Plant and Soil **257:** 397–405, 2003.

Endophytic *Pseudomonas* spp. populations of pathogen-infected potato plants analysed by 16S rDNA- and 16S rRNA-based denaturating gradient gel electrophoresis

Birgit Reiter[1], Nicola Wermbter[2], Stephen Gyamfi[1], Helmut Schwab[3] & Angela Sessitsch[1,4]
[1]*ARC Seibersdorf research GmbH, Division of Environmental and Life Sciences, A-2444 Seibersdorf, Austria.* [2]*Univ. of Hohenheim, Inst. of Soil Science, D-70593, Stuttgart, Germany.* [3] *Institute of Biotechnology, University of Technology, A-8010 Graz, Austria.* [4]*Corresponding author**

Received 3 October 2002. Accepted in revised form 11 July 2003

Key words: DGGE, endophytes, *Erwinia carotovora* subsp. *atroseptica*, potato, *Pseudomonas*, 16S rDNA, 16S rRNA

Abstract

The diversity of abundant and metabolically active pseudomonads in potato plants was analysed using a culture-independent approach. The effect of two plant varieties, Agria and Bionta, as well as the presence of a plant pathogen *Erwinia carotovora* ssp. *atroseptica* on this bacterial group was tested. A combination of *Pseudomonas*-specific PCR, DGGE analysis, cloning and sequencing of partial 16S rDNA genes was performed using DNA and RNA extracted from potato stem tissue. Sequence analysis revealed a high species diversity, with the most prominent ones being *Pseudomonas stutzeri* and *Pseudomonas gingeri*. Some species showed high rRNA contents indicating high metabolic activity. Both, highly abundant and metabolically active *Pseudomonas* populations were more affected by the plant genotype than by the presence of *E. carotovora.*

Introduction

The term endophyte refers to microorganisms that colonize intercellular spaces and vascular tissues of plants without exhibiting pathogenicity. Initially, endophytic bacteria were regarded as latent pathogens or as contaminants from incomplete surface sterilization (Thomas and Graham, 1952), but since then various reports have demonstrated that bacterial endophytes are able to promote plant growth and health (Benhamou et al., 2000; Chen et al., 1995; Nowak, 1998; Reiter et al., 2002; Sturz and Matheson, 1996). In general, endophytes are believed to originate from the rhizosphere or phylloplane microflora (Hallmann et al., 1997), although endophytes of sugarcane have been shown to exist predominantly within plant tissue and they have not been found in soils (Döbereiner, 1993).

Various studies indicated that pseudomonads frequently colonize plants internally. *Pseudomonas fluorescens* and *Pseudomonas viridiflava* were identified as main endophytic colonizers of field-grown pea plants (Elvira-Recuenco and van Vuurde, 2000), and *Pseudomonas* is one of the most dominant genera found in potato tubers (Sturz, 1995). Furthermore, Sturz and colleagues (1998) reported that about 57% of bacterial isolates obtained from red clover and potato tubers were pseudomonads. Recently, Garbeva et al. (2001) monitored endophytic populations in potato plants by PCR-DGGE that showed the occurrence of mixed populations of bacteria colonising the plant tissue. Their results indicated a preference of certain bacteria for specific plant compartments, for example pseudomonads were abundantly detected in interior stem tissues. Among the most frequently isolated endophytes Garbeva et al. (2001) found three *Pseudomonas* species, *P. aureofaciens*, *P. corrugata* and *P. putida.* Similarly, cultivation-dependent and independent ana-

* FAX No: +43-0-50550-3444.
E-mail: angela.sessitsch@arcs.ac.at

lysis indicated the occurrence of various endophytic *Pseudomonas* spp. such as *P. alcaligenes, P. fluorescens* and *P. putida* in potato (Reiter et al., 2002; Sessitsch et al., 2002). Furthermore, plant growth promoting properties have been reported for several endophytic pseudomonads (e.g. Duijff et al., 1997; Kloepper et al., 1983).

As the growth requirements of many microbial species are not known and due to the presence of cells that have entered a viable but not culturable status (Tholozan et al., 1999), culture-dependent population analysis leads to the underestimation of microbial diversity. Molecular techniques applying the 16S RNA gene as phylogenetic marker provide a powerful approach to circumvent drawbacks related to cultivation (Amann et al., 1995). Methods such as terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997; Sessitsch et al., 2001) or denaturing gradient gel electrophoresis (DGGE) (Heuer et al., 1997; Muyzer et al., 1993; Smalla et al., 2001) in combination with sequence analysis of 16S rRNA genes proved to be powerful tools for a rapid characterisation of microbial communities. However, the detection of 16S rDNA does not necessarily reflect the metabolic activity or prove the viability of the corresponding organisms. RNA-based community analysis is more suitable to describe the metabolically active members of a bacterial population, since the amount of rRNA produced by cells roughly correlates with the growth activity of bacteria (Wagner, 1994).

