Survival of *gfp*-tagged antagonistic bacteria in the rhizosphere of tomato plants and their effects on the indigenous bacterial community

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Abstract

The survival and colonization patterns of *Pseudomonas putida* PRD16 and *Enterobacter cowanii* PRF116 in the rhizosphere of greenhouse-grown tomato plants and the effects of their inoculation on the indigenous bacterial community were followed by selective plating, molecular fingerprinting, and confocal laser scanning microscopy (CLSM) over 3 weeks. Both strains, which showed *in vitro* antagonistic activity against *Ralstonia solanacearum*, were previously tagged with *gfp*. Seed and root inoculation were compared. Although plate counts decreased for both *gfp*-tagged antagonists, PRD16 showed a better survival in the rhizosphere of tomato roots independent of the inoculation method. Analysis of 16S rRNA gene fragments amplified from total community DNA by denaturing gradient gel electrophoresis and CLSM confirmed the decrease in the relative abundance of the inoculant strains. Pronounced differences in the *Pseudomonas* community patterns for plants inoculated with PRD16 compared to the control were detected 3 weeks after root inoculation, indicating a longer-lasting effect. Analysis by CLSM showed rather heterogeneous colonization patterns for both inoculant strains. In comparison with seed inoculation, root inoculation led to a much better colonization as evidenced by all three methods. The colonization patterns observed by CLSM provide important information on the sampling strategy required for monitoring inoculant strains in the rhizosphere.

Introduction

Ralstonia solanacearum is a soil-borne pathogen that naturally infects roots and invades and multiplies in the xylem vessels. *Ralstonia solanacearum* has an extremely wide host range, with over 200 plant species belonging to more than 50 botanical families, and is a major plant pathogen in subtropical and tropical areas. *Ralstonia solanacearum* (biovar 2/race 3), which causes bacterial wilt in solanaceous plants such as potato and tomato, has become adapted to more temperate climates. Biological control of *R. solanacearum* could be of great interest in tropical and subtropical regions where *R. solanacearum* is endemic and leads to important crop losses; however, fields infested with quarantine organism *R. solanacearum* need to be taken out of production in Europe and thus biological control would not be an appropriate control measure.

Mechanisms of bacterial antagonism towards plant pathogens include competition for nutrients and space, production of antibiotics, and production of cell wall-degrading enzymes or induced resistance (Lugtenberg & Dekkers, 1999; Bloemberg & Lugtenberg, 2001). An important prerequisite for effective *in vitro* selected antagonists is their ability to colonize the rhizosphere. One of the difficulties in developing rhizobacteria as a viable alternative to chemical pest control is that many biological control agents are too variable in their performance. According to Raaijmakers & Weller (2001) variable expression of genes involved in disease suppression and poor root colonization are the major contributors to this inconsistency. Therefore, the rhizosphere competence and factors affecting the establishment of potential biocontrol strains in the rhizosphere should be first studied under standardized greenhouse conditions.

From a collection of bacterial isolates from the rhizosphere of maize grown in the subtropics (Brazil), which showed *in vitro* antagonistic activity towards *R. solanacearum* (biovar 2/race 3: strains 1609 and B3B), two strains were selected. To localize the inoculant strains along the roots of greenhouse grown plants they were recently *gfp*-tagged by introducing the IncQ plasmid pSM1890. The stability of the IncQ plasmid in both strains under rhizosphere conditions was shown in a previous greenhouse experiment (Peixoto *et al.*, 2004). The aim of this study was to compare the rhizosphere competence of the two *gfp*-tagged *in vitro* antagonists in the tomato rhizosphere under greenhouse conditions and to determine how much the establishment of the strains was influenced by the inoculation method (seed inoculation vs. root dipping). The fate of the inoculant strains was monitored by selective cultivation. *In situ* detection by confocal laser scanning microscopy (CLSM) was used to localize the strains along the root. To study potential effects of the inoculant strains on the indigenous microbial community, changes in the relative abundance bacterial ribotypes after the inoculation were followed by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments amplified from total community DNA.

Materials and methods

Bacterial strains

Strains PRD16 and PRF116 both originated from the rhizosphere of maize grown in Brazil. Recently, Peixoto *et al.* (2004) identified strain PRD16 as *Pseudomonas putida* A by fatty acid methylester analysis. Sequencing of 700 bp of the 16S rRNA gene revealed that strain PRF116 was 98.6% similar to *Enterobacter cowanii* strain (AJ508303). Both strains produced siderophore compounds and antibiotics but proteolytic activity was detected only for PRD16 (Peixoto *et al.*, 2004). To facilitate monitoring of the strains in the phase of greenhouse experiments, plasmid pSM1890 (Haagensen *et al.*, 2002) was recently introduced in triparental matings as described by Peixoto *et al.* (2004). The presence of the IncQ plasmid pSM1890 was confirmed by PCR with primers targeting the IncQ *oriV* as described by Götz *et al.* (1996) and strain identity was confirmed by BOX-PCR according to Rademaker *et al.* (1999). The *gfp*-labeled antagonists were again tested for their *in vitro* antagonistic activity against *R. solanacearum* 1609 and strain B3B according to van Overbeek *et al.* (2002).

Quantification of green fluorescence

Emission of the green fluorescence of *P. putida* PRD16 and *E. cowanii* PRF116 was quantified as follows. Cells of the *gfp*-labeled strains as well as their parental strains grown overnight in LB medium were harvested by centrifugation. The pellets were resuspended in the same volume of 0.85% NaCl, and the green fluorescence was measured with a fluorometer (Fluoroscan II, Labsystems, Helsinki, Finland) at an excitation wavelength of 485 nm and an emission detection of 538 nm. The fluorescence of at least three replicates per strain was measured. The relative fluorescence activity of the cells was calculated as measured fluorescence values per turbidity (OD_{600}). The green autofluorescence of the cells was taken into account by determining the relative fluorescence activities of the parental strains grown under the same conditions and subtracting these values from those of the *gfp*-labeled strains.

