

Root Colonization by *Pseudomonas* sp. DSMZ 13134 and Impact on the Indigenous Rhizosphere Bacterial Community of Barley

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Received: 11 August 2009 / Accepted: 28 June 2010
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Abstract Over the last few decades, the ability of rhizosphere bacteria to promote plant growth has been considered to be of scientific, ecological, and economic interest. The properties and mechanisms of interaction of these root-colonizing bacteria have been extensively investigated, and plant protection agents that are based on these bacterial strains have been developed for agricultural applications. In the present study, the root colonization of barley by *Pseudomonas* sp. DSMZ 13134, that is contained in the commercially available plant protection agent Proradix[®], was examined using the fluorescence in situ

hybridization method with oligonucleotide probes and specific *gfp*-tagging of the inoculant strain in combination with confocal laser scanning microscopy. In the first phase of root colonization, the inoculant strain competed successfully with seed and soil-borne bacteria (including *Pseudomonads*) for the colonization of the rhizoplane. *Pseudomonas* sp. DSMZ 13134 could be detected in all parts of the roots, although it did not belong to the dominant members of the root-associated bacterial community. *Gfp*-tagged cells were localized particularly in the root hair zone, and high cell densities were apparent on the root hair surface. To investigate the impact of the application of Proradix[®] on the structure of the dominant root-associated bacterial community of barley, T-RFLP analyses were performed. Only a transient community effect was found until 3 weeks post-application.

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Introduction

The root colonization and plant growth promotion activities of bacteria of the genus *Pseudomonas* have been intensively studied for many years [7, 42]. During root colonization, fluorescent pseudomonads produce a variety of inhibitory substances. Both siderophores and antibacterial and antifungal secondary metabolites are considered to play a major role in the biological control of plant pathogens by pseudomonads [17, 26, 57]. Regarding this biocontrol activity, Haas and Defago [26] described three main modes of action: antibiosis, elicitation of induced systemic resistance and formation of specific pathogen-antagonist interactions. Therefore, plant growth-promoting rhizobacteria (PGPR) are considered to be highly relevant for modern agricultural applications, including biological plant protection and growth stimulation.

Plant protection agents containing rhizobacteria are increasingly used to ensure both high yields and superior food quality. The first inoculations of plants with beneficial bacteria can be dated back to the end of the 19th century [28]. Scientists have continued to search for plant growth-stimulating bacteria and applicable microbial antagonists of phytopathogenic microorganisms. In this field, the experiments with *Azospirillum* [4, 48], *Pseudomonas fluorescens*, or *Pseudomonas putida* strains [53, 61] are widely considered to be research milestones. Nowadays, there are several global, commercially available biological plant growth stimulants that contain different strains of e.g. *Bacillus*, *Azospirillum*, and *Pseudomonas*. In Germany, agents such as “FZB24®” (*Bacillus subtilis*), RhizoVital® 42 (*Bacillus amyloliquefaciens*), and “Proradix®” have been available for use in agriculture since 1997.

Proradix® is a plant protection agent that contains the naturally occurring *Pseudomonas* sp. strain DSMZ 13134 as its biologically active component. According to the information of the manufacturer (Sourcon-Padena GmbH & Co. KG, Tübingen, Germany), this strain colonizes the surface of plant roots and acts possibly by stimulating the plant’s systemic defense mechanisms, by plant growth-promoting effects that are due to enhanced root development and by displacement of soil-borne pathogens by nutrient and habitat competition. Proradix® application has been suggested for the cultivation of potatoes, vegetables (for example carrots, salad, radish, and tomato), grass, and lupin. However, there have been no investigations yet into the application of *Pseudomonas* sp. DSMZ 13134 for the growth stimulation of cereals and into the colonization of barley roots. We could demonstrate that Proradix® application to barley plants resulted in growth stimulation under both greenhouse and field conditions (Fröhlich et al., in preparation). The successful and efficient establishment of introduced microorganisms in the rhizosphere plays a central role for the use of PGPR within the agricultural practice [6, 8]. High root colonization competence depends on the ability of a bacterium to colonize roots and to compete with the indigenous rhizosphere microbiota. Therefore, the aim of this study was to investigate the colonization behavior of *Pseudomonas* sp. DSMZ 13134 following its application to barley plants. Experiments were carried out both in the laboratory and in the greenhouse. Detection of the strain on the root surface was performed using the fluorescence in situ hybridization (FISH) method with fluorescently labeled oligonucleotide probes in combination with confocal laser scanning microscopy (CLSM). To further facilitate detection, barley was also inoculated with the *gfp* (green fluorescent protein)-tagged *Pseudomonas* sp. DSMZ 13134 strain. Furthermore, the question arose to what extent the introduced strain influences the composition of the root-associated bacterial community. To answer this

question, terminal restriction fragment length polymorphism (T-RFLP)-analysis was applied and clone libraries were constructed in order to identify T-RFLP results.

Methods

Bacterial Strains and Culture Conditions

Pseudomonas sp. DSMZ 13134 was provided by the Proradix® producing company Sourcon-Padena GmbH & Co. KG. This strain was cultivated at 30°C in King’s B medium [35] adjusted to a pH of 7.2. The *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 strain harboring plasmid pJBA28 [3] was grown in King’s B medium that contained 50 µg/ml kanamycin.

Plant Material, Plant Growth Conditions, Surface Sterilization of Seeds and Inoculation

The summer barley cultivar Barke (seed breeding Josef Breun GdbR, Herzogenaurach, Germany) was used in this study. In laboratory experiments, barley plants were cultivated in sealed glass tubes (30 mm diameter, Schott AG, Mainz, Germany) filled with 70 g of washed and autoclaved quartz sand with a particle size of 1.0 to 2.5 mm in diameter and 10 ml of sterile Murashige & Skoog medium [46]. Inoculated barley seedlings were introduced into the quartz sand and grown for up to 4 weeks and to a length of about 30 cm.

For greenhouse experiments, loamy soil with a pH of 6.3 from an agricultural field in Scheyern, Germany, was sieved (<10 mm) and added to pots. Three plants were included in each 2.5 l pot. The plants were grown for a period of 10 weeks under greenhouse conditions, with temperatures around 16°C during the day and 12°C at night, a photoperiod of 12 h and 50% to 70% humidity (for T-RFLP analysis). For colonization studies, single plants were cultivated in 400 cm³ pots. The plants were watered twice a week with deionized H₂O.

To cultivate barley axenically, seeds were surface sterilized with 70% ethanol for 2 min, incubated in 2% sodium hypochlorite solution (6–14% Cl active; Riedel-de-Haën, Seelze, Germany) for 15 min and washed six times with sterile deionized water. Subsequently, seeds were incubated in a solution that contained 600 µg/ml penicillin and 250 µg/ml streptomycin for 30 min. After incubating the seeds for 2 days at 30°C in the dark on NB plates, seedlings without visible contamination were chosen for inoculation.

