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Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and *Actinomycetes*-specific PCR of 16S rRNA genes

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Abstract

Endophytic bacteria are ubiquitous in most plants and colonise plants without exhibiting pathogenicity. Studies on the diversity of bacterial endophytes have been mainly approached by characterisation of isolates obtained from internal tissues. Despite the broad application of culture-independent techniques for the analysis of microbial communities in a wide range of natural habitats, little information is available on the species diversity of endophytes. In this study, microbial communities inhabiting stems, roots and tubers of three potato varieties were analysed by 16S rRNA-based techniques such as terminal restriction fragment length polymorphism analysis, denaturing gradient gel electrophoresis as well as 16S rDNA cloning and sequencing. Two individual plant experiments were conducted. In the first experiment plants suffered from light deficiency, whereas healthy and robust plants were obtained in the second experiment. Plants obtained from both experiments showed comparable endophytic populations, but healthy potato plants possessed a significantly higher diversity of endophytes than stressed plants. In addition, plant tissue and variety specific endophytes were detected. Sequence analysis of 16S rRNA genes indicated that a broad phylogenetic spectrum of bacteria is able to colonise plants internally including α-, β-, and γ-Proteobacteria, high-GC Gram-positives, microbes belonging to the *Flexibacter/Cytophaga/Bacteroides* group and Planctomycetales. Group-specific analysis of *Actinomycetes* indicated a higher abundance and diversity of *Streptomyces scabiei*-related species in the variety Mehlige Mühlviertler, which is known for its resistance against potato common scab caused by *S. scabiei*.

Keywords: Potato; Endophyte; Terminal restriction fragment length polymorphism analysis; Denaturing gradient gel electrophoresis; 16S rRNA; *Actinomycetes*

1. Introduction

Endophytic bacteria reside in plant tissues mainly in intercellular, rarely in intracellular spaces and inside vascular tissues without causing symptoms of disease. For a long time endophytic microorganisms were regarded as latent pathogens or as contaminants from incomplete surface sterilisation [1], but recent reports have shown that bacterial endophytes are able to promote plant growth and to act as plant pathogen antagonists [2–6]. In general, endophytes are believed to originate from the rhizosphere or phylloplane microflora [7], although endophytes of sugarcane have been shown to exist predominantly within plant tissue and they have not been found in soils [8]. Infection via seed-borne bacteria has been suggested to be a common route for the transmission of bacterial endophytes [9], whereas other proposed mechanisms by which endophytes enter the plant include local cellulose degradation [10] and entrance via cracks in lateral root junctions [11].

Studies on the species diversity of bacterial endophytes have been mainly approached by cultivation-based methods [12–14], where over 129 species have been isolated from internal plant tissues [7]. *Pseudomonas*, *Bacillus*, *Enterobacter* and *Agrobacterium* have been found to be the most abundant bacterial genera isolated [7]. Few studies, however, deal with the microbial diversity within potato plants. Sturz [15] categorised endophytic bacteria from potato tubers as plant growth promoting, plant growth

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retarding or plant growth neutral. These groupings were similarly distributed throughout the different genera isolated, which were *Pseudomonas*, *Bacillus*, *Xanthomonas, Agrobacterium, Actinomyces*, and *Acinetobacter*. Recently, 28 bacterial genera affiliated with the phyla Proteobacteria, Firmicutes and *Flexibacter/Cytophaga/Bacteroides* were isolated from 640 potato tubers [14]. In that study, almost 50% of the isolates obtained belonged to the genus *Pseudomonas*. According to a recent study, dominant endophytic isolates obtained from potato were characterised as *Pseudomonas* spp., *Agrobacterium radiobacter, Stenotrophomonas maltophila* and *Flavobacterium resinovorans* [16]. However, a range of bacteria is not accessible to cultivation methods [17], because of their unknown growth requirements or their entrance into a viable but not culturable state [18]. Therefore, the 16S rRNA gene (rDNA) has become a frequently employed phylogenetic marker to describe microbial diversity in natural environments without the need of cultivation [19,20]. Methods that rely on the analysis of the 16S rRNA gene include denaturing or temperature gradient gel electrophoresis (D/TGGE) [19,21–23], terminal restriction fragment length polymorphism (T-RFLP) [24–26], PCR-single-strand-conformation-polymorphism (SSCP) [27], and 16S rDNA cloning [20,23]. Recently, Garbeva et al. [16] monitored endophytic populations by PCR-DGGE that indicated the occurrence of a range of organisms falling into several distinct phylogenetic groups. Their results also suggested the presence of non-culturable endophytes in potato.

In this paper, we combined T-RFLP analysis, DGGE and 16S rDNA cloning and sequencing as cultivation-independent approaches in order to study the diversity of endophytic populations within three potato cultivars. Plant varieties were examined regarding their overall diversity of endophytic eubacteria as well as their diversity of *Actinomycetes*. Furthermore, we compared community structures of endophytes colonising plants that were grown under different conditions. In order to obtain information on the origin of endoplant bacteria, their populations were compared with those of their adjacent rhizospheres.

