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Gnotobiotic System for Studying Rhizosphere Colonization by Plant Growth-Promoting *Pseudomonas* Bacteria

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A gnotobiotic system for studying tomato rhizosphere colonization by *Pseudomonas* bacteria was developed. The system is based on sterile seedlings that are inoculated with one or two strains and subsequently grown in a sterile glass tube containing quartz sand. After 7 days of growth in a climate-controlled growth chamber, the number of bacteria present on the root tip was analyzed. The system was optimized with respect to root morphology, inoculation of the seedling, and isolation of root tip bacteria. With this system, rhizosphere colonization on tomato, radish, wheat, and potato was analyzed. For detailed analysis of tomato rhizosphere colonization by some representative plant growth-promoting rhizobacteria, the colonization of known poor, moderate, and good potato root-colonizing *Pseudomonas* strains and of four *Rhizobium* strains was determined. All strains colonized the root tips when inoculated as single strains. When inoculated in competition with the efficient root colonizer *P. fluorescens* strain WCS365, many strains were outcompeted. Mutants of *Pseudomonas* biocontrol bacteria lacking flagella or the O-antigen of lipopolysaccharide (LPS), which were isolated in previous studies and shown to be impaired in potato rhizosphere colonization in field soil systems, showed a reduced colonization ability in the gnotobiotic system also. The gnotobiotic system was used to screen a collection of 300 random *P. fluorescens* WCS365:: Tn*5* mutants for colonization-impaired mutants. Three novel mutants were found that were outcompeted by the wild-type strain in tomato root tip colonization but were not impaired in known colonization traits such as motility, amino acid auxotrophy, and presence of the O-antigenic side chain of LPS. One strain appeared to be a thiamine auxotroph, suggesting that the root does not secrete a sufficient amount of thiamine to support growth of this strain. The other two mutants had a reduced growth rate in laboratory media, suggesting that growth rate is an important colonization factor. As the system is gnotobiotic and devoid of field-soil variables, it can also be used to study the effects of selected biotic and abiotic factors on colonization.

Additional keyword: colonization mutant.

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Colonization of the plant root system is the very first step in nearly all interactions between plants and soilborne microbes. The action of both plant-beneficial and plant-deleterious soilborne micro-organisms depends on their ability to establish in the rhizosphere. For example, unsuccessful biocontrol field experiments are often correlated with poor colonization of the root system by the biocontrol bacteria (Weller 1988; Schippers et al. 1987). Moreover, population size and biocontrol activity of *Pseudomonas fluorescens* strain 2-79 toward *Gaeumannomyces graminis* var. *triciti*, the take-all disease pathogen of wheat, were shown to be positively correlated (Bull et al. 1991). Hardly anything is known about the molecular basis of rhizosphere colonization, despite its importance for microbe-plant interactions.

A few years ago we initiated a study to elucidate the molecular basis of rhizosphere colonization by *Pseudomonas* bacteria. Our first approach was to predict bacterial traits that might be involved in colonization. After mutants defective in such a trait were isolated, the hypothesis was tested by analyzing the colonization ability of these mutants in competition with the wild type. In this way it was shown that the presence of flagella (de Weger et al. 1987b), the presence of the O-antigen of lipopolysaccharide (LPS) (de Weger et al. 1989a), and the ability to synthesize amino acids (M. Simons et al., submitted) are important colonization traits. Since this approach is limited by the knowledge of bacterial physiology and the imagination of the researchers, we initiated another approach, in which individual random mutants are tested for their ability to colonize the rhizosphere in competition with the wild type. Nucleotide sequencing of the corresponding wild-type colonization gene should then lead to novel colonization traits. In a soil system, the complexity of the plant-microbe interaction is immense and hardly anything is known about the sum of factors that lead to reliable biocontrol agent application (Kloepper et al. 1989). We therefore have developed a less complex and therefore presumably more reproducible system in which sterile seedlings are inoculated with only one or two bacterial species (Kloepper et al. 1981; Voisard et al. 1989). Since commercial inoculants are mostly applied to the seed or are applied in the furrow (Zablotowicz et al. 1991) where the bacteria can reach the young seedling, we have chosen to inoculate young seedlings. For effective biocontrol the young root parts must be

colonized by the inoculant strain. Therefore, we have specifically measured the ability of the inoculant bacteria to reach the root tip. In this paper we describe the development and optimization of a system for tomato root colonization that also appeared to be applicable to other crop plants such as radish, wheat, and potato. The system was used to screen 300 random mutants for their ability to colonize the tomato root tip. This yielded three novel colonization mutants. The system also has great promise for studying individual biotic and abiotic factors that play a role in root colonization in soil.

RESULTS

Optimizing the system for plant growth and inoculation.