Inoculation of tomato seeds with *gfp*-labeled antagonists

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Moneymaker) were surface sterilized by a 1 min treatment in 70% ethanol followed by 15 min in sodium hypochloride (~12% active chlorine) and by three subsequent washing steps with sterile water for at least 15 min. The *gfp*-labeled antagonists were grown on LB medium supplemented with rifampicin (50 μg mL^{-1}) and gentamicin (10 μg mL^{-1}) for 18 h at 28 °C. The bacterial cells were pelleted by centrifugation at 8000 ***g*** for 15 min and resuspended in fresh LB medium without the addition of any antibiotics. The cell suspension was adjusted to an optical density corresponding to a cell density of ~10^9 cells mL^{-1}. *c.* 200 seeds were mixed with the cell suspensions of each antagonist and incubated at room temperature for *c.* 10 min. Seeds that were incubated in sterile LB medium 10 min served as control. Air-dried seeds were sown into potting soil [turf substrate/clay granulate No. 4230, Klasmann-Deilmann GmbH, Geeste, Germany, sieved (2 mm mesh width) and mixed with 80% weight in weight (w/w) sand]. The pots were kept in the greenhouse at 28 °C, 30% humidity and daylight. If the daylight intensity was below 10 000 lux m^{-2} between 7 am and 7 pm, plants received an additional 5000 lux m^{-2} of light.

Inoculation of the roots of tomato plantlets with *gfp*-tagged antagonists

Prior to root inoculation, the *gfp*-labeled antagonists were grown and harvested as described above. The bacterial cell pellets were resuspended in sterile saline (0.85%) resulting in 8.78×10^9 and 1.63×10^9 CFU mL^{-1} for PRD16 and PRF116, respectively. Two-week-old tomato plantlets were removed from their pots. Soil not tightly adhering to the roots was carefully removed before dipping the roots into the cell suspensions of *gfp*-labeled antagonists for *c.* 20 min. Control plantlets were dipped in sterile saline for the same time. Control and root-inoculated plantlets were imme-

diately planted in potting soil and kept under greenhouse conditions as described above.

Sampling and sample processing

Samples were taken 8, 15, 22, and 29 days after sowing the inoculated seeds (seeds were germinating approximately 5 days after sowing) and 3, 7, 13, and 21 days after root inoculation. Four rhizosphere samples were analyzed per treatment and sampling time. Each rhizosphere sample consisted of the total root system with tightly adhering soil of four individual plants, which were thoroughly mixed and immediately processed for CFU counts or kept frozen for DNA extraction. To detach bacterial cells from the rhizosphere soil and the rhizoplane, root samples were resuspended in sterile saline (0.85%) and treated mechanically. For the seed inoculation experiment, aliquots of rhizosphere samples were placed into microcentrifuge tubes (2 mL) containing 0.2 g of glass beads (0.1–0.11 mm) and processed by vortexing four times for 10 s. Samples from the root inoculation experiment were processed by Stomacher blending for 3 min at medium speed. To determine the CFU counts of the antagonists, serial dilutions of the cell suspension were plated on King's B medium (King *et al.*, 1954) supplemented with gentamicin ($10\,\mu g\,mL^{-1}$) and rifampicin ($50\,\mu g\,mL^{-1}$). Total aerobic counts were determined by plating of serial dilutions on R2A medium (Difco, Detroit, MI). Cycloheximide was added to all media ($100\,\mu g\,mL^{-1}$) to prevent fungal growth.

At least three plants were sampled at each sampling time for CLSM analysis. The plants were carefully shaken to remove soil not tightly adhering to the roots, gently washed with phosphate-buffered saline (PBS: 100 mM NaH_2PO_4, 100 mM Na_2HPO_4, 1.3 M NaCl in H_2O, pH 7.2–7.4) to remove soil particles from the roots, and then fixed in a 4% paraformaldehyde solution in PBS at 4 °C overnight (Amann, 1995). For long-term storage the roots were subsequently placed in an ethanol–PBS mixture (1 : 1) at − 20 °C.

DNA extraction

Two-milliliter microcentrifuge tubes were filled with 0.2 g of root material and 0.4 g of sterile glass beads (0.1–0.11 mm) and processed for DNA extraction as described by Gomes *et al.* (2004).

PCR-amplification of 16S rRNA gene fragments and DGGE analysis

Recently, described primer systems and PCR conditions were used to amplify the 16S rRNA gene fragments of *Betaproteobacteria* (Gomes *et al.*, 2001) and *Pseudomonas* (Milling *et al.*, 2004) from total community DNA. The amplicons obtained with group-specific primers were reamplified with the bacterial primers F984-GC and R1378 as described by Heuer *et al.* (1997). To generate bacterial community fingerprints, the latter primer system was used directly to amplify 16S rRNA gene fragments from total community DNA. DGGE analysis was done with a double gradient gel containing 6–9% acrylamide with a gradient of 26–58% of denaturant and as recently described by Gomes *et al.* (2004). The run was performed in 1 × Tris-acetate-EDTA buffer at 58 °C at a constant voltage of 220 V for 6 h. The DGGE gels were silver-stained according to Heuer *et al.* (2001).

Cluster analysis

Bacterial community profiles were analyzed with the software package GELCOMPAR 4.0 program (Applied Maths, Kortrijk, Belgium). Background was subtracted by a rolling disk method with an intensity of 10 (relative units), and the lanes were normalized. A dendrogram was constructed by the Pearson correlation index for each pair of lanes within a gel and cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA).

Cloning and sequencing

Pseudomonas DGGE PCR products from non-inoculated rhizosphere samples (third sampling time) were joined in one microcentrifuge tube and purified using Geneclean Spin Kit (Bio 101). The amplicons were cloned using the pGEM-T Easy Vector System I (Promega Corporation, Madison, WI) according to the manufacturer's instructions, and white colonies were screened by PCR-DGGE for their electrophoretic mobility in the DGGE. One clone with mobility identical to band A (Fig. 1) in the *Pseudomonas* patterns was sent for sequencing. The sequence retrieved is available under GenBank accession number DQ099497.