As the genus *Pseudomonas* frequently colonizes the plant apoplast, the objective of this investigation was to study the diversity of endophytic *Pseudomonas* populations in healthy plants and plants infected with the blackleg pathogen *Erwinia carotovora* ssp. *atroseptica*. An additional important aim was to identify *Pseudomonas* spp. that are numerically abundant as well as those that exhibit high metabolic activity. A cultivation-independent approach was applied by specifically amplifying *Pseudomonas* 16S rRNA genes by PCR and subsequently analysing them by DGGE, cloning and sequencing.

Materials and methods

Greenhouse experiment

Two potato varieties, Agria and Bionta, were used for the analysis of bacterial endophytes in healthy and diseased plants. Potato tubers were planted in pots filled with standad growth substrate (TKS2 soil substrate; Knauf Perlite G.m.b.H.; 200–500 mg L^{-1} N, 200–500 mg L^{-1} K_2O, and 300–600 mg L^{-1} P_2O_5). After 2 weeks, plants were wounded with a sterile scalpel at the stem base and inoculated with 2×10^6 cells of a mid-exponential phase culture of *Erwinia carotovora* ssp. *atroseptica* SCRI 1039 I [DSM No. 30184] (Eca). Control plants were not infected with Eca. Six weeks after planting lower (approx. 2–5 cm above ground) and upper stem (approx. 20–25 cm above ground) sections were harvested. Two plants of each genotype and treatment were sampled for subsequent analysis. Plants were not or only weakly affected by the phytopathogen, although Eca could be isolated from all plants.

DNA and RNA isolation

In order to avoid the isolation of DNA from surface bacteria, stems were soaked in 5% bleach for 10 min, rinsed 4 times with sterile, distilled water, rinsed with ethanol, finally flamed and peeled aseptically. Stems were tested for their sterility on TSA plates. After 2 days incubation at 30 °C, no growth was observed. DNA isolation was performed as described previously (Sessitsch et al., 2002). Briefly, plants were ground in liquid N_2, 0.1 mm glass beads were added and cells were lysed by bead-beating. Samples were extracted with phenol/chloroform and DNA was precipitated with ethanol. The method we used for RNA isolation is based on the protocol described by Chang et al. (1993), but a bead-beating step was included to lyse bacterial cells. To 0.3 g plant tissue 1 mL extraction buffer (2% CTAB, 2% PVP K30, 100 m*M* Tris-HCl, pH 8.0, 25 m*M* EDTA, pH 8.0, 0.5 g L^{-1} spermidine), which was freshly amended with 2% β-mercaptoethanol, was added. The sample was frozen in liquid nitrogen, two steel beads (5 mm) were added, and plants were ground in a mixer mill (Type mm2000, 220V, 50 Hz, Retsch GmbH & Co KG, Haam, Germany). Then 0.3 g glass beads (Sigma, 0.1 mm) were added and bead-beating was performed 3 times for 1 min in a mixer mill. The sample was extracted twice with chloroform followed by addition of 0.25 vol. of 10 *M* LiCl to the aqueous phase. The RNA was precipitated at 4 °C overnight and centrifuged at 10 000 rpm for 1 h at 4 °C. The resulting pellet was resuspended in 500 μl SSTE (1 *M* NaCl, 0.5% SDS, 10 m*M* Tris-HCl, pH 8.0, 1*M* EDTA, pH 8.0). After chloroform extraction, RNA was precipitated again with 2 vol. ethanol at –70 °C for 30 min. The pellet was washed with 70%

ethanol, dried and resuspended in 30 μL RNase-free water.

PCR and RT-PCR conditions

For the amplification of 16S rRNA genes of *Pseudomonas* spp. a nested PCR was performed. In the first step eubacterial partial 16S rDNA sequences were amplified with a thermocycler (PTC-100^{TM}, MJ Research, Inc.) using an initial denaturing step of 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 1 min annealing at 52 °C and 2 min extension at 72 °C. The PCR reaction mixtures (50 μL) contained 1 x reaction buffer (Gibco, BRL), 200 μM each dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$ and 2.5 U Taq DNA polymerase (Gibco, BRL), 0.15 μM primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards et al., 1989) and 926r (5′-CCGTCAATTCCTTT(AG)AGTTT-3′) (Liu et al., 1997) and 0.5 to 1 μL extracted DNA. The same PCR conditions were used in order to check DNase–treated RNA for the presence of contaminating DNA. RT-PCR amplification was performed with 1–2 μL extracted RNA, the primer set 8f and 926r and the PCR conditions described above applying the Superscript^{TM} One-Step^{TM} RT-PCR System (Gibco, BRL) according to the manufacturer's instructions. PCR reactions obtained in the first step were purified using the NucleoTraPCR kit (Macherey-Nagel) and amplicons were used as templates for the second step of the nested PCR, in which *Pseudomonas* sequences were amplified. Specific PCR reactions were carried out as described by Johnsen et al. (1999). For cloning the primer 8f (Edwards et al., 1989) in combination with the *Pseudomonas* genus specific primer PSMGx (5′-CCTTCCTCCCAACTT-3′) (Braun-Howland et al., 1993) was used, whereas for DGGE analysis 8f carried a GC-clamp at the 5′-end. RT-PCR and PCR amplification products were confirmed by electrophoresis in 1% agarose gels.