The gnotobiotic system was varied to find optimal conditions for plant growth and root system development. Tap water was insufficient to support growth of plantlets for 7 to 14 days (data not shown), therefore a plant nutrient solution was added to prevent plant stress. In order to study root colonization per unit of root tip length, simple and reproducible root morphology was desired. As root branching is generally dependent on plant species, plant age, and soil characteristics, we varied sand particle size, compaction, moisture content, and composition of the plant nutrient solution. Quartz sand of 0.1 to 0.3 mm proved optimal for root growth and isolation. With smaller quartz sand particles, more roots were found at the sand/glass interface. Coarser particles caused quick dehydration of the root system. The isolated roots contained a layer of adhering sand particles. We assumed that this sand contained mucigel and constituted part of the rhizosphere, therefore this sand was not removed. The rapid settlement of the sand particles in water made isolation of root bacteria easy in comparison with the use of smaller sand particles, which float in solution and thus block the tubing of the Spiral Plater. Stronger compaction of the sand column slowed down growth of the root and enhanced root branching and root growth at the sand/glass interface. The plant growth system considered optimal for colonization assays is described in detail in Materials and Methods.

For inoculation, sterile seeds or seedlings were soaked for 5 to 15 min in washed and diluted overnight cultures of bacterial strains (Table 1). In initial experiments tomato seeds instead of seedlings were inoculated. Seeds were planted in the tube immediately after soaking in the bacterial suspension. These experiments resulted in low bacterial numbers on the root tip and in extremely high variations in colonization of the roots by cells of *P. fluorescens* strain WCS365 (data not shown), possibly caused by ineffective transfer of bacteria from the seed coat to the young root or by remaining traces of sodium hypochlorite in the seed coat, causing death of inoculant bacteria. Furthermore, germination in the tube is variable in that it takes between 2 and 4 days, during which period the inoculant bacteria age on the seed coat. Moreover, since only 80% of the seeds germinate there is loss of screening productivity. For these reasons we decided to inoculate germinated seeds.

Table 1. Bacterial strains used in this study

Strain[z]	Relevant characteristics	Reference or source
Escherichia.coli		
S17-1	MM294, RP4-2 Tc::Mu-Km::Tn*7* chromosomally integrated.	Simon et al. 1983
Plasmids		
pCIB100	A suicide plasmid in *Pseudomonas,* harboring Tn*5lacZ* in which the *lacZ* is constitutively expressed in *Pseudomonas*	Lam et al. 1990
pSUP2021	A suicide plasmid in *Pseudomonas,* harboring Tn*5*	Simon et al. 1986
Rhizobium		
1021	*R. meliloti* wild type	Meade et al. 1982
248	*R. leguminosarum* bv. *viciae*	Josey et al. 1979
LPR5020	*R. leguminosarum* bv. *trifolii* wild type	Hooykaas et al.1981
ANU843	*R. leguminosarum* bv. *trifolii* wild type	Rolfe et al. 1982
Pseudomonas		
WCS307	*P. fluorescens,* wild-type biocontrol strain, poor colonizer of potato roots	Geels and Schippers 1983
WCS315	*Pseudomonas* spp., wild-type biocontrol strain, poor colonizer of potato roots	de Weger et al. 1986
WCS358	*P. putida,* wild-type biocontrol strain, moderate colonizer of potato roots	Geels and Schippers 1983
PCL1397	WCS358::Tn*5* lacking flagella, nonmotile	L. A. de Weger, unpublished
LWP58-43	WCS358 mutant lacking the O-antigenic side chain of lipopolysaccharide (LPS)	de Weger et al. 1989b
WCS365	*P. fluorescens* wild type, efficient colonizer of the potato rh izosphere Biocontrol strain in a cucumber/*Pythium* system (J. Postma, unpublished)	Geels and Schippers 1983 de Weger et al. 1986
PCL1500	WCS365::Tn*5lacZ* marked derivative. Shows wild-type characteristics, except that it is kanamycin resistant and produces β-galactosidase	van der Bij et al. 1996
PCL1075	WCS365::Tn*5* colonization-impaired mutant	This work
PCL1076	WCS365::Tn*5* colonization-impaired mutant	This work
PCL1078	WCS365::Tn*5* colonization-impaired mutant, nonmotile	This work
PCL1079	WCS365::Tn*5lacZ* thi$^-$ mutant	This work
PCL1080	WCS365::Tn*5*, lacks the O-antigenic side chain of LPS	L. A. de Weger, unpublished
WCS374	*P. fluorescens* wild-type biocontrol strain. Moderate colonizer of potato roots	Geels and Schippers 1983
LWM74-29	Nonmotile WCS374::Tn*5* mutant	de Weger et al. 1987b
LWP74-30	WCS374 mutant, lacking the O-antigenic side chain of LPS	de Weger et al. 1989b
WCS379	*Pseudomonas* spp. wild type. Moderate colonizer of potato roots	de Weger et al. 1986

[z] Wild-type strains with the prefix WCS have been isolated from potato roots at the Willie Commelin Scholten Phytopathological Laboratory, Baarn, The Netherlands.

Distribution of bacteria on the tomato root and influence of inoculum density and mixed inoculum ratio on colonization of the tomato root system.