Inoculation of seeds with *Pseudomonas* sp. DSMZ 13134 was performed using vacuum infiltration with Proradix® by the Proradix® producing company. A

suspension containing 8×10^{10} CFU/kg seeds was sprayed onto unsterile seeds, and the vacuum was applied. Thereafter, the seeds were dried at 27°C for 4 h. Alternatively, sterile and unsterile seedlings were inoculated with *Pseudomonas* sp. DSMZ 13134 cells. An overnight culture was washed twice with 1×PBS and diluted to a concentration of 10^8 cells per ml 1×PBS. Unsterile seeds were germinated on water agar plates at 30°C in the dark for 2 days. Seedlings with a root length of 1 to 2 cm were incubated in the bacterial cell suspension at room temperature for 2 h. Control seedlings were incubated in 1×PBS for the same length of time.

Root Sampling and Preparation

Between 2 and 4 weeks after planting, whole plants were taken out of the glass tubes. Barley plants that were grown in agricultural soil in pots were harvested at the following sampling time points: 1, 2, 3, 4, 6, 8, and 10 weeks after planting. Roots were carefully shaken to remove adhering soil or quartz sand particles, gently washed in sterile 1×PBS in Petri dishes, separated from the shoot, and then briefly dried on sterilized paper tissue. Samples were used for immediate microscopy of *gfp*-tagged cells, for paraformaldehyde fixation, or were frozen in liquid nitrogen for nucleic acid extraction (see below).

Fluorescence In Situ Hybridization and Confocal Laser Scanning Microscopy

The FISH-method was used to detect *Pseudomonas* sp. DSMZ 13134 cells in the rhizoplane of barley plants. After 2 weeks growth in quartz sand or agricultural soil under unsterile conditions, the plants were harvested and the roots were sampled as described above. Whole root systems were fixed with 4% paraformaldehyde and 1×PBS (3:1) at 4°C for 2 h. The roots were then washed twice with 1×PBS and placed in an ethanol/1×PBS mixture (1:1) for longer storage at −20°C.

For hybridization, 2-cm root pieces were removed from all parts of the root. Hybridization was performed according to the protocols of Manz et al. [43] and Amann et al. [2]; specifically, 2 ml reaction vials with 120 µl hybridization buffer that contained a formamide concentration of 35% and 15 µl of the respective oligonucleotide probe were used. After the washing step, the root pieces were transferred to glass slides, air-dried, embedded in Citifluor (Citifluor Ltd., Canterbury, UK) and then covered with a cover slip. The following 16S rRNA-targeted oligonucleotide probes were used: an equimolar mixture of EUB-338-I [1], EUB-338-II, and EUB-338-III [13] to detect all microorganisms of the domain *Bacteria*, and PSE-225 (5'-CCG ACCTAGGCTCATCTA-3') for the specific identification of *Pseudomonas* cells. Probes were synthesized and labeled

with the fluorescent dyes Cy3 and Fluos by Thermo Electron GmbH (Ulm, Germany).

Root colonization was followed using a confocal laser scanning microscope LSM-510-Meta (Zeiss, Oberkochen, Germany) that was equipped with two helium neon lasers for excitation of the fluorophores Cy3 and Cy5 at wavelengths of 543 and 633 nm, and an argon laser for excitation of Fluos at a wavelength of 488 nm. Bacterial cells were localized with a 63× water immersion objective. Cy3- and Fluos-labeled oligonucleotide probes provided red and green fluorescent signals, respectively. The combination of EUB-338-mix-Fluos and PSE-225-Cy3 resulted in a yellow color. After binding of both probes to *Pseudomonas* sp. DSMZ 13134, these bacteria could be identified as yellow fluorescent cells. Images were taken and processed using LSM Image Browser software version 2.80 (Zeiss).

Detection of *gfp*-tagged Cells

Barley roots inoculated with the *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 strain were harvested as described above. After sampling, pieces from the washed roots were immediately placed on glass slides and embedded in Citifluor. *Gfp*-tagged cells were visualized using the confocal laser scanning microscope with the same three lasers, and with *Gfp* fluorescing at an excitation wavelength of 488 nm.

DNA Extraction, PCR Amplification, and T-RFLP Analysis

To extract the total community DNA of the bacterial fraction from the root surface, the washed roots were frozen in liquid nitrogen and pulverized using a mortar and pestle. Three roots were pooled for each treatment and each sampling time. Subsequently, 500 mg root material was transferred into 2 ml screw-cap vials that contained 1 g of a mixture of ceramic and silica particles. For each sample, three separate DNA extractions were performed using the Fast DNA® SPIN Kit for Soil (MP Biomedicals, Eschwege, Germany) according to the manufacturer's instructions. After measurement of the DNA concentration using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, USA), the DNA extracts were stored at −20°C.

T-RFLP analysis of bacterial communities was done as previously described [41]. Partial 16S rRNA genes were amplified using the primers 27f-FAM [18] and 907r [38]. The reaction mixtures (50 µl) contained 10 ng of extracted DNA as a template, 5 µl tenfold reaction buffer (Fermentas GmbH, St. Leon-Rot, Germany), 3 µl DMSO, 200 µM of each deoxynucleoside triphosphate, 3 mM MgCl₂, 25 pmol of each primer and 0.5 U of *Taq* DNA-polymerase (recombinant, 1 U/µl; Fermentas GmbH). Amplifications included the following reactions: initial denaturation at 95°C for 5 min, 23 cycles of denaturation at 94°C for 1 min,

annealing at 53°C for 30 s, elongation at 72°C for 2 min and a final extension at 72°C for 10 min. Polymerase chain reaction (PCR) products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) as recommended by the manufacturer. The concentrations of DNA fragments were determined using the NanoDrop spectrophotometer. For MspI digestion, reaction mixtures that contained 50 ng DNA in a 10 µl volume were incubated at 37°C for 2 h. The electrophoretic separation of desalted digests was performed on an automated DNA sequencer (ABI 3730, Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) and the lengths of terminal restriction fragments (T-RFs) were determined using GeneMapper® v3.5 software (Applied Biosystems). Signals with a peak height of more than 100 fluorescence units were included in further analyses [49].

T-RFLP analyses were performed for each triplicate DNA extract, and the peak heights of each of the three separate T-RFLP analyses were recorded for every rhizoplane sample. T-RFs with variations in fragment sizes over a range of ±1–2 bp were considered to be identical [24]. The relative abundance of each T-RF was calculated as the respective signal height of this fragment divided by the total peak height of all fragments of the whole T-RFLP profile. Ratios were converted to percentages. Fragments that were either smaller than 50 bp or that had a relative abundance below 0.25%, and fragments that were derived from chloroplast and mitochondrial rRNA genes [24, 56], were excluded from the analyses. To compare T-RFLP analyses, a data matrix containing the fragment lengths and the appropriate relative abundances was created, and principal component analysis (PCA) was performed using CANOCO 4.5 software (Microcomputer Power, Inc., Ithaca, NY, USA).