2. Materials and methods

2.1. Potato varieties and plant growth conditions

Three potato varieties – Bionta, Achirana Inta and Mehlige Mühlviertler – were used for the analysis of endophytic bacteria in two individual experiments. Achirana Inta is a medium to late maturing cultivar, which was first registered in Argentina, but is also cultivated in many Asian and American countries. The Austrian varieties, Bionta and Mehlige Mühlviertler, are late maturing and highly tolerant towards several potato pathogens such as *Phytophthora infestans* and various potato viruses. In addition, Mehlige Mühlviertler is also highly resistant to common scab caused by *Streptomyces scabiei* [28]. Mehlige Mühlviertler is an old, robust Austrian landrace, whereas the high-yielding cultivar Bionta has been available since 1992.

Potatoes were grown in tissue cultures on MS medium [29] at 22°C. At a plant height of about 10 cm, plants were transplanted in small containers filled with standard growth substrate (Frux ED 63 not pasteurised soil substrate; Gebr. Patzer GmbH and Co.KG, Sinntal-Jossa, Germany; 100–250 mg l^{-1} N, 100–250 mg l^{-1} potassium oxide, and 100–200 mg l^{-1} phosphorpentoxide, 85% peat, pH 5–6.5) and transferred to a greenhouse. After 2 weeks they were transplanted in bigger pots filled with the same standard growth substrate. The first experiment was set up in autumn and due to disadvantageous light conditions most plants did not produce tubers and showed some disease symptoms. Roots, stems and the rhizospheres were harvested after 13–14 weeks. In order to compare endophyte communities from stressed plants to those from healthy plants, the experiment was replicated in spring. Healthy roots, stems and tubers were harvested after 13–14 weeks at the early tuber production stage. Two individual plants of each potato variety were analysed.

2.2. DNA isolation

DNA was isolated individually from all tissues, using a protocol based on bead beating to disrupt bacterial cells. In order to avoid the isolation of surface bacterial DNA, stems and tubers were peeled aseptically. As it was not possible to peel roots, 0.2–0.5 g root material was shaken vigorously in 0.9% NaCl solution containing 0.3 g acid-washed glass beads (Sigma; 0.1 mm) for 20 min in order to dislodge cells from roots. Then, plants were rinsed five times with sterile H_2O and tested for their sterility on TSA plates. No growth was observed.

For the isolation of DNA, 0.2–0.5 g plant tissue were amended with 0.8 ml TN150 (10 mM Tris–HCl pH 8.0; 150 mM NaCl), frozen in liquid nitrogen and pulverised in a mixer mill (Type MM2000, 220 V, 50 Hz, Retsch GmbH and Co KG, Haam, Germany) in the presence of two sterile stainless steel beads (5 mm) at thawing. Then 0.3 g of 0.1 mm acid-washed glass beads (Sigma) were added and bead beating was performed twice for 1 min at full speed in a mixer mill. After extracting with phenol and chloroform, DNA was precipitated with 0.1 volume 3 M sodium acetate solution and 0.7 volume iso-propanol for 20 min at −20°C. DNA was centrifuged for 10 min at 14 000 rpm, washed with 70% ethanol and dried. Finally, the DNA was resuspended in 60 μl TE buffer containing RNase (0.1 mg ml^{-1}).

For the isolation of DNA from rhizospheres a protocol described by van Elsas and Smalla [30] was used. DNA isolated from 0.12 g rhizosphere soil was resuspended in 80 μl TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). For

further purification, spin-columns were prepared containing Sepharose CL-6B (Pharmacia) and polyvinylpyrrolidone (20 mg ml^{-1} CL-6B). In general, passage through two columns was needed to remove all PCR-inhibiting substances.

2.3. T-RFLP analysis

All rhizosphere and tissue samples of two individual plants of each potato variety were subjected to T-RFLP analysis. The eubacterial primers 8f [31] labelled at the 5′ end with 6-carboxyfluorescein (6-Fam; MWG) and 518r [24] were used to amplify approximately 530 bp of the 16S rRNA gene. Reactions were carried out with a thermocycler (PTC-100™, MJ Research, Inc.) using an initial denaturation step of 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 1 min annealing at 54°C and 2 min extension at 72°C. PCR reactions (50 μl) contained 1×reaction buffer (Gibco BRL), 200 μM each dATP, dCTP, dGTP and dTTP, 0.2 μM of each primer, 3 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Gibco BRL) and 20 ng template DNA. PCR product (100 ng) was digested for 4 h with a combination of the restriction enzymes *Hha*I and *Hae*III (Gibco BRL). Preliminary experiments with several restriction enzymes with 4-bp recognition sites (*Alu*I, *Msp*I, *Rsa*I, *Hha*I and *Hae*III; Gibco BRL) demonstrated that a combination of *Hae*III and *Hha*I yielded a higher number of T-RFs than other enzymes. Aliquots (0.5 μl) were mixed with 1 μl of loading buffer (deionised formamide+loading dye, 5+1) and 0.3 μl of DNA fragment length standard (Genescan 500 Rox; Perkin-Elmer). Reaction mixtures were denatured at 92°C for 2 min and chilled on ice prior to electrophoresis. Samples (1.75 μl) were applied on 6% denaturing polyacrylamide gels and fluorescently labelled terminal restriction sizes were analysed using an ABI 373A automated DNA sequencer (PE Applied Biosystems Inc., Foster City, CA, USA). Lengths of labelled fragments were determined by comparison with the internal standard.