DGGE analysis

DGGE analysis was performed according to Muyzer et al. (1993) using the DCode^{TM} Universal Mutation Detection System (Bio-Rad). PCR samples were loaded onto 6% polyacrylamide gels in 1 x TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA [pH 7.4]) with a denaturing gradient ranging from 40 to 60% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed for 16 h at 70 V and 30 mA (Bio-Rad Power PAC 300). The temperature was set at 60 °C. After electrophoresis, the DNA bands were visualised by silver staining. Scanned gels were analysed using the image analysis software Geldoc Quantity One 4.0.1 (Bio-Rad Laboratories). After subtracting background of lanes (Rolling disc size 50) bands were detected and quantified (peak intensity). The position and intensity of each band was transferred in a data matrix for further analysis. Cluster analysis was performed by STATISTICA software (complete linkage, 1-Pearson r).

Cloning and clone analysis

Pseudomonas 16S rDNA PCR and RT-PCR products from the lower stem samples of all treatments were used to create clone libraries. Amplicons were purified using the NucleoTraPCR kit (Macherey-Nagel) according to the manufacturer's instructions. DNA fragments were ligated into the pGEM-T vector (Promega) utilising T4 DNA ligase (Promega) and ligation products were transformed into NovaBlue Singles competent cells (Novagen, Madison, WI) as recommended by the manufacturer. Hundred DNA- and RNA-derived recombinants, appearing as white colonies on indicator plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galac topyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) , were picked, resuspended in 80 μL TE buffer and boiled for 10 min. Subsequently, cells were centrifuged for 5 min at 10 000 g and supernatants (1 μL) were used in PCR reactions using the primers PSMGx and 8f or 8f-GC, respectively, and the conditions described above to amplify cloned inserts. PCR products without GC clamp (7 μL) were digested with 5 units of the restriction endonucleases *Alu*I or *Hha*I (Gibco, BRL). Restriction fragments were separated by gel electrophoresis on 2.5% agarose gels. The resulting RFLP groups were further resolved by DGGE separation of GC-tagged PCR amplicons. Clones that showed different mobilities in DGGE gels were used for sequence analysis. In order to assign sequenced clones to specific bands in *Pseudomonas* community profiles, 1.5 μL of GC-tagged PCR product of each clone were mixed with 4–6 μL of total community PCR product and analysed on DGGE gels as described above. Clones could be assigned to DGGE patterns by selecting bands that showed strongly increased intensities as compared to total community profiles.

Sequence analysis

For sequencing, cloned partial 16S rDNA genes were amplified by PCR using the primers M13for (5′-TGTAAAACGACGGCCAGT-3′) and M13rev (5′-GGAAACAGCTATGACCATG-3′) and the conditions described above, but setting the annealing temperature at 50 °C. PCR products were purified using the NucleoTraPCR kit (Macheroy-Nagel) according to the manufacturer's instructions and applied as templates in sequencing reactions. Sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) with the primers M13for and M13rev, respectively, using an ABI 373A automated DNA sequencer and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems Inc., Faster City, CA, USA). Sequences were subjected to a BLAST analysis (Altschul et al., 1997) with the NCBI database.

Nucleotide sequence accession numbers

The sequences determined in this study were deposited in the GenBank database with the accession numbers AF453293 to AF453310.

Results

DGGE analysis of Pseudomonas *communities*

For detection of *Pseudomonas* specific 16S rRNA gene sequences in complex endophytic communities, a nested PCR was performed. All tested samples gave positive signals although the RNA-derived PCR products were weaker than those derived from DNA.

DGGE analysis of *Pseudomonas* 16S rDNA PCR amplification products (Figure 1) showed complex banding patterns. Community fingerprints of two individual plants as well as those of the upper and lower stem sections were identical. We identified a total number of 14 bands with different mobilities (Figure 1). Cluster analysis showed that both active and numerically dominant *Pseudomonas* communities of Eca infected and control plants differed from each other (Figure 2). The plant genotype had a higher impact on the *Pseudomonas* populations than the plant pathogen.