In order to gain insight into the distribution of *P. fluorescens* strain WCS365 on tomato roots after 7 days of growth, 10 roots, 8 to 9 cm long, from independent plantlets were cut into 1-cm segments. From each segment, the number of bacteria was determined. The $\log_{10}$(CFU+1) transformed numbers of each segment and the average of $\log_{10}$(CFU+1) transformed numbers at each depth are shown in Table 2. The $\log_{10}$ of the total number of bacteria was calculated after adding the numbers of each segment. Usually a gradient is observed in the number of bacteria along the root, in which numbers decrease toward the root tip. The numbers on the tip are approximately two orders of magnitude lower than on the root base. Occasionally, root segments between the root tip and base contain too few bacteria to be detected. Since we chose to investigate root tip colonization after inoculation of seedlings, the number of bacteria on the root tip were counted instead of the total number of bacteria, despite the higher standard deviation on the lower parts.

Inoculation of tomato seedlings with a 1:1 mixture of *P. fluorescens* strain WCS365 and its Tn*5lacZ*-marked derivative PCL1500 has been examined repeatedly and has always resulted in a 1:1 ratio of the inoculant strains at the root tip after 7 days of plant growth (data not shown). To determine the influence of the inoculum density on root tip colonization and on the WCS365/PCL1500 ratio, tomato seedlings were inoculated with a 1:1 mixture of increasing total cell concentrations of 10^3 to 10^8 CFU/ml. After 7 days of growth, unbranched, 8- to 10-cm-long tomato roots were sampled and the numbers of WCS365 and PCL1500 bacteria on the root tip were determined. At an inoculum concentration of 10^3 CFU/ml, only one of nine root tips was colonized, whereas at 10^4 to 10^6 CFU/ml, 50% of the root tips were colonized. At 10^7 and 10^8 CFU/ml, 80 to 90% of the root tips were colonized. Independent of the inoculum density, colonized root tips had an average number of 3.6 ± 0.5 to 4.5 ± 1.6 $\log_{10}$(CFU+1)/cm WCS365 and PCL1500 cells. All colonized root tips had a 1:1 ratio of WCS365 and PCL1500. Based on these results, we decided to inoculate seedlings in a 10^7 to 10^8 CFU/ml suspension for further studies because of the higher incidence of colonized roots.

In the experiments described above we observed that WCS365 and the WCS365::Tn*5lacZ* derivative PCL1500 show indistinguishable rhizosphere colonization. For screening of mutants, it is desirable to use a marked wild type both as an internal standard and a competing strain. The concentration of the inoculum is determined by measuring the optical density, since this is the most convenient method. However, this method may cause slight variations in the cell concentrations of each of these strains. Therefore, we determined the influence of inoculant ratio errors by varying the inoculum ratio of WCS365 and PCL1500 from 1:10 to 10:1 with a total inoculum density of approximately 5×10^7 CFU/ml. After 7 days of growth, the tomato root tips were sampled and the numbers of WCS365 and PCL1500 were determined (Table 3). The ratios at the root tip and in the inoculant mixture were comparable over a wide range. Therefore, mixed inocula were composed with optical density measurements.

Colonization by *Pseudomonas* and *Rhizobium* wild-type strains.

Previous research in our laboratory on potato rhizosphere colonization by *Pseudomonas* wild-type strains in a field soil system revealed that *P. fluorescens* strain WCS307 and *Pseudomonas* sp. strain WCS315 are poor colonizers whereas *P. putida* WCS358, *P. fluorescens* strain WCS374, and *Pseudomonas* sp. WCS379 are moderate colonizers in comparison with *P. fluorescens* strain WCS365 (Brand et al. 1990; Glandorf 1992). *Rhizobium* is known to nodulate leguminous plants in a host-specific symbiotic interaction. One of the first steps in this process is colonization of the root. Four *Rhizobium* strains, differing in host-specificity of nodulation, were selected for gnotobiotic colonization analysis. In order to gain insight into the gnotobiotic colonization behavior of these *Pseudomonas* and *Rhizobium* strains, germinated tomato seedlings were inoculated with single strains as well as with 1:1 mixtures of a test strain in competition with PCL1500, the Tn*5lacZ*-marked derivative of WCS365 (Table 4). Comparison of the bacterial numbers at the tomato root tip shows that, except for strains WCS315 and WCS358, all strains reach a level similar to that of the reference strain PCL1500 when inoculated as a single strain. In competition with PCL1500, however, the poor potato root-colonizing strains WCS307 and WCS315 and the moderate colonizer *P. putida* WCS358 were

Table 2. Distribution of cells of *Pseudomonas fluorescens* strain WCS365 on tomato roots after 7 days of growth in the gnotobiotic system