The eubacterial primers used to amplify 16S rDNA are also homologous to chloroplast 16S and mitochondrial 18S rRNA genes resulting in two T-RF peaks of 303 bp and 197 bp, respectively, in T-RFLP fingerprints. These peaks were not shown in endophyte community fingerprints. Terminal fragments (T-RFs) were only scored positive, when they had more than 50 fluorescence units. Fragment sizes between 35 and 500 bp were analysed, which was the range of the size marker that could be determined reliably.

2.4. Partial 16S rDNA clone libraries

Clone libraries were created from partial 16S rRNA genes amplified from DNA of Mehlige Mühlviertler and Achirana Inta stems and roots (first experiment). Primers and PCR conditions were used as described above for the T-RFLP analysis. A high percentage of chloroplast-derived sequences was expected and therefore, PCR products were digested with *Pvu*II (Gibco BRL) as this enzyme possesses a restriction site in chloroplast 16S rDNA sequences that is not found in most eubacterial 16S rDNA genes. Undigested fragments were excised from an agarose gel using the Concert Nucleic Acid Purification System (Gibco BRL) according to the manufacturer's instructions and ligated into the pGEM-T vector (Promega). Ligation products were cloned into electrocompetent *Escherichia coli* DH5α cells. One hundred clones of each potato variety and type of tissue that did not show β-galactosidase activity were further analysed. Positive clones were resuspended in 80 μl TE buffer, boiled for 10 min and centrifuged for 5 min at 13 000 rpm. Supernatants (0.5 μl) were used in PCR reactions with 0.15 μM each of the primers M13uni and M13rev and the conditions described above to amplify cloned inserts. Following PCR amplification, 8 to 10 μl of 16S rDNA from each of the clones were digested separately with *Alu*I and *Hae*III. Digests were electrophoresed in 2.5% agarose gels. Restriction patterns were compared and indistinguishable patterns were grouped. Each phylotype was defined as a group of sequences with identical *Alu*I and *Hae*III restriction patterns.

2.5. DGGE and sequence analysis of Actinomycetes

All tissue samples obtained from the first experiment were subjected to a DGGE analysis of *Actinomycetes*. A nested PCR approach was used to amplify 16S rDNA sequences derived from *Actinomycetes*. First, a PCR reaction was carried out as described above using the eubacterial primers 8f and pH [31]. Products were purified with a NucleoTraPCR kit (Macheroy-Nagel) and used as a template for a second PCR with the *Actinomycete*-specific primer pair F243-R518GC [22]. PCR reactions and DGGE analyses were carried out as described by Heuer et al. [22]. For sequence analysis the latter PCR reaction was carried out without GC-clamp using 16S rDNA PCR products from Mehlige Mühlviertler stem DNA as template. Purified products were cloned into pGEM-T vector (Promega) and ligation products were cloned into electrocompetent *E. coli* DH5α cells. Twenty clones that did not show β-galactosidase were submitted to DGGE analysis in order to select sequences with different running distances. Inserts that showed different mobilities were sequenced as described below.

2.6. DNA sequence analysis

Using the Quantum Prep plasmid miniprep kit (Bio-Rad) plasmids of each phylotype were isolated. Plasmid DNA (500 ng) was used as template in sequencing reactions. DNA sequencing was performed using an ABI 373A automated DNA sequencer (PE Applied Biosystems Inc.,

Foster City, CA, USA) and the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Perkin-Elmer). Sequences were subjected to a BLAST analysis [32] with the National Center for Biotechnology Information database and were compared with sequences available in the Ribosomal Database Project (RDP) [33]. Alignments with related sequences were done with the Multalin alignment tool available in the web site (http://www.toulouse.inra.fr/multalin.html) [34]. The TREECON software package [35] was used to calculate distance matrices by the Jukes and Cantor [36] algorithm and to generate phylogenetic trees using nearest-neighbour criteria.

2.7. Nucleotide sequence accession numbers

The sequences obtained in this study have been deposited in GenBank under accession numbers AF424745–AF424757 (partial eubacterial 16SrDNA sequences) and AF424758–AF424762 (partial *Actinomycetes* 16S rDNA sequences).

3. Results

3.1. T-RFLP profiles of the first experiment

In pre-experiments, DGGE and T-RFLP were compared regarding their suitability to analyse bacterial endophytes (data not shown). Despite the high abundance of plant organelle ribosomal sequences, both profiling methods, DGGE and T-RFLP, allowed the detection of bacterial endophytic communities in potato tissues. However, as silver staining of DGGE gels is less sensitive than laser detection of fluorescently labelled T-RFs [26], more endophyte-derived bands were detected by T-RFLP than by DGGE.