16S rDNA community profiles generated by PCR-DGGE revealed the presence of 10 – 13 bands for individual stem samples from healthy and infected potato plants, whereas 16S rRNA analysis resulted in 4 – 9 bands with different mobilities (Figure 1). Five bands (bands B, E, F, I and L) were exclusively detected in DNA-based profiles but not in those based on RNA. In addition, monitoring of the metabolically dominant pseudomonads revealed one band (band C) that was not detected in DNA based banding patterns. The 16S rDNA DGGE profiles of the two potato lines differed in only one band (band I), whereas 16S rRNA DGGE profiles of Agria and Bionta differed by presence and absence of a total number of 3 bands (bands D, J and N).

The DNA derived DGGE profiles of Eca treated and control plants were highly similar. Only a single DGGE band (band M) revealed to be specifically induced upon infection with the blackleg pathogen in Bionta but was found for both treatments in Agria plants. RNA-based analysis revealed two bands (bands C, D) that were only found in the presence of the pathogen. Band C was present in both plant lines, whereas D was specific for Agria. One more band (band M) was specifically found in infected Bionta plants, however, this band was present in infected as well as in control plants of Agria.

Analysis of clone library

Eight clone libraries were created, which derived from DNA (PsDNA) and from RNA (PsRNA) isolated from 4 treatments. We picked in total 52 PsDNA and 48 PsRNA clones, which could be grouped in 20 types by PCR-RFLP and DGGE analysis. One clone of each type was sequenced. We found a total number of 2 non-bacterial sequences. One PsDNA- and one PsRNA-clone showed highest homologies to potato leukoplast small subunit rRNA genes and one PsDNA clone was identified as a mitochondrial 18S rRNA gene. These clones were excluded from further analysis. The remaining clones showed highest similarities (98–100%) to 16S rRNA genes of the genus *Pseudomonas*. Based on about 440 bp sequence information the 18 different clones comprised 12 *Pseudomonas* species. The clone library PsDNA consisted of four species, *P. fragi*, *P. gingeri*, *P. putida* and *P. stutzeri*, whereas the PsRNA clone library revealed a far higher diversity, comprising 11 species (*P. borealis*, *P. citronella, P. corrugata*, *P. fluorescens*, *P. fragi*, *P. gingeri*, *P. brassicacearum*, *P. pseudoalcaligenes*, *P. pavonaceae*, *P. stutzeri* and *P. tolaasii*). In Table 1 we summarised the results of the sequence analysis. The clones were aligned with the DGGE