Construction and Analyses of 16S rDNA Clone Libraries

To identify T-RFs, four clone libraries of the root-associated bacterial communities of soil-grown barley plants 2 and 4 weeks after planting were constructed. Amplification was performed using 50 pmol of each primer of the primer pair 616f (*Escherichia coli* 16S rDNA positions 8 to 27) [33] and 1492r (*E. coli* 16S rDNA positions 1492 to 1513) [37] and with the conditions described above resulted in full-length PCR products of about 1,500 bp. PCRs were carried out with 20, 24, and 28 cycles for each rhizoplane sample, and these PCR products were pooled equally after purification. The PCR products were eluted from agarose gels using the NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany). Amplicons were cloned using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) that contained chemocompetent *E. coli* TOP10 cells and pCR® 2.1-TOPO as vector, according to the manufacturer's instructions. Positive clones were selected and checked for

correct insert size by colony PCR using the primers 616f and M13r. Plasmids were isolated using the NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany) and quantified with the help of the NanoDrop spectrophotometer. Thereafter, PCR-RFLP analysis of the 16S-rDNA inserts was performed with the restriction endonucleases MspI and RsaI (Fermentas GmbH). Restriction fragments were analyzed in 3.5% (wt/vol) agarose gels (PeqLab). Sequencing of both strands was performed using the BigDye Terminator (BDT) v3.1 sequencing kit (Applied Biosystems) on an ABI 3730 sequencer (Applied Biosystems). To obtain complete sequences, different primers were used: M13f, M13r, 609f, 616f, 612r, 907r.

Sequence data were assembled and verified using Lasergene® software (DNASTAR, Madison, WI, USA). The 16S rRNA gene sequences were checked by the chimera test program Bellerophon (<http://foo.maths.uq.edu.au/~huber/bellerophon.pl>) [31] and suspected chimeric sequences were excluded from the analysis. Phylogenetic analysis was performed with the ARB software package (<http://www.arb-home.de>) [40]. Sequences were imported into an existing database of small-subunit rRNA gene sequences using the fast alignment tool of the ARB software. Alignments were checked and corrected manually by comparing them to homologous positions of related sequences. Construction of phylogenetic trees was performed by applying maximum likelihood [19], maximum parsimony [20] and neighbor joining [51] methods that were implemented in the ARB software package.

The calculation of the relative phyla abundances in each of the four clone libraries was performed basing on results of the PCR-RFLP analysis using the restriction endonuclease MspI. For every bacterial phylum, the number of distinct RFLP patterns was divided by the total number of RFLP patterns of all phyla within the clone library. Statistical analysis was done using the R-package vegan (vegdist). A permutational multivariate analysis of variance using distance matrices—ADONIS—was performed to test the significance of differences between the clone libraries related to distinct sample time points and treatments. This analysis uses a permutation test with pseudo-F ratios. The distance matrix was generated from Bray–Curtis' index. The number of permutation selected was 1,000. When $P < 0.05$, differences between clone libraries are denoted significant.

Results

In situ Detection of *Pseudomonas* spp. Colonizing Barley Roots Using FISH-analysis

To visualize *Pseudomonas* spp. in the rhizoplane of barley, fluorescence in situ hybridization was carried out using the

oligonucleotide probe PSE-225 that detects several *Pseudomonas* spp. (Table 1). On the root surface of seedlings that were treated with Proradix® by vacuum infiltration, *Pseudomonas* cells were found in great numbers colonizing first of all the root hair zones of plants that were grown in the quartz sand system (Fig. 1a). However, besides other bacteria, Pseudomonads could also be visualized in the rhizoplane of unsterile control plants that had been denied Proradix® treatment (Fig. 1b). Thus, Pseudomonads originating from the seeds effectively colonized the roots of germinating seeds. Therefore, a germ-free axenic (monoxenic) system with barley (Fig. 1c) was used in order to examine the colonization behavior of *Pseudomonas* sp. DSMZ 13134 specifically. After inoculation of surface sterilized barley seeds with *Pseudomonas* sp. DSMZ 13134, root hairs were heavily colonized with this bacterial

strain (Fig. 1d). In soil-grown plants, bacterial cells could barely be localized on the roots using the FISH-protocol, because of the strong autofluorescence of root adhering soil particles.

Localization of *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 Cells

To obtain detailed information on the ability of *Pseudomonas* sp. DSMZ 13134 to efficiently colonize barley roots in the presence of other bacteria, germinated seeds were inoculated with *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 cells. The application of the *gfp*-tagged strain enabled the visualization of the bacterial cells on intact roots immediately after sampling; thus, the physical and chemical influences that occurred during paraformaldehyde fixation and FISH could

Table 1 Sequence comparison between the sequence of the oligonucleotide probe PSE-225 and available target regions

Oligonucleotide probe PSE-225 Sequence of the probe	5'-CCGACCTAGGCTCATCTA-3'
Sequence of the target site	5'-UAGAUGAGCCUAGGUCGG-3'
<i>Pseudomonas</i> sp. DSMZ 13134	UCACGCUAU-----AUUAGCUAG
<i>Pseudomonas fluorescens</i>	UCACGCUAU-----AUUAGCUAG
<i>Pseudomonas oryzae</i>	UCACGCUAU-----AUUAGCUAG
<i>Agrobacterium agile</i>	UCGCGCCAU-----AUUAGCUAG
<i>Pseudomonas stutzeri</i>	UUGCGCUAA-----AUUAGCUAG
<i>Pseudomonas flavescens</i>	UUGCGCUAA-----AUUAGCUAG
<i>Azomonas insignis</i>	UUGCGCUAA-----AUUAGCUAG
<i>Pseudomonas psychrotolerans</i>	UCACGCUAU-----AUUAGCUAG
<i>Pseudomonas pseudoalcaligenes</i>	UCGCGCCAU-----AUUAGCUAG
<i>Pseudomonas guinea</i>	UCGCGCCAU-----AUUAGCUAG
<i>Pseudomonas oleovorans</i>	UCACGCUAU-----AUUAGCUAG
<i>Pseudomonas anguilliseptica</i>	UCGCGCCAU-----AUUAGCUAG
<i>Pseudomonas aeruginosa</i>	UCACGCUAU-----AUUAGCUAG
<i>Pseudomonas mendocina</i>	UCGCGCCAU-----AUUAGCUAG
<i>Pseudomonas argentinensis</i>	UUGCGCUAA-----AUUAGCUAG
<i>Pseudomonas straminea</i>	UUGCGCUAA-----AUUAGCUAG
<i>Pseudomonas putida</i> F1	UUGCGCUAU-----AUUAGCUAG
<i>Pseudomonas putida</i>	UUGCGCUAU-C-----AUUAGCUAG
<i>Pseudomonas aeruginosa</i>	UCACGCUAU-C-----AUUAGCUAG
<i>Pseudomonas monteilii</i>	UUGCGCUAU-C-----AUUAGCUAG
<i>Pseudomonas poae</i>	UUGCGCUAU-C-----AUUAGCUAG
<i>Pseudomonas</i> sp. L54	UUGCGCUAU-C=N-----AUUAGCUAG
<i>Pseudomonas aeruginosa</i>	UCACGCUAU-C=N-----AUUAGCUAG
<i>Pseudomonas</i> sp. K94.23	UUGCGCUAU-C=N-----AUUAGCUAG
<i>Pseudomonas</i> sp. S3-24	UUGCGCUAU-C=N-----AUUAGCUAG
<i>Flavobacterium lutescens</i>	UCACGCUAU-CN-----AUUAGCUAG
<i>Pseudomonas syringae</i>	UUGCGCUAU-C=N-----AUUAGCUAG
<i>Pseudomonas entomophila</i>	UUGCGCUAU-C=N-----AUUAGCUAG
<i>Pseudomonas</i> sp. C9B1	UCACGCUAU-g-g-----AUUAGCUAG
<i>Azotobacter chroococcum</i>	UCACGCUAU-Cg-----AUUAGCUAG