T-RFLP analysis was applied to analyse endophytic bacteria in stems and roots of three potato varieties. In our analyses a combination of the restriction enzymes *Hae*III and *Hha*I was used to generate T-RFLP fingerprints. Because of the high abundance of plant organelle-derived T-RFs, T-RFLP data were used only qualitatively, i.e. the absence or presence of bands was recorded. Furthermore, a PCR bias due to preferential annealing to particular primer pairs [37] cannot be excluded. Four to eleven T-RFs representing the endophytic bacterial community, depending on the plant variety and the type of tissue, were detected (Table 1). Few variations were found among replicate plants, and potato varieties possessed endophytic populations with comparable diversities.

In the first experiment T-RFs of 151 bp, 201 bp and 312 bp were present in all cultivars and plant compartments (Table 1). Several T-RFs were found predominantly in stem tissues such as a 60 bp, 132 bp, 191 bp, 297 bp and a 388 bp fragment (Table 1). The 191-bp and the 297-bp fragments were present in all potato cultivars, whereas fragments of 132 bp and 388 bp were found exclusively in stems of Achirana Inta. A fragment of 388 bp was detected only in stems of Austrian cultivars, whereas a 204-bp T-RF was observed exclusively in root tissues but was present in all varieties (Table 1).

The potato variety Mehlige Mühlviertler hosted a unique endophyte as indicated by the presence of a T-RF of 337 bp (Table 1). Additional differences were found among Austrian and American varieties. The South American potato cultivar Achirana Inta showed two T-RFs of 83 bp and 132 bp that were not present in the Austrian varieties. Fragments that were found in both

Table 1
T-RFs obtained after *Hae*III+*Hha*I digestion of 16S rDNA PCR products obtained from DNA of roots and stems of three potato varieties (first experiment)

Variety	T-RF length (bp)																
	39	42	60	83	132	145	148	151	156	191	201	204	224	297	312	337	388
Achirana Inta																	
A-root1								□[b]			■[a]	■			■		
A-root2		■		■				□			■				■		
A-stem1			■		□			□	□	■	■			□	■		
A-stem2	■		■		□			□	□	■	■		■	□	■		
Bionta																	
B-root1						■	■	□			■	■			■		
B-root2						■	■	□			■	■			■		
B-stem1						■	■	□	□	■	■			□	■		■
B-stem2						■	■	□	□	■	■			□	■		■
Mehlige Mühlviertler																	
M-root1						■	■	□	□		■	■	■	□	■	□	
M-root2		■				■	■	□	□		■	■			■	□	
M-stem1						■		□	□	■	■			□	■		■
M-stem2	■		■			■	■	□	□	■	■			□	■		■

Chloroplast- and mitochondrial-derived T-RFLP fragments are not included.
[a]■ T-RFs found in planta as well as in the rhizosphere.
[b]□ T-RFs not found in the rhizosphere.

Table 2
T-RFs obtained after *Hae*III+*Hha*I digestion of 16S rDNA PCR products obtained from DNA of roots, stems and tubers of three potato varieties (second experiment)

Variety	T-RF length (bp)															
	60	145	148	151	163	176	191	201	276	297	308	312	318	335	337	388
Achirana Inta																
A-root1	■	■	■	■	■		■		■	■	■	■		■	■	
A-stem1	■	■	■	■	■				■	■	■	■		■	■	■
A-stem2	■	■	■	■	■				■		■	■		■	■	■
A-tuber1	■	■	■	■	■	■			■		■	■	■	■	■	
A-tuber2	■	■	■	■	■		■		■	■	■	■	■	■	■	
Bionta																
B-root1	■	■	■	■	■			■			■	■		■	■	
B-root2	■	■		■	■		■	■		■	■	■		■	■	
B-stem1					■		■	■	■	■	■	■		■	■	■
B-stem2		■			■			■	■		■	■		■	■	■
B-tuber1	■	■	■	■	■	■		■	■		■	■	■	■	■	
B-tuber2	■	■	■	■	■	■		■		■		■		■	■	
Mehlige Mühlviertler																
M-root1	■	■	■	■	■				■		■	■	■	■	■	■
M-root2	■	■	■	■	■		■			■	■	■			■	
M-stem1	■	■	■	■	■		■		■	■	■	■		■	■	■
M-stem2	■	■	■	■	■				■		■	■		■	■	■
M-tuber1	■	■	■	■	■						■	■	■	■	■	
M-tuber2	■	■	■	■	■						■	■		■	■	

Chloroplast- and mitochondrial-derived T-RFLP fragments are not included.

Austrian varieties, but not in the American one, included 145 bp, 148 bp, and 388 bp.

T-RFLP fingerprints were used to compare endophytic and rhizosphere bacterial communities. Most endophyte T-RFs were also detectable in the rhizosphere, however, some fragments were exclusively found in planta such as T-RFs of 132 bp, 151 bp, 156 bp, 297 bp and 337 bp (Table 1).