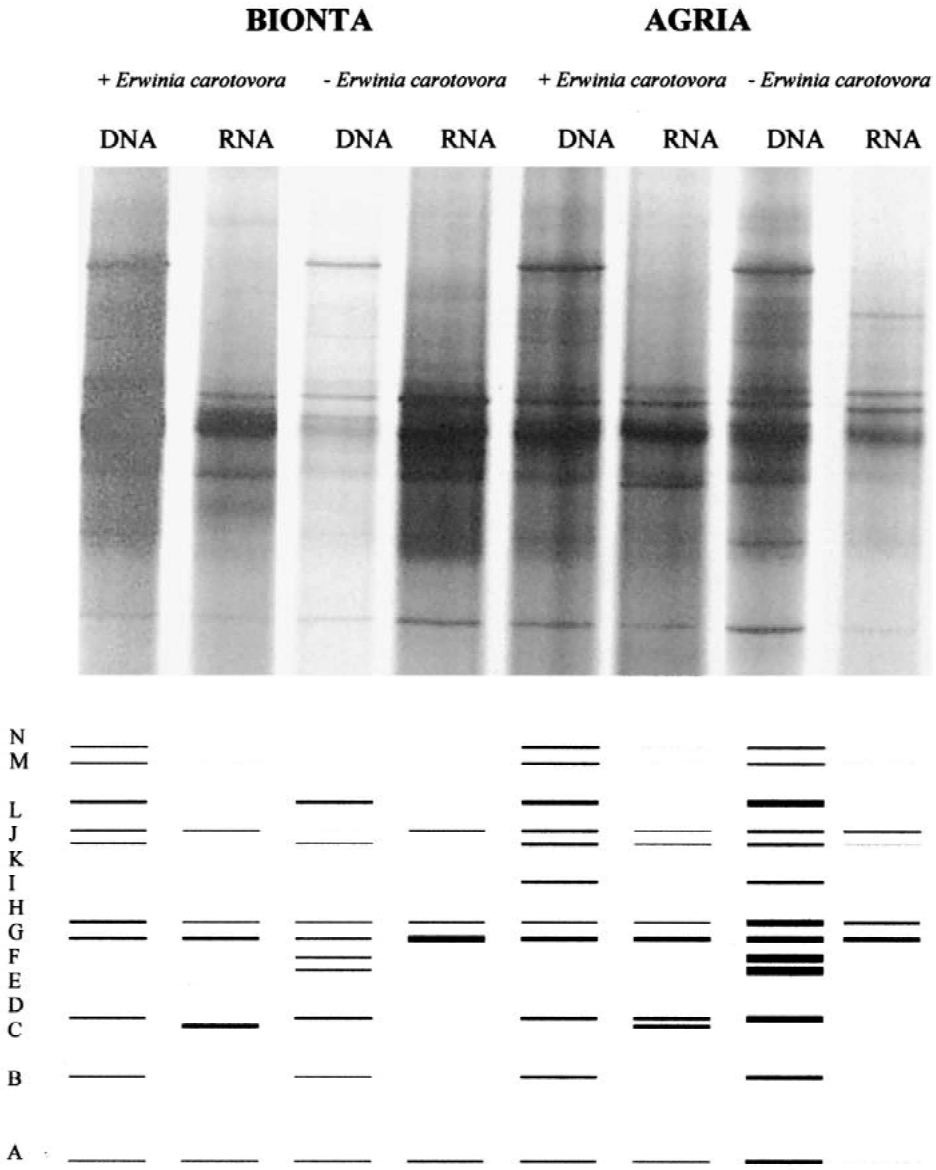

Figure 1. *Pseudomonas*-DGGE patterns produced from 16S rDNA and 16S rRNA templates isolated from stems of potato plants infected with Eca and control plants. Samples were taken from two potato lines, Bionta and Agria. Replicas of each treatment showed identical banding patterns. The schematic presentation shows the identification of single bands in the DGGE patterns based on the comparison of migration behaviour of cloned sequences and the total pseudomonads profiles.

profiles and assigned to a certain band. All clones showed migration distances equal to bands in the *Pseudomonas* community fingerprints.

Several species were detected in DNA-derived as well as RNA-derived DGGE profiles of both plant varieties (matching lanes A, G, H, K and M). Band A matched with the soil bacterium *Pseudomonas* sp. C34A. Four clones with highest 16S rRNA gene similarity to *P.* sp. NZTK7, *P.* sp. DGG18, *Bacterium* 7 (most closely related to *P. corrugata*) and *P.* clone M3sb9 showed the same mobility in DGGE gels as band G. The latter clone insert sequence was identical with a *Pseudomonas* sp. sequence that was recently found in stem tissue of the potato cultivar Mehlige Mühlviertler that was grown in the same soil substrate as used in this study (Sessitsch et al., 2002). Band H may consist of three *Pseudomonas* spp., which showed highest homology to *P. gingeri*, *P. brassicaearum*, and

Table 1. Results of sequence analysis and tentative phylogenetic affiliations of bands

DGGE-band	Closest database match (NCBI)	Identity [%]	Acession no.	Most closely related species
A	*Pseudomonas* sp. C34A	99	AF408932	*P. citronellolis*
B	*Pseudomonas tolaasii* strain NCPPB 2193	99	AF320989	*P. tolaasii*
C	*Pseudomonas sp.* clone Pseudo3d	99	AY081757	*P. borealis*
D	*Pseudomonas fluorescens*	100	AF375844	*P. fluorescens*
F	*Pseudomonas* sp. ADP	99	AF326383	*P. putida*
Ga*	*Pseudomonas* sp. NZTK7	98	AF408871	*P. stutzeri*
Gb	*Bacterium* A7	100	AF393452	*P. corrugata*
Gc	*Pseudomonas* sp. clone M3sb9	99	AF424753	*P. pavonaceae*
Gd	*Pseudomonas* sp.DGG18	99	AY082368	*P. pseudoalcaligenes*
Ha	*Pseudomonas gingeri*	99	AF320991	*P. gingeri*
Hb	*Pseudomonas brassicacearum* Am3	98	AY007428	*P. brassicacearum*
Hc	*Pseudomonas sp.* clone M3sb9	100	AF424753	*P. pavonaceae*
I	*Pseudomonas gingeri*	99	AF320991	*P. gingeri*
J	*Pseudomonas* sp. PDB	99	AF323493	*P. fragi*
K	*Pseudomonas gingeri*	99	AF320991	*P. gingeri*
L	*Pseudomonas stutzeri*	99	PSU65012	*P. stutzeri*
M	*Pseudomonas stutzeri*	99	PSU65012	*P. stutzeri*
N	*Pseudomonas stutzeri*	99	PSU65012	*P. stutzeri*

* The small letters were used for different co-migrating *Pseudomonas* spp. clones.