Bacterial strains showing up to two mismatches within their target region are listed. The calculation was performed with the tool “probe match” of the software package ARB [40]

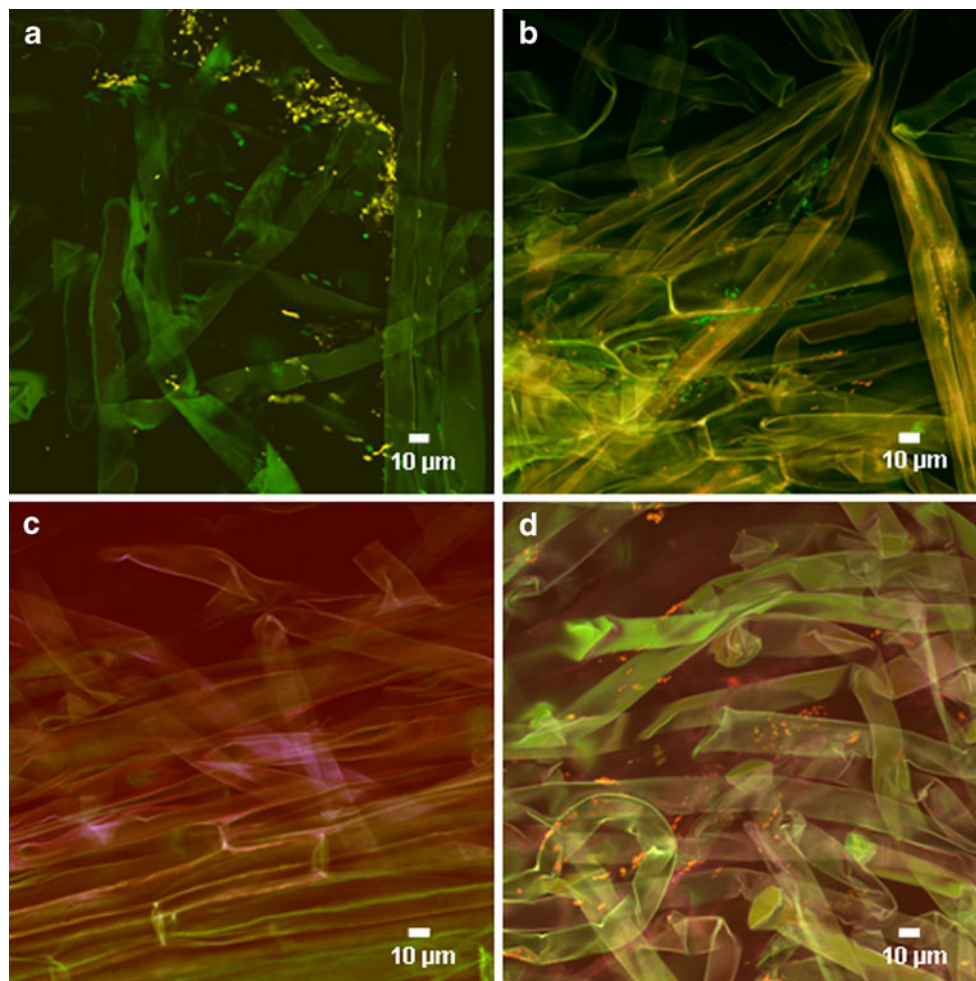


Figure 1 In situ detection of *Pseudomonas* spp. on the surface of barley roots using FISH in combination with CLSM. FISH-analysis was performed using the oligonucleotide probes EUB-338-mix labeled with Fluos and PSE-225 labeled with Cy3. Roots were harvested after 2 weeks of growth. All images were taken from the root hair zone. **a** Barley was grown in a quartz sand system. Unsterile seeds were treated with Proradix[®] by vacuum infiltration. *Pseudomonas* cells appeared in yellow, and other bacteria were visible with green fluorescent signals. **b** Plants were grown in the quartz sand