3.2. T-RFLP profiles of the second experiment

Potato plants obtained in healthy plants possessed more diverse endophytic populations than those obtained in the first experiment (Tables 1 and 2) and numbers of endophytic T-RFs detected ranged from 9 to 13. In general, the majority of peaks could be detected in both experiments, however, six T-RFs (163 bp, 176 bp, 276 bp, 308 bp, 318 bp and 335 bp) were found exclusively in healthy plants. Two peaks, 176 and 318 bp, represented endophytes that mainly colonised tubers, which were not analysed in the first experiment. T-RFs that were present in roots, stems and tubers of all potato plants included fragments of 151 bp and 312 bp as in stressed plants as well as additional fragment sizes of 163 bp, 335 bp and 337 bp. In addition, most healthy plants contained T-RFs of 60 bp, 145 bp and 148 bp. Again, the T-RF of 388 bp was predominantly present in stem tissues.

3.3. Analysis of 16S rRNA clones

Bacterial endophytes of the first experiment were analysed by 16S rDNA cloning and sequencing. In total, 400 clones obtained from the varieties Mehlige Mühlviertler and Achirana Inta were screened for the presence of eubacterial 16S rRNA genes. The majority of clones contained mainly mitochondrial and to a lower extent chloroplast small subunit rRNA sequences, whereas 13 clones were of bacterial origin (Table 3). Ten of these sequences derived from the cultivar Mehlige Mühlviertler, whereas only three were obtained from Achirana Inta. Names and accession numbers of most closely related organisms, their percent similarities calculated by BLAST, as well as their tentative phylogenetic placements by the RDP Sequence Match function, are given in Table 3.

Our clones fell into six different lineages of the eubacterial domain: the *Flexibacter/Cytophaga/Bacteroides* phylum, the α, β, and γ subdivisions of the Proteobacteria, Gram-positive organisms with a high GC content as well as Planctomycetales. Two clones derived from Mehlige Mühlviertler, M3rb1 and M4rb3, which fell into the *Flexibacter/Cytophaga/Bacteroides* phylum, however, showed only 89% and 93% sequence homology, respectively, to unidentified 16S rRNA genes within the NCBI database. Phylogenetic analysis demonstrated that both clones cluster with a range of as yet uncultivated bacteria that showed the highest sequence similarity with *Flexibacter flexilis* (Fig. 1). However, as only partial 16S rRNA gene sequences were used, this plylogenetic placement is tentative. Three Mehlige Mühlviertler sequences, M4rb3, M4rb4 and M3sb7, grouped with different *Streptomyces* species, and the remaining sequences showed high homology (96–99%) to Proteobacteria. The clones M4rb6, M3sb9 and M4sb10 showed highest similarity with members of the γ-Proteobacteria, whereas M4rb5 was highly

Table 3
Sequence analysis of clones containing eubacterial 16S rDNA sequences obtained from roots and stems of the potato varieties Mehlige Mühlviertler and Achirana Inta[a]

Clone	Closest database match	Similarity (%)	Putative phylum	RDP[c]	T-RF length (bp)
Mehlige Mühlviertler root					
M3rb1	unidentified eubacterium AF010069[b]	89	*Flexibacter/Cytophaga/Bacteroides*	unclassified	39
M3rb2	clone TBS17 AJ005988	93	*Flexibacter/Cytophaga/Bacteroides*	unclassified	95
M4rb3[d]	*Streptomyces lincolnensis* X79854	98	High-GC Gram-positives	*S. mashuensis* sg	224
M4rb4[d]	*Streptomyces turgidiscabies* AB026221	98	High-GC Gram-positives	*S. scabiei* sg	224
M4rb5	*Sphingomonas subterranea* AB025014	99	α-Proteobacteria	*Sph. subarctica* sg	81
	Sphingomonas aromaticivorans AB025012	99			
M4rb6[d]	*Pseudomonas fluorescens* AF134705	99	γ-Proteobacteria	*P. stutzeri* sg	200
Stem					
M3sb7	*Streptomyces scabies* AB026214	98	High-GC Gram-positives	*S. scabiei* sg	225
M4sb8	β-proteobacterium A0640 AF236010	96	β-Proteobacteria	*Rub. gelatinosus* sg	217
M3sb9[d]	*Pseudomonas* sp. PsK AF105389	99	γ-Proteobacteria	*P. amygdali* sg	39
M4sb10	*Cellvibrio* sp. AJ289164	97	γ-Proteobacteria	unclassified	39
Achirana Inta root					
A2rb11[d]	uncult. bacterium OSW4 AF018068	98	γ-Proteobacteria	*Acinetobacter* g	200
Stem					
A3sb12[d]	uncultured planctomycete clone 40 AF271331	94	Planctomycetales	*Pirellula* g	186
A3sb13[d]	uncultured eubacterium WD259 AJ292672	98	γ-Proteobacteria	*Pseudomonas* sg	40

[a]Tentative phylogenetic placement and percent similarity values were determined by using BLAST and are based on approximately 500 bp of the 16S rRNA gene sequence for each clone.
[b]Accession numbers of closest database matches are given.
[c]The tentative phylogenetic placement was determined by using the Sequence Match option in the RDP.
[d]Sequenced from both ends of the PCR product.