	Log_{10}(CFU+1)/cm root segment										
Depth (cm)	A[w]	B	C	D	E	F	G	H	I	J	Ave.[x]
1	6.6	5.0	5.6	6.2	5.0	5.6	6.5	6.5	6.0	5.2	5.8 ± 0.6 a
2	4.5	3.5	5.8	5.8	4.8	4.9	5.7	5.8	4.9	4.6	5.0 ± 0.7 b
3	4.9	5.6	5.7	4.7	4.5	3.9	4.9	3.4	5.3	4.5	4.8 ± 0.7 b
4	3.9	3.0	5.0	4.0	5.0	4.1	5.7	5.4	4.7	5.1	4.6 ± 0.8 b
5	4.6	3.8	4.5	3.1	5.0	4.4	4.6	5.0	4.8	4.6	4.4 ± 0.5 b
6	3.0	0.0[y]	4.9	4.8	3.8	3.9	5.7	4.9	4.4	3.4	3.9 ± 1.5 b
7	4.7	2.4	4.7	4.7	3.6	3.9	4.8	5.6	3.6	4.5	4.3 ± 0.9 b
8	4.4	2.4	4.9	4.6	4.6	4.1	0.0	5.4	2.8		3.7 ± 1.6 b
9	4.2		0.0			4.0	3.9	4.6			3.4 ± 1.7 b
Total[z]	6.6	5.7	6.3	6.4	5.7	5.7	6.7	6.7	6.1	6.0	

[w] A through J represent single roots of independent 7-day-old tomato plants.
[x] Numbers in this column with the same letter are not statistically different ($P = 0.05$) according to the Wilcoxon-Mann-Whitney test.
[y] 0.0. = not detected. Detection limit is 2.4 log CFU/cm.
[z] Total numbers of bacteria are not statistically different ($P = 0.05$) according to the Wilcoxon-Mann-Whitney test.

hardly able to reach the tomato root tip. The other moderate potato colonizing strains WCS374 and WCS379 are moderate colonizers in this system in competition with PCL1500. *R. meliloti* 1021 colonizes the root tip as a single strain 10 times lower than *P. fluorescens* PCL1500, and is 10 times reduced in number by the presence of PCL1500 as a coinoculant. *R. leguminosarum* strain 248 and *R. leguminosarum* bv. *trifolii* strains LPR5020 and ANU843, when inoculated as single strains, colonized the tomato root tip about one to two orders of magnitude lower than *P. fluorescens* PCL1500 (Table 4). The presence of strain PCL1500, the Tn*5lacZ*-marked derivative of the efficient colonizing *P. fluorescens* strain WCS365, has no detectable influence on the colonization by these latter *Rhizobium* strains under the conditions we tested.

Tomato root tip colonization by known potato root tip colonization-impaired mutants.

From studies by de Weger et al. (1987b) it is known that the presence of flagella and the O-antigen of LPS (de Weger et al. 1989a) are essential for potato rhizosphere colonization. To investigate whether this also applies in our gnotobiotic tomato system, nonmotile mutants and mutants lacking the O-antigen of LPS of *P. putida* strain WCS358 and *P. fluorescens* strains WCS365 and WCS374 were analyzed for tomato root tip colonization in competition with their wild-type strains (Table 5). The three *Pseudomonas* wild-type strains, *P. putida* WCS358 and *P. fluorescens* strains WCS365 and WCS374, colonize the tomato root tips to levels similar to those in the experiment described previously. The colonization-impaired, nonmotile or O-antigen-lacking, mutants of WCS358 and WCS374 were not found on the tomato root tip whereas the mutants of WCS365 were severely impaired in root tip colonization.

Colonization of *P. fluorescens* strain WCS365 on other crops.

To study whether the system that is used for tomato growth is also suitable for the growth of other crops, root colonization by *P. fluorescens* strain WCS365 on radish, wheat, and potato was also tested. Tomato was included as a reference. Wheat and radish were inoculated as sterile germinated seeds, potato was inoculated as sterile stem cuttings. The colonization of 25 root tips of each of the four crops was comparable: 4.1 ± 0.7, 4.3 ± 0.7, 4.8 ± 0.6, and 4.6 ± 1.0 in $\log_{10}$(CFU+1)/cm root tip for tomato, radish, wheat, and potato, respectively. These results indicate that the gnotobiotic tube system can be used for other crops as well. It also allows the use of potato when sterile stem cuttings are used as the starting material.

Isolation of colonization-impaired mutants.

One of the reasons to develop a gnotobiotic colonization system was to screen for mutants that are hardly or not able to reach the root tip. A set of 300 WCS365::Tn*5* transconjugants was screened on two tomato plants each for tomato root tip colonization in competition with the Tn*5lacZ*-marked derivative PCL1500. Putative mutants resulting from this initial screening were analyzed for tomato root tip colonization on 10 plants. The mutants selected in this way were subsequently analyzed for the known colonization traits motility, presence of O-antigen, and amino acid auxotrophy to test whether mu-

Table 3. Influence of inoculum ratio of strains WCS365 and PCL1500 on root tip colonization ratio

Ratio of WCS365 and PCL1500[z]		
In inoculum	At root tip	Number of plants
0.12	0.17	9
0.14	0.16	8
0.28	0.18	8
0.63	1.66	8
1.11	0.40	6
1.25	0.91	7
7.2	8.1	7

[z] CFU/ml of WCS365 divided by CFU/ml of PCL1500. Total inoculum density was approximately 5×10^7 CFU/ml for all combinations.