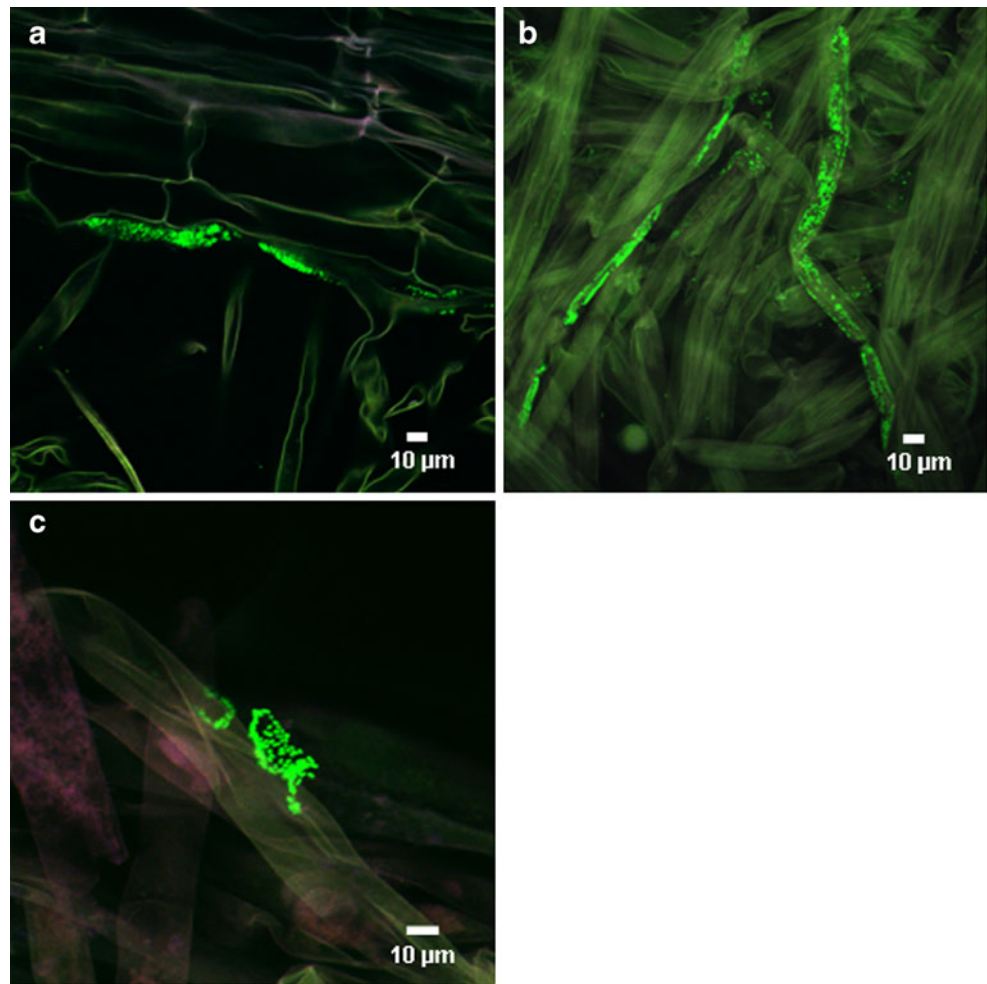
system. Seeds were not surface sterilized and seedlings were not inoculated. *Pseudomonas* cells were detectable in orange, and other bacteria appeared with green fluorescent signals. **c** Plants were grown in a germ-free axenic system without *Pseudomonas* sp. DSMZ 13134 inoculation. After FISH-analysis, no bacterial cells were visible in this sterile system. **d** Plants were grown in an axenic system. Surface sterilized 2-day-old seedlings were inoculated with a *Pseudomonas* sp. DSMZ 13134 bacterial suspension (10^8 cells/ml). *Pseudomonas* sp. DSMZ 13134 cells appeared in red

be avoided. The surface of axenically grown barley seedlings was efficiently colonized by *Pseudomonas* sp. DSMZ 13134 (images not shown). For detailed analysis of roots that were grown in quartz sand and in soil under unsterile conditions, samples were analyzed by confocal laser scanning microscopy at time points of 1, 2, 3, and 4 weeks after planting. In many different root preparations, *gfp*-tagged cells could be detected on the root surface and in all parts of the roots that were grown in the quartz sand system. *Pseudomonas* sp. DSMZ 13134 cells were not evenly distributed along the root; instead, they were found as microcolonies located either at the surface of the root (Fig. 2a) or on root hairs (Fig. 2b). Strong colonization could mostly be observed in older basal

root parts and in the root hair zone, although colonization was also evident at the root tip in some samples.

Due to the strong *gfp*-fluorescence, *Pseudomonas* sp. DSMZ 13134 cells could clearly also be localized on the root surface, and in particular in the root hair zone of plants that were grown in agricultural soil (Fig. 2c). Even 4 weeks after sowing, *gfp*-marked cells could still be found; however, the number of detectable cells decreased after 3 weeks. There was a considerably lower degree of root colonization by *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 cells in the soil system than in the quartz sand system; this was due to the larger number of indigenous competitive root-colonizing bacteria.

Figure 2 In situ detection of *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 cells on the surface of barley roots. Two-day-old seedlings were inoculated with a *Pseudomonas* sp. DSMZ 13134 bacterial suspension (10^8 cells/ml). Plants were grown in quartz sand (**a**, **b**) or in agricultural soil (**c**). Roots were harvested after 2 (**c**) or 3 (**a**, **b**) weeks of growth. All images were taken from the root hair zone. **a** Numerous *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 cells could be localized on the surface of epidermal cells. **b** *Gfp*-tagged *Pseudomonas* sp. DSMZ 13134 colonized the surfaces of root hairs of a young root part at a high cell density. **c** Image of a microcolony consisting of *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 cells attached to the surface of a root hair



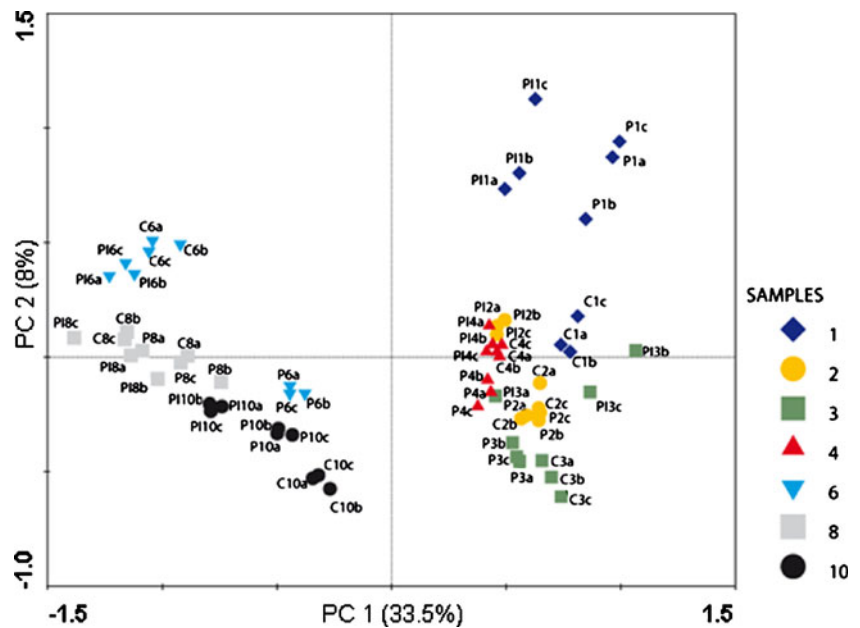
T-RFLP Analysis of the Whole Root-Associated Bacterial Community after Inoculation with *Pseudomonas* sp. DSMZ 13134

T-RFLP analysis of 16S rRNA gene fragments was used to examine the impact on the indigenous bacterial community of the barley rhizosphere as a result of inoculation with *Pseudomonas* sp. DSMZ 13134. For this purpose, three plants were grown per 2.5 l pot in agricultural soil (Scheyern) in the greenhouse. Untreated control plants (C) and two different Proradix[®] treatments were compared with each other. Either Proradix[®] was applied by vacuum infiltration alone (P), or vacuum-treated seeds were further inoculated with *Pseudomonas* sp. DSMZ 13134 at a rate of 10^8 cells per ml inoculum solution (PI). Barley roots were sampled at time points of 1, 2, 3, 4, 6, 8, 10 weeks after planting. Three separate T-RFLP analyses were carried out for each sample.