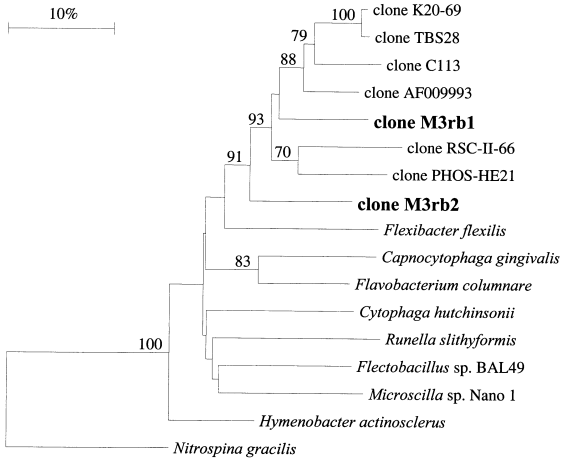

Fig. 1. Neighbour-joining phylogenetic tree based on 437 nucleotides of the 16S rRNA gene of clones showing highest similarity with bacteria belonging to the *Cytophaga/Flexibacter/Bacteroides* division. Sequences obtained in this study are printed in bold letters. Percent of 100 bootstrap replicates are shown at the left nodes when at least 70%. As only partial 16S rRNA sequences were determined this tree presents tentative rather than definitive phylogenetic relationships. Accession numbers of the 16S rDNA sequences used are: AF145860 (clone K20–69), AJ0059990 (clone TBS28), AF013535 (clone C113), AF009993 (clone AF009993), AJ252690 (clone RSC-II-66), Af314419 (clone PHOS-HE21), M62794 (*F. flexilis*), L14639 (*Capnocytophaga gingivalis*), AB016515 (*Flavobacterium columnare*), M58768 (*Cytophaga hutchinsonii*), M62786 (*Runella slithyformis*), AF182020 (*Flectobacillus* sp. BAL49), AB015937 (*Microscilla* sp. Nano1), and Y17356 (*Hymenobacter actinosclerus*). L35504 (*Nitropsina gracilis*) was used as outgroup.

related to α-Proteobacteria and M4sb8 to β-Proteobacteria. Two Achirana Inta clones, A2rb11 and A3sb13, showed 98% sequence homology to γ-Proteobacteria, whereas clone A3sb12 fell into the phylum Planctomycetales.

3.4. Analysis of Actinomycetes

The species diversity of endophytic *Actinomycetes* in potato samples obtained from the first experiment was assessed by DGGE as well as by cloning and sequencing. PCR with *Actinomycetes*-specific PCR primers yielded reproducibly higher amounts of amplified products with plant material of the cultivar Mehlige Mühlviertler than with other cultivars. Particularly stems of Bionta and Achirana Inta contained only low concentrations of *Actinomycetes*-derived PCR product. The composition of *Actinomycetes* populations was characterised by DGGE analysis. The number of DGGE bands ranged from 0 to 4 bands depending on the type of tissue and potato cultivar tested. *Actinomycetes* populations in stems and roots were highly different, with only the variety Mehlige Mühlviertler showing identical banding patterns in both tissues (Fig. 2).

Cloning of *Actinomycetes*-derived partial 16S rRNA genes and DGGE analysis of 20 clones revealed 5 clones with different mobilities. Three of them showed the same running distances as bands obtained in the population