Table 4. Tomato root tip colonization by some *Pseudomonas* and *Rhizobium* wild-type test strains, alone and in competition with the Tn*5lacZ*-marked *P. fluorescens* strain WCS365 derivative PCL1500

Tested strain	Competition with PCL1500	Tested strain $\log_{10}$(CFU+1)/cm	PCL1500 $\log_{10}$(CFU+1)/cm
Pseudomonas			
WCS307	–	3.2 ± 2.1	
WCS307	+	0.0 a[z]	4.3 ± 0.7 b
WCS315	–	2.1 ± 1.0	
WCS315	+	0.4 ± 1.1 a	3.4 ± 0.5 b
WCS358	–	2.4 ± 1.2	
WCS358	+	1.4 ± 0.8 a	4.6 ± 1.2 b
WCS365	–	4.5 ± 1.1	
WCS365	+	4.2 ± 0.9 a	4.3 ± 1.0 a
WCS374	–	3.6 ± 0.9	
WCS374	+	2.8 ± 1.5 a	5.0 ± 1.1 b
WCS379	–	3.6 ± 1.3	
WCS379	+	2.7 ± 1.2 a	4.4 ± 1.1 a
PCL1500	–	4.4 ± 0.8	
Rhizobium			
1021	–	3.5 ± 0.3	
1021	+	2.2 ± 1.5 a	4.7 ± 0.8 b
248	–	2.4 ± 1.1	
248	+	2.8 ± 0.6 a	4.5 ± 0.6 b
ANU843	–	2.9 ± 0.2	
ANU843	+	3.0 ± 0.5 a	4.1 ± 0.8 b
LPR5020	–	2.3 ± 0.6	
LPR5020	+	2.6 ± 0.6 a	4.8 ± 0.5 b

[z] For each wild type tested against PCL1500, numbers in the same row with the same letter are not statistically different ($P = 0.05$) according to the Wilcoxon-Mann-Whitney test.

Table 5. Tomato root tip colonization by known potato root colonization-impaired *Pseudomonas* mutants tested in competition with their wild types

Mutants	Corresponding wild-type strain	Mutant[y] $\log_{10}$(CFU+1)/cm	Wild-type strain $\log_{10}$(CFU+1)/cm
O-antigen			
LWP58-43	WCS358	0.0 a[z]	3.6 ± 0.6 b
PCL1080	WCS365	1.7 ± 1.5 a	4.6 ± 0.9 b
LWP374-30	WCS374	0.0 a	4.1 ± 1.2 b
Motility impaired			
LWM58-2	WCS358	0.0 a	3.9 ± 0.7 b
PCL1078	WCS365	0.7 ± 1.1 a	4.2 ± 0.8 b
LWM74-29	WCS374	0.0 a	4.5 ± 1.0 b

[y] Mutant and wild-type characteristics are summarized in Table 1.

[z] For each mutant/wild type pair, numbers with the same letter are not statistically different ($P = 0.05$) according to the Wilcoxon-Mann-Whitney test.

tants in novel colonization traits were present. Finally, the cell envelope protein pattern of the latter mutants was analyzed to test whether the mutants are derivatives of WCS365. Three mutants appeared to be nonmotile, one was an amino acid auxotroph, and one (PCL1079) was a non-amino acid auxotroph, whereas two mutants, PCL1075 and PCL1076, had no discernible defects. Three mutants, PCL1075, PCL1076, and PCL1079, were analyzed in more detail (Table 6). PCL1079 turned out to require thiamine. In comparison with both the wild-type strain WCS365 and the *lacZ*-marked wild-type PCL1500, mutants PCL1075 and PCL1076 had higher generation times in both complex KB and synthetic SSM medium (data not shown).

DISCUSSION

Rhizosphere colonization by micro-organisms is a very important but poorly understood process, mainly due to the large number of biotic and abiotic factors that determine the outcome. In this paper a simple gnotobiotic quartz sand system is described, in which colonization of inoculated germinated seeds was studied after 7 days of incubation under standard conditions. Determination of the numbers of CFUs of segments of individual roots showed a substantial decrease of bacterial numbers from root base to root tip, with an approximately 100-fold difference between the extremes (Table 2).

Some of the root segments in between the root base and the root tip were either not colonized or colonized to a very low level, indicating a non-uniform distribution of bacteria in the tomato rhizosphere (Table 2). This is consistent with data on root colonization by *lux*-marked *Pseudomonas* bacteria (de Weger et al. 1991; Shaw et al. 1992). Loper and Schroth (1984) demonstrated a lognormal distribution of rhizobacteria, therefore the data were $\log_{10}$(CFU+1)/cm transformed before any statistical treatment, as described by Kloepper and Beauchamp (1992).

Crucial questions that should be solved are under which conditions bacteria grow in the rhizosphere, and how fast they grow. In fact, the observed gradient in number of bacteria from root base to root tip and the poor colonization of regions of some roots (Table 2) is consistent with the notion that only a minority of the bacteria located at the upper root segments take part in colonization of the lower part of the root. If this is indeed the case, the cells recovered from the root tip must have undergone many generations, e.g., 6 in case 0.1% of the population is in a colonization-competent state. It is clear that insight on growth conditions and the population dynamics of a bacterial population in the rhizosphere is required before the question can be answered of how many cell divisions have occurred between inoculation and appearance of a cell at the root tip.