Based on T-RFLP analyses, differences between the control plants and plants subjected to the Proradix[®] treatments were not apparent at first sight. To reveal and

interpret the information contained in these T-RFLP results, a PCA was performed (Fig. 3). The root-associated bacterial community of both types of Proradix[®] treatments (P, PI) was separated from the control at the first sampling time (after 1 week). After 2 weeks of growth, the composition of the community did not markedly differ between the control and the Proradix[®]-treated plants (P). However, differences between the control (C) and additional inoculation (PI) could be observed during the first 3 weeks (Fig. 3). Furthermore, because the T-RFLP patterns of the earlier sampling points after 1, 2, 3, and 4 weeks grouped together (see right side of Fig. 3), a clear general shift of the community patterns with time could be detected. In contrast, the remaining patterns formed a group that is clearly distinct. To be able to detect the *Pseudomonas* sp. DSMZ 13134-specific fragment in the rhizosphere, a T-RFLP analysis of a *Pseudomonas* sp. DSMZ 13134 culture was performed and the respective T-RF with a length of 143 bp was determined. This fragment could only be found in the barley rhizosphere of the PI-treatment at the first sampling date.

Figure 3 Principal component analysis of T-RFLP profiles of 16S rRNA genes that were derived from the total root-associated bacterial community. Barley was grown in agricultural soil in the greenhouse and roots were sampled 1, 2, 3, 4, 6, 8, and 10 weeks after planting. The colored symbols were named with the appropriate treatment (C control, P Proradix® treatment by vacuum infiltration, PI vacuum infiltration+ inoculation with *Pseudomonas* sp. DSMZ 13134) together with the number indicating the sampling time and a, b, c marking the three separate replicates. Different symbols and colors were chosen for every sampling week shown in the legend



Identification of Root-Associated Bacteria in Barley

To identify members of the rhizobacterial community of barley, four clone libraries were generated from the washed roots. Full-length 16S rRNA gene sequences were obtained from root-associated bacteria of barley plants at 2 weeks after planting, at 4 weeks after planting, from those that were treated with Proradix® by vacuum infiltration, and from those that were denied Proradix® treatment. A total of 150 clones from each clone bank were verified for the correct insert size after cloning, and a total of 466 clones were analyzed by RFLP analysis. One clone was chosen from each RFLP-group with identical restriction patterns for sequencing. Finally, 152 sequences were phylogenetically analyzed after the exclusion of chimeric sequences and sequences that were derived from chloroplast or mitochondrial rRNA genes. Sequences showed similarity to both known and unknown bacteria that belonged to: α -Proteobacteria (19 clones), β -Proteobacteria (43), γ -Proteobacteria (14), δ -Proteobacteria (14), Acidobacteria (3), Actinobacteria (10), Bacteroidetes (23), Chloroflexi-Thermomicrobia (7), Firmicutes (6), Gemmatimonadetes (1), Planctomycetes (1), OTU Spam (1), Spirochaetes (2), and Verrucomicrobiae (8) (Fig. 4). Comparisons with the National Center for Biotechnology Information database by BLAST searches revealed that 77 sequences were related to uncultured bacteria.

The relative phyla abundances in each of the four clone libraries (C2, P2, C4, and P4) are shown in Table 2. The result of ADONIS showed that there was no significant difference between the clone libraries regarding the Proradix® treatment (C2/C4 versus P2/P4; $P=0.177$) and regarding the sample

time point (C2/P2 versus C4/P4; $P=0.69$). This is in line with the results obtained with T-RFLP-analysis for the time points 2 and 4 weeks after inoculation.

Two clones with a high relatedness to known *Pseudomonas* sequences and similarity values of 99.7% and 99.3% were identified within the clone libraries of Proradix® treated plants. These two clones (P2III14, P4III8) exhibited sequence similarities of 97.5% and 96.4%, respectively with the 16S rRNA gene sequence of *Pseudomonas* sp.

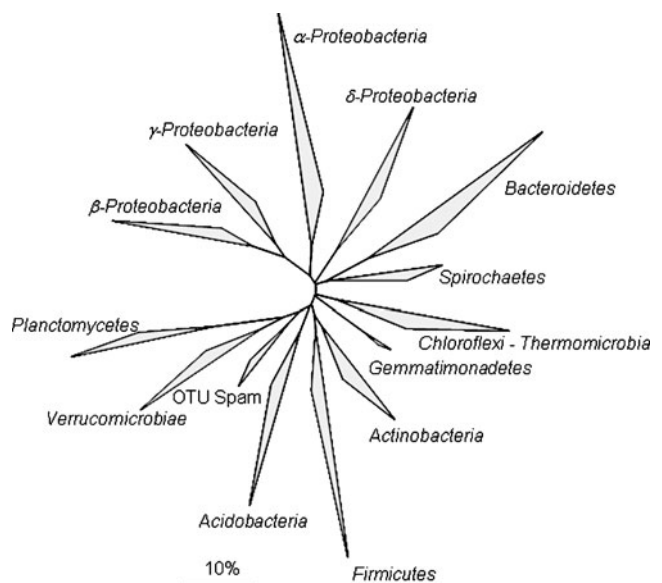


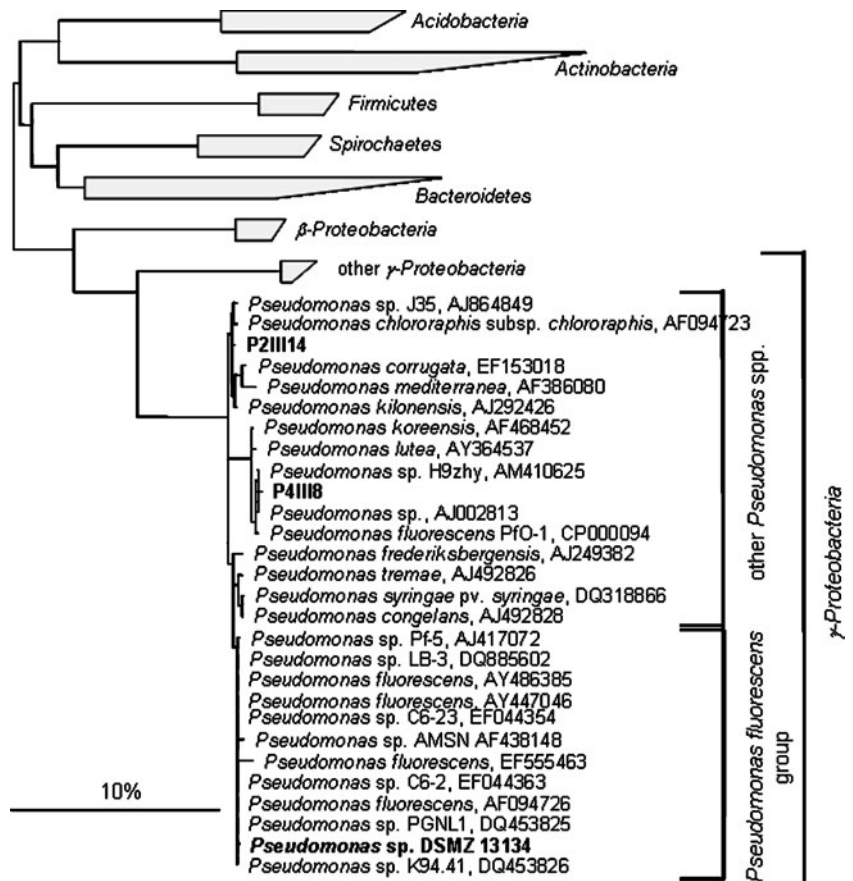
Figure 4 Radial 16S rRNA-based phylogenetic tree showing all major lineages of the Bacteria, which include sequences from the four clone libraries that were analyzed in this study. The length bar indicates 10% sequence difference