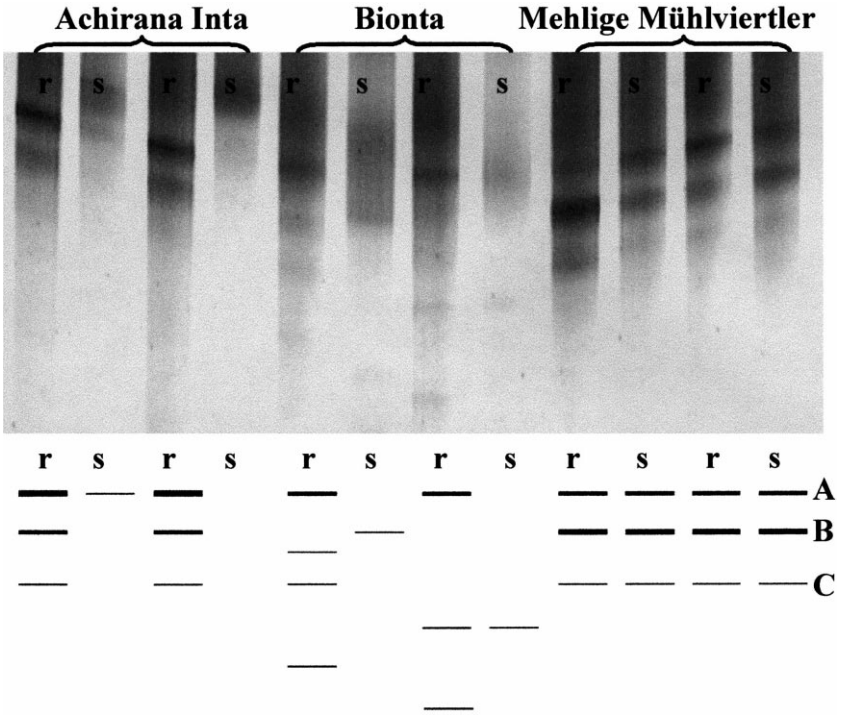

Fig. 2. DGGE profiles of endophytic *Actinomycetes* populations within roots (r) and stems (s) of three potato varieties (first experiment).

analysis, whereas the remaining clones were not detected in DGGE profiles. These two clones showed similar mobilities as other clones and may have co-migrated with other bands in *Actinomycetes* DGGE profiles. Sequence analysis indicated the presence of several endophytic *Streptomyces* species of the *S. scabiei* subgroup, only one sequence showed higher similarity to bacteria belonging to the *S. coelicolor* subgroup (Table 4). Sequences were identical or highly similar to *Streptomyces* sequences that were found in 16S rDNA clone libraries. As a smaller portion of the 16S rRNA gene was analysed with sequences derived from *Actinomycetes*-specific PCR, BLAST analysis resulted in closest database matches that are different to clones obtained with eubacterial PCR primers. However, the sequence represented by the 16S rDNA clone M4rb3, which showed 98% similarity to *S. lincolnensis*, was not found among *Actinomyectes*-derived clones. *Actinomycetes* sequences showed 1–5 nucleotide differences demonstrating the high resolution of DGGE analysis.

4. Discussion

Endophytic bacterial communities of three potato varieties were examined by applying a 16S rRNA-based cultivation-independent approach. Two individual plant experiments were conducted. In the first experiment, plants suffered from light deficiency resulting in lower photosynthesis rates and therefore probably preventing the transformation of carbon into starch. As a consequence no tubers were produced. Additionally, plants were weakened by the presence of white flies and thrips in the greenhouse. The second experiment provided robust and healthy plants. Plants obtained from both experiments showed distinct endophytic communities, although the majority of endophytic T-RFs were found in both stressed and robust plants. Interestingly, healthy plants of the second experiment possessed a higher diversity of endophytes than stressed plants of the first experiment. Growth of Achirana Inta was particularly affected by the unfavour-

Table 4
Sequence analysis of clones containing partial *Actinomycetes* 16S rDNA sequences obtained from Mehlige Mühlviertler stems

Clone	Closest database match[a]	Similarity (%)	RDP[c]	Designation in DGGE
ActinM3s7	*Streptomyces cyaneus* AJ310927[b]	99	*S. scabiei* sg	A
ActinM3s5	*Streptomyces kathirae* AY015428	99	*S. scabiei* sg	B
ActinM3s4	*Streptomyces nodosus* AF114036	99	*S. coelicolor* sg	C
ActinM3s1	*Streptomyces cyaneus* AJ310927	99	*Streptomyces scabiei* sg	n.d.
ActinM3s20	*Streptomyces cyaneus* AJ310927	100	*S. scabiei* sg	n.d.

[a]Tentative phylogenetic placement and percent similarity values were determined by using BLAST and are based on approximately 285 bp of the 16S rRNA gene sequence for each clone.
[b]Accession numbers of closest database matches are given.
[c]The tentative phylogenetic placement was determined by using the Sequence Match option in the RDP.

able conditions of the first experiment and this cultivar showed a lower endophyte diversity compared to the Austrian potato varieties. It is well known that biotic and abiotic stressors of plants induce a cascade of reactions leading to the formation of several enzymes such as peroxidases, catalases, and superoxide dismutases, as well as the synthesis of stress proteins. Typical stress responses also include the synthesis of stress metabolites including H_2O_2, phytoalexins, and stress signals such as absicic acid, jasmonic acid and salicylic acid [38], which can create a hostile environment for bacteria, and may explain the lower species diversity found in stressed plants. Although it has been postulated that the low stress tolerance of axenic plants may partly result from the absence of endophytic microorganisms [7], it remains unclear, whether the higher diversity found in healthy plants contributed to their better performance.

Several T-RFs were predominantly abundant in robust plants of the second experiment such as a 60 bp fragment and 337 bp fragment. Of the known bacterial 16S rRNA sequences only lactobacilli possess a theoretical T-RF of 337 bp, whereas the 60 bp T-RF is characteristic for bacteria belonging to the *Rhizobium–Agrobacterium* group. Both, the latter group as well as lactobacilli are known to live in association with plants and they also have been isolated from internal plant tissues [3,6,13,14,39]. Stressed plants had T-RFs of 156 bp, 204 bp and 224 bp that were not found in robust plants of the second experiment. The latter fragment probably derived from bacteria belonging to the genus *Streptomyces*, as also sequence analysis of amplified 16S rRNA gene sequences indicated the presence of *Streptomyces* species. Endophytic *Streptomyces* strains have been isolated from a variety of plants including *Ficus, Dieffenbachia, Allium porrum, Brassica oleracea, Quercus* sp., and others [40,41].