Our results strongly suggest that many of the data obtained with the gnotobiotic system, both for wild-type strains and for mutants, could be valid in soil. Firstly, wild-type *Pseudomonas* strains, reported to colonize potato grown in field soil with various efficiencies were studied here (Brand et al. 1990; Glandorf 1992). The results obtained in the gnotobiotic system were very similar to those found in field soil: (i) the poor potato root-colonizing strains WCS307 and WCS315 are outcompeted by the efficient colonizer *P. fluorescens* strain WCS365 (Table 4); and (ii) the moderately potato root-colonizing strains WCS358, WCS374, and WCS379 are reduced in numbers on tomato in comparison with WCS365 (Table 4). Secondly, poor potato root-colonizing mutants that lack flagella or the O-antigen of LPS were shown previously to be colonization-defective in field soil (de Weger et al. 1987b, 1989a). In the gnotobiotic system they also show a reduced colonization ability compared with their respective parental strains (Table 5). Therefore, we conclude that these previously described colonization-impaired mutants are also impaired in colonization in our gnotobiotic system. A screening procedure using the latter system would have yielded these flagella-less and O-antigen-defective mutants as colonization-impaired mutants.

The observed competition between various *Pseudomonas* wild-type strains (Table 4) suggests competition for nutrients or niches. Some competition with PCL1500 was observed also for *R. meliloti* strain 1021. In contrast, *Rhizobium leguminosarum* bv. *trifolii* strains LPR5020 and ANU843 and *R. leguminosarum* bv. *viciae* strain 248 seem not to compete with *P. fluorescens* strain PCL1500, suggesting either the use of different nutrients from the exudate and/or occupation of different niches. We are currently analyzing which parts of the roots are being colonized by these strains.

In comparison with colonization studies in soils, the gnotobiotic system has various advantages. The system is simple and relatively reproducible. It mimics results obtained in soils with various wild-type strains (Table 4) and mutants (Table 5). Moreover, it allows testing of the influence of individual biotic and abiotic factors, such as pathogens, resident microorganisms, amoeba, pH, chemicals, and soil types, on the outcome of the colonization process. Future work will be partly directed to testing the influence of such factors.

Three hundred WCS365::Tn*5* mutants were tested in competition with the parental strain in the gnotobiotic system for their ability to colonize tomato root tips. The parental strain was included because (i) it served as an internal standard, and (ii) based on the data obtained with poorly colonizing wild types (Table 4) one may expect that the colonization defect of at least some mutants is more pronounced in competition. The system identified mutants known to be defective in colonization, e.g., nonmotile mutants (de Weger et al. 1987b) and

Table 6. Properties of some WCS365::Tn*5* mutants impaired in tomato root tip colonization

		Tomato root tip colonization $\log_{10}$(CFU+1/cm)		
Strain	**Characteristics**	**Mutant alone**	**Mutant competition**	**Wild type PCL1500**
PCL1500	Tn*5lacZ* marked wild type			4.6 ± 0.8
PCL1075	Low growth rate	1.4 ± 1.3 a[z]	0.0 a	4.2 ± 0.6 b
PCL1076	Low growth rate	1.2 ± 1.1 a	0.5 ± 0.9 a	4.3 ± 0.9 b
PCL1079	Thiamine requiring	1.7 ± 1.0 a	2.3 ± 0.9 a	6.0 ± 0.6 b

[z] For each mutant, numbers with the same letter are not statistically different ($P = 0.05$) according to the Wilcoxon-Mann-Whitney test.

amino acid auxotrophs (M. Simons et al., submitted), thus confirming that the system can be used for screening mutants defective in root colonization. One of the colonization-defective mutants appeared to be auxotrophic for thiamine (vitamin B_1), a component known to be present in the rhizosphere (West 1939; Lilly and Leonian 1939). The fact that the mutant was isolated shows that the bio-availability of thiamine in the gnotobiotic rhizosphere of tomato is very low. The growth rate of two of the isolated colonization-defective mutants was reduced in laboratory media. As these mutants are presumably affected in household genes, it is likely that these mutants will not yield specific, novel, colonization genes. However, we will clone and sequence the affected gene(s) to test this notion. The twofold correlation between poor colonization and lower growth rate suggests a causal relationship. This result is consistent with the notions that (i) the amount of exuded nutrients is limiting to bacterial growth, and (ii) strains that grow fast under these conditions outcompete poor growers.

Despite the positive results with the screening of colonization mutants (text and Table 6) it should be realized that not all possible colonization-defective mutants will be detected with this screening: (i) mutants in which the defect is only obvious through interactions with factors present in soil, but not in the gnotobiotic system, will not be detected; (ii) some mutants will be missed because the initial screening is carried out on two plants; and (iii) mutants with a relatively minor defect in colonization (e.g., 10-fold) will not be detected due to the large standard deviation. However, the system allows screening of many mutants and the results will yield a further genetic basis of the important process of rhizosphere colonization.