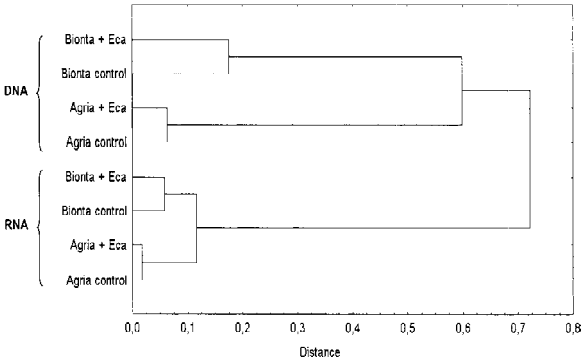

Figure 2. Dendrogram (complete linkage, 1-Pearson r) generated from 16S rDNA- and 16S rRNA-based *Pseudomonas* DGGE community profiles. For each treatment two individual plants were analyzed that resulted in identical banding patterns.

again to *P*. clone M3sb9. *P. gingeri*, which is another pathogen of *Agaricus bisporus*, causing brown blotch disease (Godfrey et al., 2001a, b), was also responsible for band K. Band M consisted of *P. stutzeri*. In addition, the plant variety Agria hosted additional two metabolically active species, which were highly similar to *P.* sp. PDB and *P. stutzeri.*

Strains of the *Pseudomonas* species *P. borealis* (DGGE band C) as well as *P. corrugata*, *P. stutzeri*, *P. pavonaceae* and *P. pseudoalcaligenes* (DGGE band G) clearly dominated the metabolically most active pseudomonads in potato stems. The numerically abundant pseudomonads were dominated by a pseudomonad related to *P. putida* (DGGE band F).

Discussion

The primary goal of this study was to compare numerically abundant and metabolically active endophytes belonging to a genus that has been frequently found in association with plants. To the best of our knowledge, this is the first attempt to investigate endophytes by RNA-based cultivation-independent analysis. Our results clearly demonstrated that at least some of the endophytic *Pseudomonas* spp. show high rRNA contents indicating high activity within potato stem tissues. Distinct populations were identified by DNA- and RNA-based analysis (Figures 1 and 2). The differences found may be at least partly explained by the different ribosome contents of microorganisms in various growth and metabolic phases (Srivastava and Schlessinger, 1990). Similar findings were reported for bacterial populations in the rhizosphere of chrysanthemum (Duineveld et al., 2001) as well as for sulphate-reducing bacteria in a stratified fjord (Teske et al., 1996). Both research groups found patterns

of DGGE bands after amplification of a selected region from small subunit ribosomal RNA, which could be clearly distinguished from a profile obtained after DNA amplification. In these studies, the banding patterns obtained when targeting the 16S rRNA were less complex than the DNA-derived profiles. Similarly, we observed a reduced number of bands in RNA-based fingerprints indicating that bacteria, which are highly abundant inside the plant were not necessarily highly active. In general, bands of RNA-based profiles were also present in DNA-based fingerprints, suggesting that endophytes with an active metabolism were also numerically abundant (Figure 1).

It has been previously shown that the majority of endophytes originate from the rhizosphere soil (Hallmann et al., 1997; Sessitsch et al., 2002). Therefore, the finding that both potato varieties hosted several pseudomonads, which were found in both varieties (Figure 1), can be explained by the fact that they were cultivated in the same soil. However, the plant variety clearly affected the *Pseudomonas* populations (Figure 2). Both, Agria and Bionta, are high-yielding potato lines, commonly used in Austrian agriculture. Bionta is known for its tolerance of several potato pathogens including viruses. In contrast, Agria is highly susceptible to infection with phytopathogens such as *Streptomyces scabies* and *Erwinia carotovora* ssp. *atroseptica* (Schiessendoppler E., pers. comm.). Genotypic and phenotypic variations may translate into different phytochemical reactions creating different microenvironments for endophytes. As endophytic life depends on the availability of nutrients provided by the plant, the viability of bacteria may be strongly influenced by the plant metabolism. Previously it was reported that endophytic eubacterial communities of potato were significantly effected by the presence of the phytopathogen *Erwinia carotovora* ssp. *atroseptica*, and that this effect was more pronounced than that of the plant genotype (Reiter et al., 2002). This suggests that distinct bacterial groups respond differently to metabolites either produced by different plant varieties or produced in pathogen-infected plants.

DGGE analysis following PCR amplification of *Pseudomonas*-specific 16S rRNA and 16S rDNA fragments allowed a detailed analysis of the endophytic microflora at the species level. Strains of the same species differing in only 3 nucleotides within the sequenced DNA fragment could be well separated with DGGE. However, some genes of various species showed the same migration distance in the DGGE, which might be due to a similar melting behaviour of different 16S rRNA genes. Gyamfi et al. (2002) used this methodology to study the microbial community in the rhizosphere of glufosinate-tolerant oilseed rape and made similar observations on the specificity of the method.