Confocal laser scanning microscopy (CLSM)

To wash away soil particles, fixed roots were gently rinsed with sterile tap water. Soil particles still adhering to the roots were removed with a forceps. Whole roots (except for roots of plants 21 days after root inoculation, which were cut under the binocular prior to the examination) were placed on glass slides, mounted with tap water and covered with a cover slip. At least three plants were analyzed for each treatment and sampling time. Root colonization was followed by CLSM [Leica TCS SP2 with Acusto Optical Beam Splitter equipped with an Argon/Krypton laser (among others, 488 nm for excitation of GFP), a diode laser (405 nm for excitation of DAPI) and detectors for simultaneous monitoring GFP (495–590 nm), DAPI (435–460 nm; data not shown) and the transmitted light picture]. Images

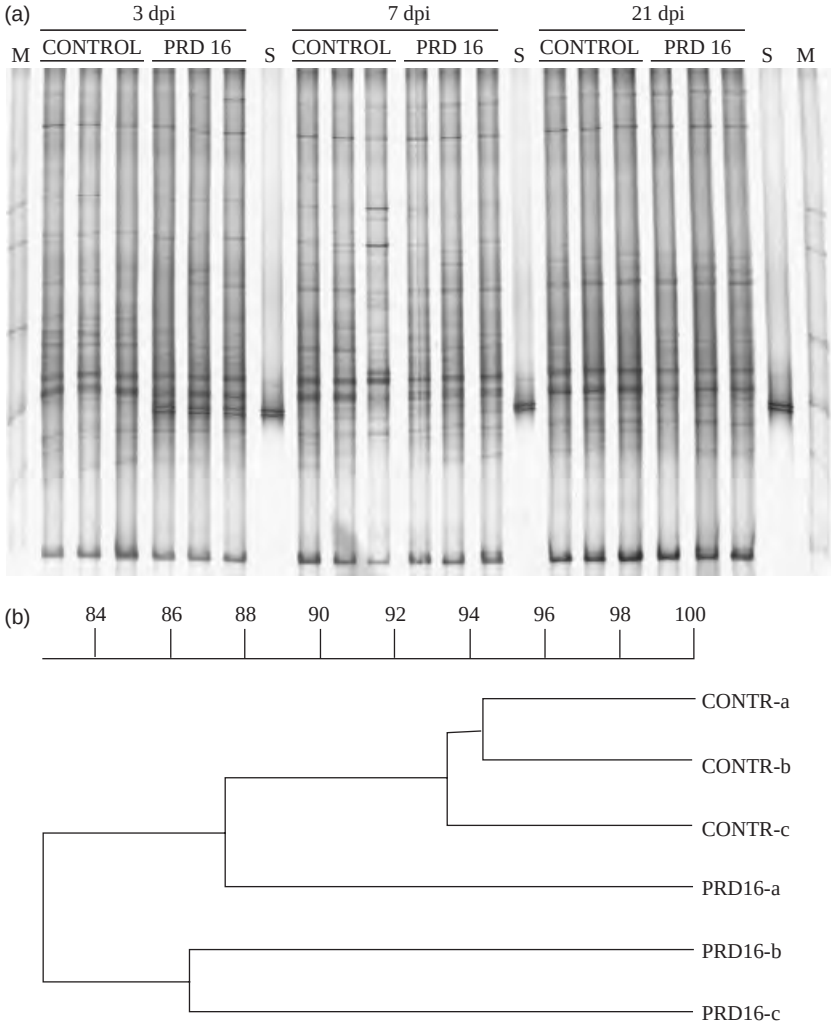

Fig. 1. (a) Comparison of bacterial community fingerprints from rhizosphere samples of control (noninoculated) plants and of tomato plants inoculated with *Pseudomonas putida* PRD16. S, 16S rRNA gene fragment amplified from PRD16; M, bacterial marker (Heuer *et al.*, 2001). (b) UPGMA based on Pearson indices for the samples taken 21 days post inoculation.

were collected in a z-series from 30–60 optical sections ranging from 0.5 to 4.0 μm in thickness. Optical sections, maximum intensity projections and overlays were generated using the Leica Confocal Software, version 2.5 (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany). Images were processed by PHOTOSHOP software (Adobe, Mountain View, CA).

Results

Characterization of *gfp*-labeled antagonists

To facilitate monitoring of the two antagonistic strains under greenhouse conditions rifampicin-resistant mutants of *Pseudomonas putida* PRD16 and *Enterobacter cowanii* PRF116 were previously tagged with *gfp* by introducing the IncQ plasmid pSM1890 in a triparental mating. All cells of PRF116 showed a bright relative fluorescence activity with relative fluorescence activity values of 1830.6 and even the colonies on the plates looked green. In contrast, overnight cultures of PRD16 showed only a few cells with bright fluorescence, most of the cells displaying a weak fluorescence (relative fluorescence activities 4.9). The rifampicin-resistant mutants as well as the *gfp*-tagged strains were retested for their antagonistic activity towards *R. solanacearum* 1609 and *Ralstonia solanacearum* B3B (Table 1). In comparison

Table 1. Antagonistic activity of *Pseudomonas putida* PRD16 and *Enterobacter cowanii* PRF116 and their *rif*- and *gfp*-mutants towards *Ralstonia solanacearum* 1609 and *Ralstonia solanacearum* B3B

	Inhibition zones (mm) of *Ralstonia solanacearum*	
	1609	B3B
PRD 16	5	10
PRD 16 rif^R	5	10
PRD 16 rif^R pSM1890	4	5
PRF 116	9	14
PRF 116 rif^R	12	15
PRF 116 rif^R pSM1890	10	10

Table 2. CFU of the antagonistic strains *Pseudomonas putida* PRD16 and *Enterobacter cowanii* PRF116 after seed and root inoculation

	CFU of the antagonists†		Total bacterial counts‡		
Sampling time*	PRD16	PRF116	Control	PRD16	PRF116
Seed inoculation					
8	6.52 ± 0.40	5.36 ± 0.23	8.99 ± 0.09	8.39 ± 0.20	8.93 ± 0.21
15	5.49 ± 0.21	4.28 ± 0.22	8.05 ± 0.10	8.27 ± 0.18	8.55 ± 0.11
22	5.01 ± 0.14	3.45 ± 0.24	8.14 ± 0.31	7.63 ± 0.16	8.43 ± 0.14
29	4.20 ± 0.17	0.79 ± 1.59			
Root inoculation					
3	8.41 ± 0.33	7.96 ± 0.26	8.20 ± 0.35	8.80 ± 0.16	8.85 ± 0.23
7	6.82 ± 0.14	6.37 ± 0.06	8.37 ± 0.24	8.75 ± 0.03	8.54 ± 0.17
13	5.87 ± 0.21	5.45 ± 0.14	8.41 ± 0.33	8.46 ± 0.17	8.80 ± 0.16
21	5.33 ± 0.12	4.43 ± 0.17	8.34 ± 0.11	8.29 ± 0.57	8.80 ± 0.12

*Days after seed and root inoculation.