MATERIALS AND METHODS

Bacterial strains.

Pseudomonas, Rhizobium, and *Escherichia coli* strains used in this study and their relevant characteristics are listed in Table 1.

Media and growth conditions.

All *Pseudomonas* strains were grown at 28°C in King's medium B (KB; King et al. 1954). Media were solidified with 1.8% agar (Bacto Agar, Difco Inc., Detroit, MI). Stock cultures of all strains were kept at –80°C in 35% glycerol. Cultures on solid media were stored at +4°C until use. Standard succinate medium (SSM; Meyer and Abdallah 1978) was modified by the addition of biotin (20 μg/ml), thiamine (20 μg/ml), and a solution of trace elements (from a 400× stock solution containing $MnSO_4$ [0.61g/liter], $ZnSO_4 \cdot 7H_2O$ [0.1 g/liter], H_3BO_3 [1.27 g/liter], Na_2MoO_4 $2H_2O$ [0.40 g/liter], $CuSO_4$ [0.04 g/liter]), and iron ions from a 400× stock solution of Fe_2EDTA (13.5 g/liter). *Rhizobium* was grown on solidified yeast mannitol broth (YMB), consisting of K_2HPO_4 (0.5 g/liter), $MgSO_4 \cdot 7H_2O$ (0.2 g/liter), NaCl (0.1 g/liter), mannitol (10 g/liter), and yeast extract (Difco; 0.4 g/liter). For colonization experiments, *Rhizobium* strains were grown in tryptone-yeast medium (TY; van Brussel et al. 1982). *E. coli* S17-1 was grown at 37°C in Luria broth (LB; Maniatis et al. 1982) supplemented with 20 μg of kanamycin per ml.

Gnotobiotic colonization assay.

A 1-cm-long piece of silicone tubing (Python tubing nr. 1749 25*35 mm, Rubber BV, Hilversum, The Netherlands) was connected to a glass tube (22 mm inner diameter, 26 mm outer diameter, 20 cm length) at 5 cm from one end. A cotton gauze was mounted over the same end and secured with a rubber band. The glass tube was mounted in a round-bottomed glass beaker (40 mm inner diameter, 95 mm length) by gentle force. The connection between the two was airtight due to the silicone ring. A mixture of quartz sand (0.1 to 0.3 mm, Wessem S50, Wessem, The Netherlands), was moistened with 10% Plant Nutrient Solution (PNS), consisting of 5mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and micronutrients (Hoffland 1992). The sand was compacted by gentle shaking. A sand column of 12 cm was constructed with 60 to 70 g of sand/PNS mixture. This system is shown in Figure 1. The open end of the tube was plugged with cotton and capped with aluminum foil to prevent dehydration after autoclaving. The tube was then autoclaved at least 24 h before use for 25 min at 121°C.

Seed sterilization and germination.

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Carmello; S&G Seeds B.V., Enkhuizen, The Netherlands) were sterilized by gentle shaking for 3 min in a solution of 5% household sodium hypochlorite. The sterilized seeds were soaked six times for 30 min in fresh, sterile, demineralized water. Radish (*Raphanus sativus* L. cv. Saxa Nova; S&G Seeds B.V., Enkhuizen, The Netherlands) and wheat (*Tricitum aestivum* L. cv. Obelisk; S&G Seeds B.V., Enkhuizen, The Netherlands) seeds were sterilized by washing with 70% ethanol for 30 s, followed by two soakings in sterile, demineralized water for 5 min. Subsequently, the seeds were shaken gently in a solution of 1.5% household sodium hypochlorite, supplemented with

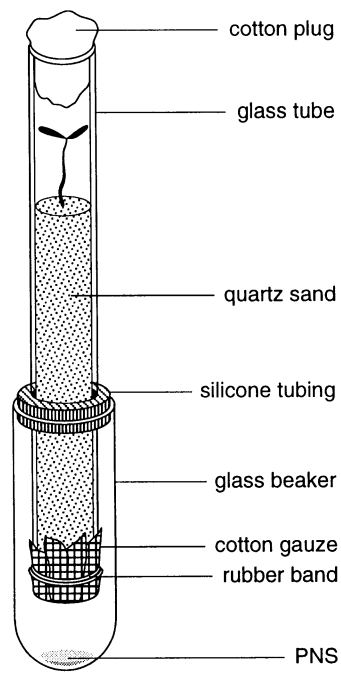

Fig. 1. Glass tube system used for the gnotobiotic colonization assay system. For explanation, see text.

0.1% Tween 80 (Sigma, Bornhem, Belgium) for 1.5 h, followed by 1.5 h shaking in sterile, demineralized water and another 1.5 h in the sodium hypochlorite–Tween solution. The sterilized seeds were soaked six times in fresh, sterile, demineralized water for 30 min. To synchronize the germination process, seeds were placed in petri dishes containing PNS solidified with 1.5% Pronarose D1 (Hispanagar, Burgos, Spain) and incubated overnight upside down in the dark at 4°C, followed by incubation at 28°C for 2 days. Seed sterilization of tomato and radish resulted in 100% sterility and 80% germination, as was observed by sampling on solidified KB medium before and after plant growth. Less than 1% of the wheat seedlings were infected. Potato (*Solanum tuberosum* L. cv. Bintje; Stichting Keuringsdienst Noordzeepolders van de N.A.K. Emmeloord, The Netherlands) was kept as sterile plantlets on modified LS medium (Linsmaier and Skoog 1965). Sterile stem cuttings were used as the starting material after storage overnight in solidified LS. For potato root colonization assays, the quartz sand in the tube was covered with 2 ml of solidified PNS.