The *Pseudomonas fluorescens* 16S rRNA gene recovered in this study was identical to the sequence of a strain previously isolated from the same plant material (Reiter et al., 2002). We obtained three additional *Pseudomonas* strains by cultivation on TSA (Reiter et al., 2002). None of the latter isolates were detected by the cultivation-independent approach used in this study. This indicates that a particular growth medium may select bacteria, which show only very low abundance in relation to the total (*Pseudomonas*) microflora. It is surprising that the majority of pseudomonads identified by a cultivation-independent approach could not be isolated as the genus *Pseudomonas* is generally assumed as culturable. The use of a rich medium (TSA) for the isolation of endophytes may have selected for fast growers, and probably a higher diversity of pseudomonads could have been obtained by using more specific media. Recently, Aagot et al. (2001) described various nutrient-poor *Pseudomonas*-selective soil extract media for the isolation of *Pseudomonas* strains from soil. Furthermore, it is known that in nature bacterial cells may enter a viable but not culturable state. Such a loss of culturability has been for example reported for the biocontrol strain *P. fluorescens* CHA0 (Troxler et al., 1997).

Sequence analysis revealed a high *Pseudomonas* species diversity in the potato plants. The *Pseudomonas* community contained species such as *P. borealis*, *P. citronellolis*, *P. corrugata*, *P. gingeri*, *P. pavonaceae* and *P. brassicacearum* that were for the first time reported to be able to colonise plants endophytically.

This investigation as well as other cultivation-dependent studies of bacterial endophytes show that the apoplast of a single plant may host several *Pseudomonas* spp. We were able to demonstrate that some of them also have the potential to exhibit high metabolic activity in planta. Furthermore, cultivation-independent community analysis targeting particular bacterial groups proved to be a promising approach to get insight into the interaction between plants, the environment and bacterial endophytic communities.

Acknowledgements

This project was financed by the Austrian Science Foundation (Fonds zur Förderung der wissenschaftlichen Forschung). A. Sessitsch received an APART fellowship funded by the Austrian Academy of Sciences. Potato tubers were obtained from the Niederösterreichische Saatbaugenossenschaft Windigsteig. We are grateful to B. Hyman of the Scottish Crop Research Institute (Invergowrie, Dundee), who kindly provided the *Erwinia carotovora* ssp. *atroseptica* strain used in this studies. We thank Ulrike Pfeifer for her excellent technical assistance.

References

Aagot N, Nybroe O, Nielsen P and Johnsen K 2001 An altered *Pseudomonas* diversity is recovered from soil by using nutrient-poor *Pseudomonas*-selective soil extract media. Appl. Environ. Microbiol. 67, 5233–5239.

Altschul S F, Madden T L, Schäffer A A, Zhang J, Zhang Z, Miller W and Lipman D J 1997 Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic. Acids Res. 25, 3389–3402.

Amann R I, Ludwig W and Schleifer K H 1995 Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Appl. Environ. Microbiol. 59, 143–169.

Benhamou N, Gagné S, Le Quéré D and Dehbi L 2000 Bacterial-mediated induced resistance in cucumber: beneficial effect of the endophytic bacterium *Serratia plymuthica* on the protection against infection by *Pythium ultimum.* Phytopathology 90, 45–56.

Braun-Howland E B, Vescio P A and Nierzwicki-Bauer S A 1993 Use of simplified cell blot technique and 16S rRNA-directed probes for identification of common environmental isolates. Appl. Environ. Microbiol. 59, 3219–3224.

Chang S, Puryear J and Cainey J 1993 A simple and efficient method for isolating RNA from pine tree. Plant Mol. Biol. Rep. 11, 113–116.

Chen C, Bauske E M, Musson E M, Rodríguez-Kábana R and Kloepper J W 1995 Biological control of *Fusarium* wilt on cotton by use of endophytic bacteria. Biol. Control 5, 83–91.

Duijff B J, Gianiniazzi-Pearson V and Lemanceau P 1997 Involvement of the outer membrane lipopolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WSC417r. New Phytol. 135, 325–334.

Döbereiner J 1993 Recent changes in concepts of plant bacteria interactions: Endophytic N_2 fixing bacteria. Cienc. Cult. 44, 310–313.

Duineveld B M, Kowalchuk G A, Keijzer A, van Elsas J D and van Veen J A 2001 Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. Appl. Environ. Microbiol. 67, 172–178.

Edwards U, Rogall T, Blöcker H, Emde M and Böttger E C 1989 Isolation and direct complete nucleotide determination of entire genes: characterization of a gene coding for 16S ribosomal RNA. Nucleic. Acids Res. 17, 7843–7853.

Elvira-Recuenco M and van Vuurde J W 2000 Natural incidence of endophytic bacteria in pea cultivars under field conditions. Can. J. Microbiol. 46, 1036–1041.