†Log10 CFU ± SD per gram root fresh weight after selective plating on King's B supplemented with gentamicin and rifampicin.

‡Log10 CFU ± SD per gram root fresh weight after plating on R2A-medium.

with the parental strains and the rifampicin-resistant mutants, both *gfp*-tagged strains showed a reduced antagonistic activity towards B3B. Interestingly, such a reduction was not seen when the *gfp*-tagged strains were tested against strain 1609 (Table 1). Strain PRD16 showed a lower *in vitro* antagonistic activity compared to PRF116. The growth behavior in nutrient broth revealed an approximate 20% fitness disadvantage compared to the plasmid-free strain for PRD16 and PRF116 (data not shown).

Survival of the *gfp*-tagged antagonists in the rhizosphere of tomato plants followed by selective plating

Quantitative data on the survival of the *gfp*-tagged antagonists in the rhizosphere of the *R. solanacearum* host plant tomato were obtained by selective plating on King's B supplemented with gentamicin and rifampicin (Table 2). No background growth was observed for rhizosphere samples from non-inoculated plants. The seed inoculation resulted in *c.* 1.3×10^6 and 2.8×10^6 CFU per seed for PRD16 and PRF116, respectively. At the first sampling time, the *gfp*-tagged antagonists were exposed to soil conditions for 8 days in the seed inoculation experiment (3 days after germination) and 3 days in the root inoculation experiment. Eight days after seed inoculation, approximately 5×10^6 CFU g^{-1} of root fresh weight were found for PRD16 but only 5×10^5 CFU g^{-1} for strain PRF116. After 29 days, the CFU counts of PRD16 were just slightly above 10^4, whereas the CFU counts of PRF116 were below 10^2 g^{-1} of root fresh weight.

Three days after root inoculation the counts of PRD16 were still *c.* 4×10^8 and the PRF116 counts 1×10^8 g^{-1} of root fresh weight. Three weeks after root inoculation the CFU counts dropped for both strains to 3×10^5 and 5×10^4 g^{-1} of root fresh weight for PRD16 and PRF116, respectively. The total CFU counts determined on R2A for the seed inoculation experiment were in the range of 5×10^8 and 10^9 g^{-1} root fresh weight for all treatments and replicates and a decrease of the total counts of approximately one order of magnitude was observed after 22 days (Table 2). The total counts in the root inoculation experiment were more stable but also in the same range. Interestingly, at the first sampling time the counts of the control were lower than of the root-inoculated treatments, indicating that the inoculant strains made up the numerically dominant population.

Effects of inoculation on relative abundance of ribotypes in tomato rhizosphere

Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene fragments amplified from total community DNA was used to detect shifts in the relative abundance of ribotypes in the tomato rhizosphere as a consequence of seed or root inoculation with antagonists. At the first sampling time, strain PRD16 could be detected in the bacterial rhizosphere patterns of both seed- and root-inoculated plants, but the bands with identical electrophoretic mobility as the inoculant strains were much less intense for a seed inoculation experiment (Fig. 1; data shown only for the root inoculation experiment). Detection of strain PRF116 in the bacterial rhizosphere patterns of seed-inoculated plants was impossible due to the presence of a band with identical electrophoretic mobility as PRF116 in the replicates of the control. However, in the root inoculation experiment an unequivocal detection of the inoculant strain PRF116 was possible at the first sampling time because this band was absent in the replicates of the control (Fig. 2). Independently of the inoculant strain and the type of inoculation, bands matching the electrophoretic mobility of the inoculant strains were not detected in the bacterial community patterns of the last sampling time in our study.

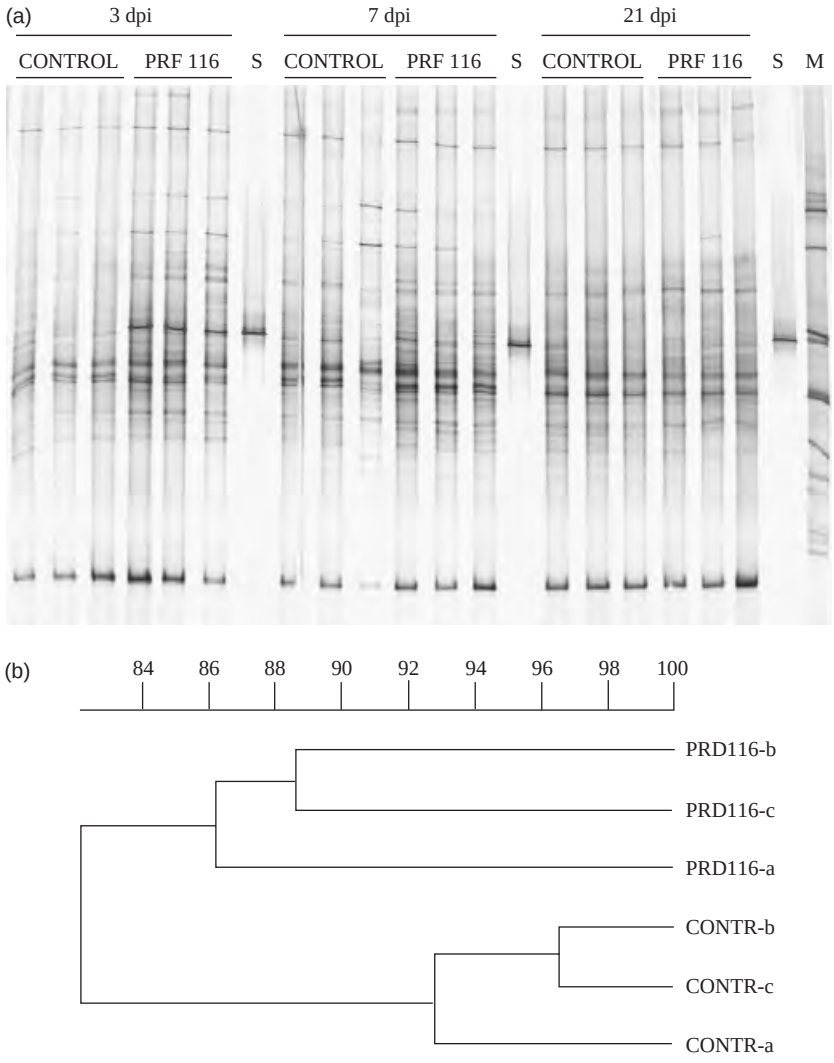

Fig. 2. (a) Comparison of bacterial community fingerprints from rhizosphere samples of control (noninoculated) plants and of tomato plants inoculated with *Enterobacter cowanii* strain PRF116. S, 16S rRNA gene fragment amplified from PRF116; M, bacterial marker (Heuer *et al.*, 2001). (b) UPGMA based on Pearson indices for the samples taken 21 days post inoculation.