Table 2 Relative phyla abundances in% of the four distinct clone libraries C2, P2, C4, and P4

	C2	P2	C4	P4
<i>Acidobacteria</i>	0	1.12	0	3.06
<i>Actinobacteria</i>	2.67	4.49	5.06	4.08
<i>Alphaproteobacteria</i>	10.67	12.36	11.39	13.27
<i>Bacteroidetes</i>	12.00	11.24	12.66	15.31
<i>Betaproteobacteria</i>	41.33	34.83	41.77	36.73
<i>Chloroflexi</i>	6.67	2.25	1.27	4.08
<i>Deltaproteobacteria</i>	6.67	8.99	10.13	6.12
<i>Firmicutes</i>	1.33	3.37	2.53	2.04
<i>Gammaproteobacteria</i>	10.67	12.36	11.39	9.18
<i>Gemmatimonadetes</i>	1.33	0	0	0
OTU Spam	1.33	0	0	0
<i>Planctomycetes</i>	0	1.12	0	1.02
<i>Spirochaetes</i>	0	1.12	0	1.02
<i>Thermomicrobia</i>	1.33	1.12	1.27	1.02
<i>Verrucomicrobiae</i>	4.00	5.62	2.53	3.06

DSMZ 13134 (Fig. 5). The sequence of *Pseudomonas* sp. DSMZ 13134 could not be found in the clone libraries. One possible reason could be the incomplete coverage of the diversity of the clone libraries.

Figure 5 Phylogenetic tree showing relatedness of *Pseudomonas* sp. DSMZ 13134 and two *Pseudomonas*-related clones that appeared in the clone libraries of Proradix® treated plants at 2 and 4 weeks after planting (designated with P2 and P4) followed by internal numbers in the clone library. The bar represents 10% estimated sequence divergence



Discussion

Efficient root colonization and rhizosphere competence are crucial factors in determining the beneficial effects and the success of inoculations with plant growth-promoting rhizobacteria. Root colonization has been most intensively studied in *Pseudomonas* [42]. In experiments with *P. fluorescens* WCS365, a robust colonization was found to occur near to the root base with decreasing cell densities towards the root apex [11]. Microcolonies could mostly be visualized in the junctions between epidermal cells and in the grooves of the epidermal surface. Similar observations were made by Gamalero et al. [21] based on investigations with *P. fluorescens* A6RI. In agreement with these published results, *Pseudomonas* sp. DSMZ 13134 cells could be localized on the root surface, in all parts of the root from the root base up to the tip. However, there was particularly strong colonization of this strain in the root hair zone, and high cell densities could be detected on the surface of root hairs. Probably due to variations in root exudation at different places within the root system [55], cell numbers of introduced bacteria are known to vary along the axis of a root [62]. In keeping with these results, *Pseudomonas* sp. DSMZ 13134 cells were also found to be unevenly distributed along the root. In addition, similar to

many previous studies with root-colonizing *P. fluorescens* strains [9–11, 15, 21], we could identify the formation of microcolonies. Regarding the rhizosphere competence, the root colonization is successful if the introduced bacteria are able to spread along the growing root, and if they can propagate and survive in the presence of competitive indigenous rhizosphere microorganisms [42, 62]. For *Pseudomonas* sp. DSMZ 13134, this ability could be demonstrated in experiments that used either quartz sand or agricultural soil.

Pseudomonas spp. and other bacteria originating from the seeds establish at the surface of the roots during germination and seedling growth (Fig. 1a, b). The inoculation with *Pseudomonas* sp. DSMZ 13134 results in additional colonization of the root surface as part of the *Pseudomonas* community. Dependent on the density of inoculation, the inoculated bacteria could be detected short or some extended time on the root surface using T-RFLP-community analysis, before they were replaced by other bacteria (Fig. 3).

Lugtenberg et al. [42] emphasized the advantages of a quartz sand system over the use of a soil system. Competition due to a large number of indigenous bacteria is high in field soil and the influencing factors are much more complex. The cell numbers of *P. fluorescens* WCS365 decreased tenfold when quartz sand was replaced by potting soil [42]. These experiences could be confirmed in the present work because the population density of *Pseudomonas* sp. DSMZ 13134 in agricultural soil was clearly lower than in quartz sand.

The number of inoculated, *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 cells decreased after 3 weeks of growth in the soil system. This could be due to an instability of the *gfp*-tagging using a plasmid system in long-term experiments without selective pressure [9, 58]. However, we did not observe any instability of the *gfp*-labeling in *Pseudomonas* sp. DSMZ 13134 under non-selective conditions (not shown). Probably, the inoculated *gfp*-labeled strain was replaced by other bacteria to levels below the detection limit of the community analysis. Van Elsas et al. [59] reported a low survival rate of *P. fluorescens* WCS374 in the rhizosphere of wheat. Schloter and Hartmann [52] reported that the number of the inoculated *Azospirillum brasilense* Wa5 strain colonizing the rhizosphere of wheat was reduced from 10^6 to 10^4 bacteria/g dried root within 14 weeks after planting. In contrast, the strain Sp245 was able to colonize the inner root tissue endophytically and reached a constant number of 10^5 bacteria/g dried root in the root interior. Jacoud et al. [32] inoculated maize seeds with *Azospirillum lipoferum* CRT1 at the level of 10^7 CFU/seed in experiments that used field conditions. Two weeks after planting, the cell numbers rapidly declined, and after 28 days the CRT1 bacterial density dropped to a concen-

tration that was lower than the detection threshold of 10^3 CFU/plant. However, despite the lack of detection of inoculated cells at later stages, plant growth-promoting effects were recorded [16].