Garbeva P, van Overbeek L S, van Vuurde J W L and van Elsas J D 2001 Analysis of endophytic bacterial communities of potato by plating and denaturating gradient gel electrophoresis (DGGE) of 16S rDNA based PCR fragments. Microbiol. Ecol. 41, 369–383.

Godfrey S A, Harrow S A, Marshall J W and Klena J D 2001a Characterization by 16S rRNA sequence analysis of pseudomonads causing blotch disease of cultivated *Agaricus bisporus*. Appl. Environ. Microbiol. 67, 4316–4323.

Godfrey S A, Marshall J W and Klena J D 2001b Genetic characterization of *Pseudomonas* 'NZI7' – a novel pathogen that results in brown blotch disease of *Agricus bisporus*. J. Appl. Microbiol. 91, 412–420.

Gyamfi S, Pfeifer U, Stierschneider M and Sessitsch A 2002 Effects of transgenic glufosinate-tolerant oilseed rape (*Brassica napus*) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere. FEMS Microbiol. Ecol. 41, 181–190.

Hallmann J, Quadt-Hallmann A, Mahaffee W F and Kloepper J W 1997 Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43, 895–914.

Heuer H, Krsek M, Baker P, Smalla K and Wellington E M 1997 Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. 63, 3233–3241.

Johnsen K, Enger O, Jacobsen C S, Thirup L and Torsvik V 1999 Quantitative selective PCR of 16S ribosomal DNA correlates well with selective agar plating in describing population dynamics of indigenous *Pseudomonas* spp. in soil hot spots. Appl. Environ. Microbiol. 65, 1786–1789.

Kloepper J W 1983 Effects of seed piece inoculation with plant-growth promoting rhizobacteria on populations of *Erwinia carotovora* on potato roots and daughter tubers. Phytopathology 73, 217–219.

Liu W T, Marsh T L, Cheng H and Forney L J 1997 Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63, 4516–4522.

Muyzer G, de Waal E C and Uitterlinden A G 1993 Profiling of complex microbial populations by denaturating gradient gel electrophoresis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Nowak J 1998 Benefits of *in vitro* 'biotization' of plant tissue cultures with microbial inoculants. *In Vitro* Cell. Dev. Biol.-Plant 34, 122–130.

Reiter B, Pfeifer U, Schwab H and Sessitsch A 2002 Response of endophytic bacterial communities in potato plants to infection with *Erwinia carotovora* subsp. *atrospetica*. Appl. Environ. Microbiol. 68, 2261–2268.

Sanger F, Nicklen S and Coulson A R 1977 DNA sequencing with the chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74, 5463–5467.

Sessitsch A, Weilharter A, Gerzabek M H, Kirchmann H and Kandeler E 2001 Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. Appl. Environ. Microbiol. 67, 4215–4224.

Sessitsch A, Reiter B, Pfeifer U and Wilhelm E 2002 Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and *Actinomycetes*-specific PCR of 16S rRNA genes. FEMS Microbiol. Ecol. 39, 23–32.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser N, Heuer H and Berg G 2001 Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. Appl. Environ. Microbiol. 67, 4742–4751.

Srivastava A K and Schlessinger D 1990 Mechanism and regulation of bacterial ribosomal RNA processing. Annu. Rev. Microbiol. 44, 105–129.

Sturz A V 1995 The role of endophytic bacteria during seed piece decay and potato tuberization. Plant Soil 175, 257–263.

Sturz A V and Matheson B G 1996 Populations of endophytic bacteria which influence host-resistance to *Erwinia*-induced bacterial soft rot in potato tubers. Plant Soil 184, 265–271.

Sturz A V, Christie B R and Matheson B G 1998 Association of bacterial endophyte populations from red clover and potato crops with potential for beneficial allelopathy. Can. J. Microbiol. 44, 162–167.

Teske A, Wawer C, Muyzer G and Ramsing N B 1996 Distribution of sulfate-reducing bacteria in a stratifies fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl. Environ. Microbiol. 62, 1405–1415.

Tholozan J L, Cappelier J M, Tissier J P, Delattre G and Federighi M 1999 Physiological characterization of viable-but-nonculturable *Campylobacter jejeuni* cells. Appl. Environ. Microbiol. 65, 1110–1116.

Thomas W D and Graham R W 1952 Bacteria in apparently healthy pinto beans. Phytopathology. 42, 214.

Troxler J, Zala M, Moenne-Loccoz Y, Keel C and Defago G 1997 Predominance of nonculturable cells of the biocontrol strain *Pseudomonas fluorescens* CHA0 in the surface horizon of large outdoor lysimeters. Appl. Environ. Microbiol. 63, 3776–3782.

Wagner R 1994 The regulation of ribosomal RNA synthesis and bacterial cell growth. Arch. Microbiol. 161, 100–106.

Section editor: K. Sivasithamparam