To analyze the effects of the inoculation on bacterial community composition, Pearson correlation coefficients were compared by UPGMA. Whereas the bacterial community patterns of PRD16-treated and untreated tomato plants did not form separate clusters at the last sampling time for either type of inoculation, a clustering of DGGE patterns of controls and PRF116-treated plants was observed in both experiments for this sampling point. To analyze the effects of the inoculation with PRD16 and PRF116 on less dominant members of the bacterial rhizosphere community, we used a seminested approach as suggested by Gomes *et al.* (2001) for the *Betaproteobacteria*, the bacterial group to which the pathogen *R. solanacearum* belongs. Two dominant bands were detectable in all treatments and at all time points (Fig. 3). At all sampling times and for both inoculant strains and inoculation methods no separate UPGMA clusters were observed for the inoculated and nontreated communities. However, one band (indicated with an arrow), which was not detectable in the replicates of the inoculated samples appeared in the patterns of all replicates of the control (Fig. 3).

In addition, the *Pseudomonas* community in the rhizosphere of tomato plants was also analyzed for the seed and root inoculation experiments with the *P. putida* strain PRD16. The *Pseudomonas* rhizosphere patterns of the seed inoculation experiment did not differ markedly from that of the control, and an unequivocal detection of the inoculant strain was again only detectable at the first sampling point. In contrast, the structural diversity of the *Pseudomonas* community in the root inoculation experiment showed the most pronounced differences between inoculated and noninoculated samples (indicated by arrows) observed in this study (Fig. 4). The inoculant strain PRD16 was clearly detectable 3 and 7 days after root inoculation. Band A, which was only detected in the rhizosphere patterns of the control, was excised and re-amplified. Unfortunately, re-

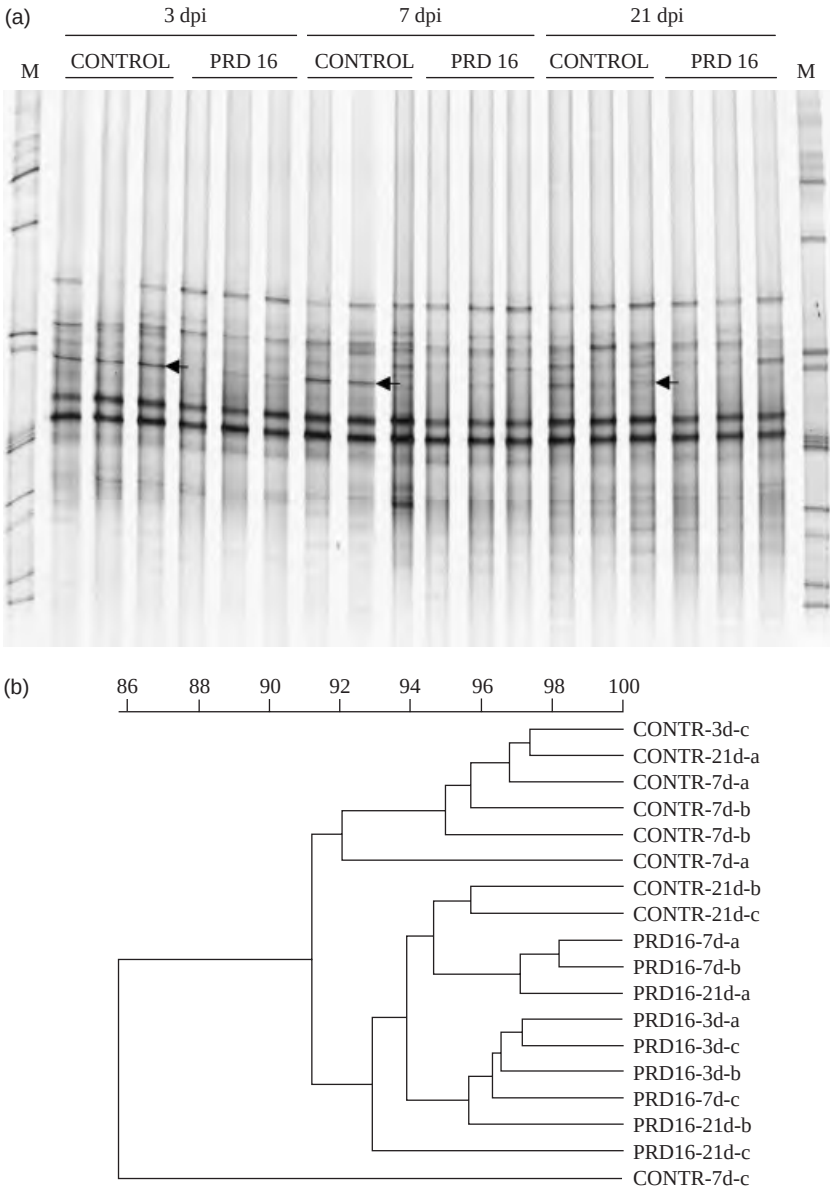

Fig. 3. (a) Comparison of *Betaproteobacterial* community fingerprints from rhizosphere samples of control (noninoculated) plants and of tomato plants inoculated with *Pseudomonas putida* PRD16; a differentiating ribotype is marked by an arrow. M, bacterial marker (Heuer *et al.*, 2001). (b) UPGMA based on Pearson indices for the samples taken 3, 7 and 21 days post inoculation.

amplification did not work and thus the PCR products obtained after *Pseudomonas*-specific PCR from non-inoculated plants were combined, cloned and screened by PCR-DGGE. One clone co-migrating with band A was sent for sequencing. Its 16S rRNA gene sequence showed 99.8% similarity with *Pseudomonas taetrolens* (D84027) and *Pseudomonas lundensis* (AB021395).