Endophytes proved to be plant tissue-sensitive, as different bacterial communities were found in different plant compartments, particularly in stressed plants. Furthermore, stems showed slightly higher diversities than roots. Similar findings were reported by Sturz et al. [42], who found different endophytic populations in roots, foliage, stems and nodules of red clover. In that study the greatest diversity was found in stems and foliage and certain bacteria were found colonising only stems and foliage, roots or nodules. In addition, cultivar-dependent differences were found. Again, this effect was more pronounced in the first experiment, where plants suffered from unfavourable conditions. Host-specific endophytic populations were also observed by cultivation-dependent approaches [43,44]. The fact that many T-RFs were found in both experiments and that variation between replicates was low indicated that the potato apoplast is a suitable niche for certain specific sets of bacteria.

A comparison of endophytic and rhizosphere microbial communities confirmed, to a certain extent, the observation of previous reports that endoplant populations represent a subset of rhizosphere bacteria [9,45,46]. However, bacteria that were not found in the rhizosphere also inhabited plants. These included microbes that were probably already present as latent infections in tissue cultures. In this regard it has been questioned whether aseptic tissue plant cultures exist and even whether it is possible to achieve plant cultures free of microbes over long time periods [47].

Sequencing of partial 16S rRNA genes revealed that a broad phylogenetic spectrum of bacteria is able to colonise plants internally including α-, β-, and γ-Proteobacteria as well as high-GC Gram-positives, microbes falling in the *Flexibacter/Cytophaga/Bacteroides* group and Planctomycetales. Garbeva et al. [16], who analysed endophytic bacterial communities of potato by plating and DGGE analysis identified similar phylogenetic groups. Although endophytic bacteria of the *Flexibacter/Cytophaga/Bacteroides* group were reported previously [16,42,48], our sequences showed only 89 and 93% similarity to 16S rDNA sequences of uncultured bacteria. Therefore, these endophytes may belong to a new branch of bacteria within this phylogenetic group and may merit further investigation. In addition, a bacterial sequence belonging to the Planctomycetales was identified. These microorganisms were originally thought to inhabit only aquatic habitats [49]. However, Derakshani et al. [50] recently recovered Planctomycetales 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms, suggesting that these bacteria may also have colonised roots internally. Our study is the first documentation of endophytic Planctomycetales in potato. Among 16S rRNA sequences, various sequences derived from *Pseudomonas* species were detected. This group of bacteria is easy to culture and cultivation-dependent studies identified *Pseudomonas* strains as frequently occurring endophytes [3,7,16].

By 16S rDNA sequence analysis, three *Streptomyces* species, members of the *Actinomycetes*, were found in the potato variety Mehlige Mühlviertler. One sequence showed 98% similarity to *S. scabiei*, the causative agent of common scab of potato, although typical disease symptoms were not detected. Two sequences showed 98% similarity to *S. turgidiscabies* and *S. lincolnensis*, respectively. As the variety Mehlige Mühlviertler is known for its resistance to scab disease development [28], *Actinomycetes* communities were further characterised in all varieties demonstrating the presence of several *Streptomyces* strains related to *S. scabiei*. Interestingly, the cultivar Mehlige Mühlviertler possessed a higher population density of *Streptomyces* than other cultivars. In addition, a high species diversity of *Streptomyces* was found in stems of Mehlige Mühlviertler, whereas in other varieties this bacterial group was mainly associated with roots. These results suggest that the reported high tolerance of Mehlige Mühlviertler against common scab [28] may be at least partly due to the ability to host endophytic *Streptomyces* strains. Doumbou et al. [51] identified *S. scabiei* as well as other

Streptomyces sp. that protected potato against common scab by utilising thaxtomin, the phytotoxin produced by the pathogen.

5. Conclusions

Community analysis by T-RFLP of 16S rRNA genes proved to be a suitable and sensitive tool to investigate endophytic microbial communities and to detect population shifts of bacteria in different plant tissues, varieties or plants grown under different conditions. Nevertheless, the presence and high concentration of organelle small subunit RNA in plants is a major drawback for the culture-independent community analysis of endophytes. This is particularly true for direct cloning and sequencing of bacterial 16S rRNA genes. We demonstrated rather high-complex community structures as well as the presence of bacteria belonging to various phylogenetic groups within plants, but our 16S rDNA clone library did not encompass the number of community members found by T-RFLP analysis. The use of group-specific PCR primers avoided the problem of chloroplast- and mitochondrial-derived sequence confusion and proved to be a valuable tool for the analysis of endophytes. We conclude that molecular techniques suitable for the analysis of endoplant bacteria will continue to improve our understanding of the role of endophytes for stress tolerance and pathogen resistance in plants.

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