (Table 1). Twenty-nine of 32 strains reached $\geq$log 2.5 cfu/g of root in all three assays. Bacterial numbers ranged from log 1.9 to 6.1 cfu/g of root. Mean cfu/g of root values among the three bacterial species were not statistically different, regardless of the assay used.

Seed colonization. Log colony-forming units per seed values ranged from 1.4 (*P. putida* W31) to 7.0 (*P. fluorescens* 17-62) (Table 2). Mean seed-colonization values for the three species were not statistically significant because of the high variability between individual strains of a species.

Generation times. Generation times for individual strains ranged from 0.8 to 1.7 hr. Mean generation times were not significantly different among the three species.

Chemotaxis. *P. putida* and *P. fluorescens* exhibited significantly greater swarm diameters in soft agar (Table 3) and higher capillary motility and chemotaxis (Table 4) than *Serratia* spp. Mean chemotactic values for *P. putida* and *P. fluorescens* were not significantly different from each other in either assay.

Correlation of root and seed colonization to bacterial characteristics. R values were obtained by regression analysis of log cfu/g of root or log cfu/g of seed values to bacterial generation time, motility, and chemotaxis values for individual strains (Tables 1–4). Correlations were made only within bacterial types. There was no significant correlation between any of the bacterial characteristics and root colonization in the pot or field assays. High root colonization in the bag assay significantly correlated with high chemotaxis on exudate agar by *P. fluorescens* ($r = 0.77$) and to low motility, low swarm diameters on exudate agar, and low capillary chemotaxis by *Serratia* spp. ($r = -0.55$, -0.54, and -0.62, respectively). No bacterial characteristics significantly correlated with seed colonization. Seed colonization did not significantly relate to root colonization.

Comparative root and seed colonization of RW3 and a nonmotile mutant of RW3. Comparative population densities of RW3 and the nonmotile mutant RW3- on soybeans were assessed in two types of experiments. In the first, there was no significant difference in seed or whole root colonization between the strains at any sampling time from 0 to 7 days (Fig. 1). In the second test, bacterial distribution on roots was not significantly different between the two strains at 7 days (Fig. 2).

DISCUSSION

The three bacterial species colonized soybean roots and seeds well, regardless of their relative generation times, motility, and chemotaxis characteristics (Tables 1 and 2) or original isolation from water or seeds. These results are encouraging to efforts designed to develop useful inoculants that require rhizosphere

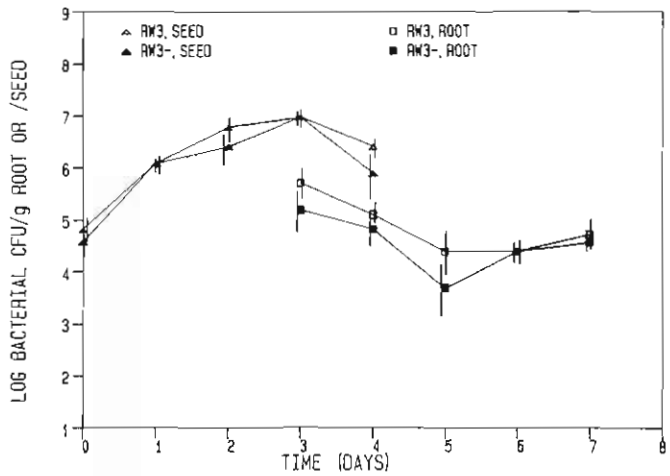

Fig. 1. Comparative soybean seed and root colonization of two strains of *Pseudomonas putida* in the bag assay. RW3 represents the motile wild type, and RW3- represents the Tn*5* nonmotile mutant. Seeds were sampled at times 0, 1, 2, 3, and 4 days. After day 2, whole root systems were separated from seed or stem and were sampled. Bars indicate standard error. There was no significant ($P = 0.05$) difference in colonization between RW3 and RW3- on seeds or roots at any sample time.

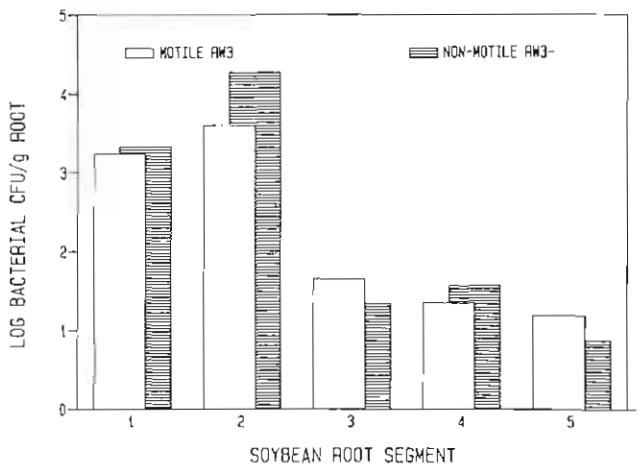

Fig. 2. Comparative distribution of *Pseudomonas putida* RW3 (motile) and RW3- (nonmotile) on soybean roots at 7 days. Segment 1 = all lateral roots from the crown; 2 = top 4-cm segment of taproot; 3 = 4- to 8-cm segment of taproot; 4 = 8- to 12-cm segment of taproot; 5 = 12- to 16-cm segment of taproot, including tip. Standard error of means ranged from 0.25 to 0.39. There was no significant ($P = 0.05$) difference in colonization between RW3 and RW3- on any root segment number.

competence, because isolation of root-colonizing strains was commonplace.

Mean generation times for the three species did not significantly relate to their overall root or seed colonization levels. However, the generation times were assessed in culture media in which the bacteria grew at a faster rate (doubling time of 0.8–1.7 hr) than reported for *Pseudomonas* in soil (5.2 hr) (3). Assuming that the relative rate differences noted here would occur in soil, we conclude that generation time differences of these magnitudes do not correlate to relative root-colonizing capacity.

P. putida and *P. fluorescens* were significantly more motile and chemically attracted to exudates than *Serratia* spp. (Tables 3 and 4). No relationship of bacterial motility or chemotaxis was found to root colonization in the pot or field assays in which overhead watering occurred. Swarm size on soft exudate agar by *P. fluorescens* showed a positive significant correlation to root colonization in the bag assay. However, this trend was not repeated in the capillary assay. *Serratia* spp. showed significantly negative correlations of motility and chemotaxis to root colonization in the bag assay. Why motility or chemotaxis would confer a disadvantage to root colonization by *Serratia* spp. is not known.

Root colonization rate and bacterial distribution by motile RW3 and the nonflagellated RW3- did not significantly differ. Thus, bacterial motility and chemotaxis may not be necessary for root colonization by *P. putida*. These results confirm those found by Howie et al (8), in which three nonmotile *P. fluorescens* mutants colonized wheat roots as well as the motile parents. However, these results contradict those found by De Weger et al (6), in which bacterial motility was required for potato root colonization by *P. fluorescens*.

Bacterial colonization of roots is a complex phenomenon and probably occurs as a result of numerous bacterial activities. Simple screens for fast-growing bacterial strains in culture media, swarm formation on soft exudate agar, or capillary chemotaxis likely would not improve the isolation and identification of bacterial root colonizers. Thus, the challenge is to identify those attributes that confer bacterial rhizosphere competence so that techniques for isolation and enhancement of root-colonizing bacteria can be developed.

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