In situ detection of *gfp*-labeled antagonists in the rhizosphere by CLSM

To obtain information on the localization of the antagonistic strains on the roots the colonization patterns of both inoculants were followed by CLSM. The strain PRF116 showed an intense fluorescence, but most cells of PRD16 exhibited only weak fluorescence and it was therefore more difficult to detect them using CLSM. Detection by CLSM was improved by storing the unfixed roots for 12 h at 4 °C to facilitate chromophore formation of GFP as suggested by Cody *et al.* (1993). This additional incubation time did not result in any cell growth (data not shown). In neither of the experiments did the roots of noninoculated controls show any fluorescent cells at any sampling time. The distribution of the labeled cells was very heterogeneous for both strains and both inoculation methods. In general, similar colonization patterns were observed for all three replicates per treatment and both strains.

After seed inoculation, PRD16 and PRF116 were most frequently detected on the primary root with decreasing

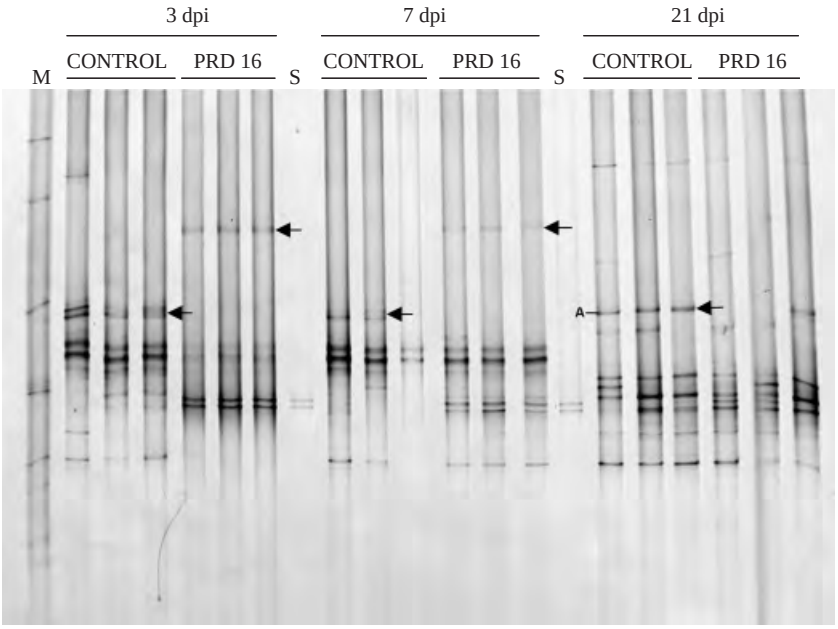

Fig. 4. Comparison of *Pseudomonas* community fingerprints from rhizosphere samples of control (noninoculated) plants and of tomato plants inoculated with *Pseudomonas putida* PRD16. Differentiating ribotypes are marked by arrows. S, 16S rRNA gene fragment amplified from PRD16; M, bacterial marker (Heuer *et al.*, 2001). Samples were taken 3, 7 and 21 days post inoculation (dpi).

density from the base (1 cm below ground) to the tip of the root. Only very few or no *gfp*-tagged cells were found on the above-ground stem base. PRD16 and PRF116 were also found on root hairs in both experiments (Fig. 5a). Some *gfp*-tagged cells of PRF116 and PRD16 were observed on soil particles at the first sampling time but, later on, only very few or no cells could be detected. The number of labeled cells of both inoculants on the root surface decreased in the course of the experiment. At each sampling time higher numbers of cells were observed for PRD16 than for PRF116, confirming the plate count results. Even on the primary root base only a few fluorescent cells could be detected for either strain 15 days after seed inoculation. At this sampling time the *gfp*-tagged cells were located predominantly along junctions between the rhizodermal cells (Fig. 5b). In general, the inoculant strains could not be detected on newly emerging side roots. Despite the CFU counts being still above 10^4 CFU g^{-1} root fresh weight for PRD16, no *gfp*-tagged cells were detectable on the roots of all three plants inspected at the last sampling time. In the case of PRF116, the CLSM data confirmed the plate counts, which were around the detection limit. Using DAPI as a counterstain, many indigenous bacteria were found, confirming that the preparation method was suitable and that bacteria were not washed away (data not shown).

After root inoculation of tomato seedlings, similar colonization patterns were found again for both antagonists as observed for seed inoculated plants. Three days after inoculation, high numbers of both strains were found on all parts of the root initially inoculated. Again, the distribution of labeled cells was very heterogeneous: closely colonized areas and weakly colonized regions were observed (Fig. 5c). On newly emerging side roots, only few or no antagonists were found (Fig. 5d). In the course of plant development the number of bacteria decreased, especially on the base of the primary roots. *Gfp*-tagged cells were found preferentially in deepenings, e.g. the borders of the root cells or damaged cells (Fig. 5e and f), where root exudates or nutrients were still available. Sporadically fluorescent cells of PRF116 were observed in the vascular bundles where PRD16 was never detected. At the end of the root inoculation experiment (21 dpi) only a few cells of PRF116 were found on the primary root; some more were located on the 'old' probably inoculated side roots, where microcolonies and dividing cells were found. *Gfp*-tagged cells of PRD16 were also found in the hairy zone and at the tip of the primary root and on probably inoculated side roots and sporadically on newly emerged side roots.

Fluorescent bacteria located in the root tissue or in the plant cells themselves indicating an endophytic growth were never observed for either strain independent of the inoculation method. The only exception was the sporadically observed occurrence of PRF116 in the vascular bundles for root-inoculated plants.