Bacterization.

Cell suspensions for bacterization were prepared from overnight cultures in KB medium. A volume of 1 ml of an overnight culture was centrifuged in an Eppendorf centrifuge. The pellet was washed with 1 ml of PNS. After vortexing, the suspension was diluted 25-fold in PNS and the A_{620} value was measured. Based on the established strain-dependent relationship between A_{620} value and number of viable cells, 1:1 mixtures of cells were prepared. From cultures of an A_{620} value of approximately 0.2, a 500-µl suspension of each culture was added to 4.5 ml of PNS. For mixed inoculations in which one of the inoculants was *lacZ* marked (Lam et al. 1990), the precise volume of each strain was adjusted, according to the established A_{620}/CFU ratio. Unless mentioned otherwise, bacterization of germinated seeds was performed by dipping germinated seeds for 15 min in a suspension of 10^7 CFU/ml. After inoculation, the seeds were aseptically placed 5 mm below the surface of the quartz sand. The plant growth tubes were kept in a climate-controlled growth chamber (18°C, 70% relative humidity, 16 h of daylight) to allow the plantlets to grow.

Plant growth and isolation of bacteria from the root tip.

After 7 days of growth in the gnotobiotic system, the root systems of tomato and radish were mostly unbranched and 6 to 10 cm in length, whereas wheat root systems consisted of 3 to 5 roots. Potato, grown for 14 days, had roots that were variable, in that single, unbranched roots and many short roots were observed. The root tip (1 to 2 cm length) was removed and shaken vigorously in the presence of adhering sand particles in 1.0 ml of PNS on an Eppendorf shaker for 15 min to remove the bacteria. The bacterial suspension was diluted tenfold and 37 µl was plated on KB plates with a Spiral Plater (Spiral Systems Inc., Cincinnati, OH). For mixed inoculations, the KB medium was supplemented with 40 µg of X-gal per ml. After 2 days of incubation at 28°C the number of yellow/white (Tn*5* mutants or nonmarked wild type) and blue (Tn*5lacZ*-marked wild type) colonies was counted. For mixed inoculations without *lacZ*-containing mutants, the mixture was plated on separate media with and without 20 µg of kanamycin per ml. *Rhizobium* strains were plated on YMB as a selective medium. The CFU/cm root tip was calculated (Davies and Whitbread 1989), and the data transformed to $\log_{10}$ (CFU+1)/cm values (Loper and Schroth 1984), after which estimates of the mean and standard deviation were calculated. Experiments were performed in tenfold, and done twice. Statistical comparison of strains in mixed inocula was done exclusively with the nonparametric Wilcoxon-Mann-Whitney test (Sokal and Rohlf 1981). For analysis of mutant behavior in mixed inocula, only plates with more than 30 colonies per plate (5×10^3 CFU/cm) were included. When the number was lower, the value was omitted. Zeroes were included for analysis of single inocula (Kloepper and Beauchamp 1992). Calculation of $\log_{10}$(CFU+1)/cm was chosen to avoid nonexistent $\log_{10}(0)$ situations.

Tn*5* and Tn*5lacZ* mutagenesis.

Transposon mutagenesis of *P. fluorescens* strain WCS365 was carried out according to the method of Simon et al. (1983). *E. coli* strain S17-1 was used as the donor strain for two-parental matings on solidified KB medium. A derivative of strain S17-1, harboring pCIB100 (Lam et al. 1990) or pSUP2021 (Simon et al. 1983) was used for Tn*5lacZ* and Tn*5* mutagenesis, respectively.

Screening WCS365::Tn*5* mutants for their ability to colonize tomato root tips.

To obtain WCS365::Tn*5lacZ* colonization-negative mutants, purified transconjugants were analyzed in the gnotobiotic system by coinoculation with the wild type in a 1:1 ratio. Three hundred mutants were analyzed, each on two plants. Data on numbers of mutant and wild-type cells on the root tips was recorded as number of CFU and percentage of mutant colonies observed on KB plates supplemented with 40 µg of X-gal per ml. Putative mutants were retested on 10 plants each. Motility was assayed on 1:20 diluted KB medium supplemented with 0.3% agar as described by de Weger et al. (1987b). Auxotrophy was tested on solidified SSM medium. To identify the mutants as derivatives of *P. fluorescens* strain WCS365, LPS ladder patterns were obtained by polyacrylamide gel electrophoresis and visualized as described by de Weger et al. (1987a) and cell envelope protein patterns were obtained by polyacrylamide gel electrophoresis as described by Lugtenberg et al. (1975).

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