Molecular fingerprinting methods, such as T-RFLP or denaturing gradient gel electrophoresis (DGGE) analysis, offer the possibility of monitoring changes within the abundances of bacteria in a rhizosphere community in response to variations in environmental conditions or to inoculations with beneficial root-colonizing bacteria [27, 49, 60]. Within the scope of the present work, T-RFLP analysis was used to investigate the effect of inoculation with *Pseudomonas* sp. DSMZ 13134 on the indigenous root-associated bacterial community. Due to its specific fragment length of 143 bp in the T-RFLP analysis, *Pseudomonas* sp. DSMZ 13134 could be detected specifically. In soil-grown barley plants that had received the Proradix® treatment by vacuum infiltration, the inoculant strain was not detectable. However, when an additional inoculation (PI) was applied, the inoculant could be detected 1 week after inoculation. This result indicates that higher inoculation densities (seed treatment+additional inoculation) cause a more effective colonization by the introduced bacterial strain in competition with seed borne and other early colonizing soil bacteria. This is consistent with the results of an inoculation study, in which Götz et al. [23] could only detect the inoculated *P. putida* strain PRD16 in the rhizosphere of tomato plants at the first sampling point 8 days after inoculation.

The application of *Pseudomonas* sp. DSMZ 13134 exerted only a transient impact on the dominant bacterial community of barley roots in the soil system. This effect was restricted to the very early stage of root development. In previous investigations, some authors have reported changes within the rhizosphere community following inoculation with selected bacterial strains [5, 22, 23, 50, 54], whereas others found no such alterations [30, 39]. Miethling et al. [44] observed only a minor effect after inoculation with *Sinorhizobium meliloti* L33 in experiments with alfalfa and no effect on the rhizosphere community of rye. Inoculations with antibiotic-producing *P. fluorescens* strains led to small and transient disturbances [5]. In DGGE analyses done by Götz et al. [23], differences between control and inoculated plants were more readily detectable when bacterial subpopulations were investigated with group-specific primers.

Our T-RFLP analyses (Fig. 3) showed clear temporal community changes that indicated a connection between the root-associated bacterial population structure and root development. Using fingerprinting analyses, Herschkovitz et al. [30] and Mougél et al. [45] also detected variations in the structure of bacterial communities in the rhizosphere that depended on the age of maize or *Medicago truncatula*

plants. Normander and Prosser [47] did not find any alterations in the bacterial community that were associated with plant age. The number of root-colonizing bacteria increases with increasing root development because the amount of root exudates also increases. Growing roots are prized as an important source of carbon [29]. Gransee and Wittenmayer [25] showed that the composition of root-derived substances depends on the age of the plant and that it is related to physiological changes that occur in the plant during development. As the amount of assimilated carbon that is translocated to the roots decreases with increasing plant age [36], the bacterial community structures on young roots were found to be different to older parts of the root [14, 63]. In our experiments, whole root systems were always harvested. At the beginning of the investigation period, young roots were harvested. As root maturation progressed, the roots that were collected contained both young and old parts and an increasing supply of habitat and of nutrients as well as the existence of specific niches for colonizing bacteria.

To identify restriction fragments that were detected in the T-RFLP analyses, four clone libraries were constructed. In agreement with the relative abundances that were calculated from the T-RFLP analyses, the clone libraries were dominated by β -*Proteobacteria*. However, as a result of the high number of clones that were related to uncultured bacteria, the unambiguous identification of all the detected T-RFs was not possible. Moreover, certain bacteria were also represented by more than a single T-RF, and a specific T-RF could also be assigned to several different clone library sequences. This problem was also reported by Yang and Crowley [63]. The presence of indigenous *Pseudomonas* was demonstrated by the identification of two *Pseudomonas* clones in both Proradix[®]-treated clone libraries. It could be possible that both strains occupied similar niches on the root surface as *Pseudomonas* sp. DSMZ 13134 and influenced the colonization of the inoculated strain by nutrient and habitat competition.

Culture-independent analyses of microbial communities based on PCR include steps that may introduce biases [34]. For example, because the structure of the bacterial cell, and in particular, the cell wall varies in different phylogenetic groups, some bacteria lyse more readily than others. Fingerprinting profiles are often dominated by gram-negative genera [5]. Although this potential source of bias may have influenced the results of clone library analyses in the present study, some gram-positive bacteria, e.g., *Lentzea albidocapillata*, *Streptomyces turgidiscabies*, *Paenibacillus alginolyticus*, *Bacillus niacini*, were identified. In addition, the copy number of rRNA genes can exhibit considerable variability in different organisms. This can be problematic when a highly diverse community is to be quantitatively analyzed [60]. Similar to previous studies [5,

30], we used general bacterial primers. The phylogenetic diversity that can be detected is considerably higher with group-specific primers than with universal primers [7, 12]. Moreover, in the present work the amplification of chloroplast and mitochondrial DNA influenced the analysis of the bacterial community; this has also been reported by other authors [24, 47, 56, 63]. As a result of the typically large amounts of plant-derived DNA, the amplification of less-dominant bacterial DNA may often be repressed [47].

The application of plant growth-promoting rhizobacteria offers several advantages compared with chemical plant protection measures. However, the reliability of the performance of plant growth promoting or biocontrol inoculants in practical field application is of central importance and an often discussed problem. An efficient early colonization of the inoculant strain is a necessary prerequisite of the development of growth-promoting effects. We have demonstrated that *Pseudomonas* sp. DSMZ 13134 could transiently colonize the rhizoplane in detectable amounts. During this early colonization, it had to compete against, e.g., *Pseudomonas* bacteria originating from the seeds. This transient colonization resulted in beneficial effects of the Proradix[®] inoculation under both greenhouse and field conditions (Fröhlich et al., in preparation). Apparently, only the introduced *Pseudomonas* sp. DSMZ13134 had the ability to induce plant growth promotion effects, while the residing *Pseudomonas* community was ineffective in this respect. This appears similar to the well known situation with *Rhizobium* inoculations to induced root nodulation and symbiotic nitrogen fixation in legumes, when effective nodulating inoculants have to compete with ineffective residing rhizobia. Consequently, to inform recommendations on the practical use of PGPRs and to develop effective and novel plant growth stimulants, an extensive investigation of the competitive colonization behavior of PGPRs and their beneficial traits is considered to be indispensable.

Acknowledgments We thank Dr. Tillmann Lüders and Sabine Schäfer, Institute of Groundwater Ecology, Helmholtz Zentrum München, Neuherberg, Germany, for their introduction to and their very helpful advice on the T-RFLP method, and Dr. Uta von Rad, Institute of Biochemical Plant Pathology, for providing the *gfp*-tagged *Pseudomonas* sp. DSMZ 13134 strain. Furthermore, we are grateful to Sourcon-Padena GmbH & Co. KG, Tübingen, Germany, for providing Proradix[®] and for performing the inoculation of the seeds by vacuum infiltration.

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