Discussion

The efficacy of biological control strategies has traditionally been monitored by the decrease of disease symptoms. It has often remained unclear why biological control measures sometimes work and in other cases fail. This inconsistency of biological control agents is assumed to be due to differences in the ability of inoculant strains to successfully colonize the rhizosphere of different plant species under

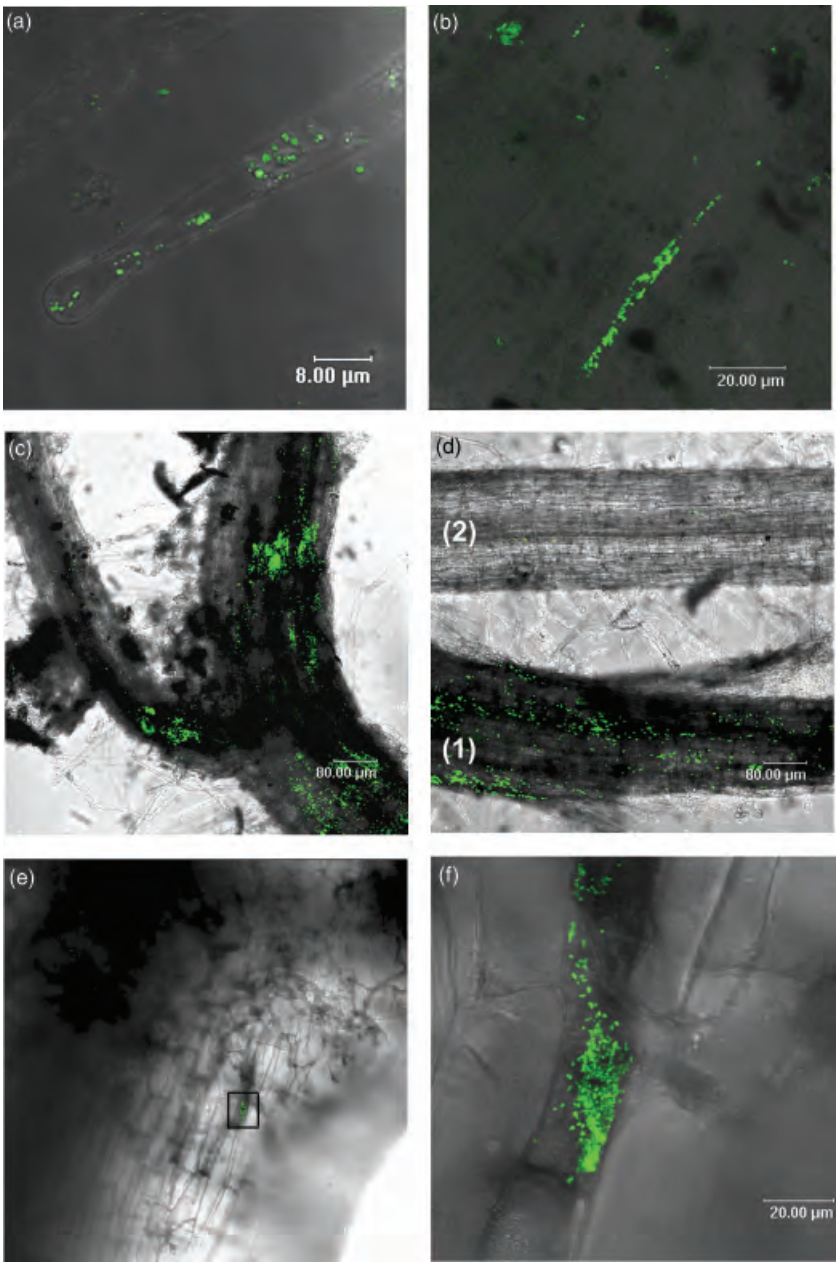

Fig. 5. Confocal laser scanning microscopy of tomato roots colonized with *gfp*-labeled antagonistic bacteria. Images are overlays of maximum intensity projections of the GFP-fluorescence (495–590 nm) and the transmitted light pictures in z-series. (a) Single bacterial cells of *Pseudomonas putida* PRD16 on root hairs of side roots 21 days after root inoculation. (b) Long-stretched microcolony of *Pseudomonas putida* PRD16 extending along the junctions of the rhizodermal cells on the primary root base. (c) Heterogeneous distribution of *Enterobacter cowanii* PRF116 on the primary root and an inoculated side root 3 days after root inoculation. (d) Strong colonization of an inoculated (1) and weak colonization of a newly emerged side root (2) with *Enterobacter cowanii* PRF116 7 days after root inoculation. (e) Weakly colonized base of primary roots with areas of high cell density of PRF116 13 days after root inoculation, and (f) Magnification of the square indicated in (e).

different conditions. Several studies demonstrated that rhizosphere competence is probably the crucial factor determining the success of biological control agents. However, the biotic and abiotic factors affecting rhizosphere competence of potential biocontrol strains are not well understood. Therefore, the development of reliable methods to monitor the fate of the inoculant strain in the rhizosphere is crucial for investigating how different factors influence the survival and colonization patterns of inoculant strains. Rhizosphere competence and the different factors affecting the establishment of inoculants can best be studied under well-defined greenhouse conditions which often fulfil the safety requirements needed to use genetically marked (e.g. *gfp*-tagged) strains. The methodological approach used in this study comprised selective cultivation, DGGE analysis of 16S rRNA gene fragments amplified from community DNA, and CLSM. The plate counts provided quantitative data on the survival of the inoculant strains. Higher CFU counts were observed for *P. putida* PRD16 at all sampling times and with both types of inoculation. Independently of the type of inoculation, the counts of both antagonists decreased during the time course. The decrease was, however, more pronounced for the enterobacterial strain, in particular for the seed inoculation experiment. To test whether the strong

expression of *gfp* in PRF116, which was advantageous for its microscopic detection, might have affected the survival ability of strain PRF116 pSM1890, competition experiments in nutrient broth were performed for the parental and the plasmid-carrying strains of PRD16 and PRF116. Interestingly, both strains (PRD16 and PRF116) showed a comparable fitness disadvantage for the plasmid-carrying strain. It would thus appear that PRD16 has a higher rhizosphere competence than PRF116. In this study the introduced *gfp*-tagged plasmids were mainly used to localize the strains in the rhizosphere and to introduce additional antibiotic resistance markers for the determination of the CFU counts by selective cultivation. Even supposing that the fitness disadvantage observed in nutrient broth studies might be less pronounced in the rhizosphere, *gfp*-labeled strains were planned to be used only to localize the strains at the plant root during greenhouse testing and not for field applications. Although we expected that the introduction of the small, low-copy number plasmid would affect the fitness less than a chromosomal insertion would, our results indicate that the generation of rifampicin-resistant mutants or *gfp*-labeling either chromosomally or by introduction of plasmids might impair the fitness of inoculant strains. Although the colonization ability of the antagonists in the rhizospheres of seed- and root-inoculated plants cannot be directly compared because sampling took place at different plant development stages, the CFU data showed that a better colonization was achieved after root inoculation for both PRD16 and PRF116. Air-drying of the seeds after inoculation with the antagonists and the time period until seed emergence occurred (3–5 days) might have been an additional stress factor. In other studies, biocontrol strains were inoculated on pregerminated seeds which might have facilitated the establishment of the inoculant strains compared to the strategy used here (Normander *et al.*, 1999; Lübeck *et al.*, 2000; Gamalero *et al.*, 2004). Due to environmental stress, the inoculant strains might have entered a state in which they are not readily accessible by plating on solid nutrient media. Gamalero *et al.* (2004) reported a remarkable difference between CFU counts and the direct counts determined by flow cytometry for a gnotobiotic system, suggesting that a considerable fraction of the inoculant was not culturable. Selective plating provided quantitative data on the culturable fraction of bacteria but not on their localization or on the composition of the total bacterial community. The rhizosphere competence of both PRD16 and PRF116 seemed to be lower than for other inoculant strains, e.g. *Pseudomonas fluorescens* SBW25 (Unge & Jansson, 2001). It can be speculated that the soil–sand mixture used might be one reason for the decline of the inoculant CFU, as in pre-experiments a better survival was observed for the same strains in potting soil without sand amendment (Peixoto *et al.*, 2004). Molecular fingerprinting methods such as the DGGE analysis of 16S rRNA gene fragments amplified from total community DNA offered the possibility to monitor shifts in the relative abundance of ribotypes in the tomato rhizosphere as a consequence of seed or root inoculation with antagonists. When the inoculant strains belong to the dominant ribotypes, bands with the same electrophoretic mobility as the inoculants should be detectable in the bacterial community patterns. Strains PRD16 (seed and root inoculation experiment) and PRF116 (root inoculation) were unequivocally detectable in the bacterial community patterns only at the first sampling time. At all further sampling times, the inoculant strains were detected as faint bands, or were not detected at all. Thus DGGE analysis confirmed the observation made by plate counts that the relative abundance of the inoculant strains decreased during the experiment. In comparison with the bacterial DGGE fingerprints, the complexity of the *Betaproteobacteria* or *Pseudomonas* patterns was reduced. The bacterial patterns revealed only transient changes in the relative abundance of certain ribotypes, but differences between the control and the inoculated plants became detectable when amplicons obtained with group-specific primers were analyzed. The *Pseudomonas* patterns revealed remarkable differences in the relative abundance of *Pseudomonas* ribotypes which would not have been detectable had only the bacterial patterns been analyzed. To our knowledge this is the first report on longer-lasting changes in the composition of *Pseudomonas* communities as a result of inoculation. DGGE analysis of PCR-amplified 16S rRNA gene fragments was also used by Lottmann *et al.* (2000) to follow the fate of inoculant strains in the rhizosphere of potato plants grown in the field and their effect on the indigenous bacterial community. In the study by Lottmann *et al.* (2000) neither of the inoculant strains could be detected in the bacterial community patterns, indicating that the inoculated strains did not belong to the numerically dominant strains.

Different methods to localize inoculant strains by microscopy *in situ* along the roots were developed in the 1990s. Combined with rapidly developing microscopy techniques, these methods have revolutionized our view of how bacterial inoculants colonize the root system (Hartmann *et al.*, 1998). *In situ* hybridization with oligonucleotides specific to the rRNA of the inoculant strain (Assmus *et al.*, 1995, 1997), fluorescently labeled specific antibodies (Schloter *et al.*, 1995, 1997) or marker and reporter genes (Jansson, 2003) were used and made *in situ* observations possible, in particular about spatio-temporal colonization patterns. However, in many studies these tools were applied in rather artificial and often gnotobiotic systems (Simons *et al.*, 1996; Gamalero *et al.*, 2004). To localize the inoculant strains along the roots of tomato plants grown in nonsterile soil up to 4 weeks, we used *gfp*-marked inoculant strains. The

differences in the brightness of GFP fluorescence between PRD16 and PRF116 were quite remarkable and made microscopic detection of PRD16 much more difficult. Unge & Jansson (2001) also reported problems in detecting fluorescent *P. fluorescens* SBW25::*gfp/lux* on wheat roots because of the high background fluorescence of the roots.

Although the potentials of CLSM were not fully exploited in this study, the observations made confirmed many of the colonization patterns reported by other authors for gnotobiotic and non-sterile systems (Chin-A-Woeng *et al*., 1997; Bolwerk *et al*., 2003; Gamalero *et al*., 2004). Overall, we found similar colonization patterns for both strains and in both experiments with *gfp*-labeled cells detected mainly on the primary root with decreasing densities from the root base to the root tip. At the later sampling times, *gfp*-tagged cells were found in string-like microcolonies predominantly in the deepenings of the root surface, e.g. junctions between the rhizodermal cells or associated with damaged cells, where more nutrients are supposed to be available. String-like microcolonies were also observed with other host plants under sterile and non-sterile conditions (Normander *et al*., 1999; Lübeck *et al*., 2000). *Gfp*-labeled cells were never found inside the roots or the plant cells except for the sporadically observed occurrence of PRF116 in the vascular bundles in the root inoculation experiment. However, it is most likely that bacteria entered the vascular bundles through lesions during the inoculation of the roots rather than exhibiting a true endophytic growth.

The heterogeneous colonization patterns observed by CLSM for *gfp*-tagged inoculants raised the question of the triggers stimulating the colonization of some sites but not of others. Are these triggers primarily nutrients, which differ in the different parts of the root or the absence of indigenous bacteria competing for the similar nutrient sources?

The polyphasic approach comprising cultivation-dependent and -independent methods enabled us to follow the spatio-temporal colonization patterns of the inoculant strains and to assess potential effects of the inoculants on the indigenous rhizosphere bacterial communities of greenhouse-grown tomato plants. This tool set will now be used to study the fate and effects of strain PRD16 after tomato root inoculation in the presence of the pathogen *R. solanacearum*. Although the CLSM data were important for future sampling strategies, the DGGE analysis showed that the resolution of bacterial patterns might be insufficient to detect potential effects of inoculants on the rhizosphere bacterial community, and thus the use of taxon-specific patterns is strongly recommended.

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