

# Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities

Angela Sessitsch, Birgit Reiter, and Gabriele Berg

**Abstract:** To study the effect of plant growth on potato-associated bacteria, the composition and properties of bacteria colonizing the endosphere of field-grown potato were analyzed by a multiphasic approach. The occurrence and diversity of potato-associated bacteria were monitored by a cultivation-independent approach, using terminal restriction fragment length polymorphism analysis of 16S rDNA. The patterns obtained revealed a high heterogeneity of community composition and suggested the existence of plant-specific communities. However, endophytic populations correlated to a certain extent with plant growth performance. Endophytes were also isolated from plants that grew well or grew poorly and were identified by partial sequencing of the 16S rRNA genes. A broad phylogenetic spectrum was found among isolates and differently growing plants hosted different bacterial populations. In an approach to investigate the plant-growth-promoting potential of potato-associated bacteria, a total of 35 bacteria were screened by dual testing for in vitro antagonism towards (i) the fungal pathogens *Verticillium dahliae*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Phytophthora cactorum* and (ii) the bacterial pathogens *Erwinia carotovora*, *Streptomyces scabies*, and *Xanthomonas campestris*. The proportion of isolates with antagonistic activity was highest against *Streptomyces* sp. (43%) followed by those against *Xanthomonas* sp. (29%). As all plants showed more or less severe disease symptoms of scab disease caused by *Streptomyces scabies*, we assume that the presence of the pathogen induced the colonization of antagonists. The antifungal activity of the isolates was generally low. The biotechnological potential of endophytic isolates assessed by their antagonistic activity and by in vitro production of enzymes, antibiotics, siderophores, and the plant growth hormone indole-1,3-acetic acid was generally high. Overall, seven endophytes were found to antagonize fungal as well as bacterial pathogens and showed a high production of active compounds and were therefore considered promising biological control agents.

**Key words:** T-RFLP, 16S rRNA, siderophores, IAA, biocontrol.

**Résumé :** Afin d'étudier l'impact de la croissance végétale sur les bactéries associées à la pomme de terre, la composition et les propriétés des bactéries colonisant l'endosphère de pommes de terres cultivées dans le champs ont été analysées à l'aide d'une approche multiphasique. La fréquence et la diversité des bactéries associées aux pommes de terre ont fait l'objet d'un suivi via une approche indépendante de la culture par l'analyse de T-RFLP de l'ADNr 16S. Les patrons obtenus ont mis en évidence une importante hétérogénéité de la composition des communautés et ont signalé l'existence de communautés spécifiques aux plantes. Toutefois, les populations endophytes ont corrélé dans une certaine mesure avec la performance de croissance des plantes. Des endophytes ont également été isolés de plantes poussant mieux ou moins bien et ont été identifiés par séquençage partiel des gènes de l'ARNr 16S. Un large spectre phylogénétique a été constaté parmi les divers isolats et des plantes croissant différemment étaient l'hôte de populations bactériennes distinctes. Une méthode a été élaborée visant à étudier le potentiel de stimulation de la croissance des plantes chez les bactéries associées aux pommes de terre. Un total de 35 bactéries ont été criblées par l'analyse simultanée de l'antagonisme in vitro envers les champignons pathogènes *Verticillium dahliae*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* et *Phytophthora cactorum*, et envers les bactéries pathogènes *Erwinia carotovora*, *Streptomyces scabies* et *Xanthomonas campestris*. La proportion des isolats ayant une activité antagoniste était la plus élevée contre *Streptomyces* sp. (43 %) suivi par *Xanthomonas* sp. (29 %). Puisque toutes les plantes ont manifesté des symptômes plus ou moins graves de gale provoqués par *Streptomyces scabies*, nous supposons que la présence du pathogène entraîne la colonisation d'antagonistes. L'activité antifongique des isolats était généralement basse. Le potentiel biotechnologique d'isolats endophytes tel qu'évalué par leur activité d'antagonisme et leur production in vitro d'enzymes, d'antibiotiques, de sidérophores et de l'hormone de croissance végétale, l'acide indole-1,3-acétique, était généralement élevé. En tout, sept

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endophytes se sont avérés capables d'antagoniser autant les pathogènes fongiques que bactériens et ont démontré une production élevée de composés actifs; ils sont par conséquent perçus comme des agents de lutte biologique prometteurs.

*Mots clés* : T-RFLP, ARNr 16S, sidérophores, AIA, lutte biologique.

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## Introduction

The study of plant-associated bacteria and their antagonistic potential is important not only for understanding their ecological role and interaction with plants but also for biotechnological applications, e.g., biological control of plant pathogens or isolation of significant compounds (Weller 1988; Emmert and Handelsman 1999; Bloemberg and Lugtenberg 2001).

Endophytic bacteria have been defined by Hallmann et al. (1997) as bacteria that can be isolated from surface-disinfected plant tissues or extracted from within the plant, and additionally, do not visibly harm the plant. Recently, it has been demonstrated that bacterial endophytes may have beneficial effects on host plants, such as growth promotion and biological control of pathogens (Frommel et al. 1991; Chen et al. 1994; Sturz et al. 1997; Downing and Thomson 2000; Adhikari et al. 2001). It has been suggested that bacteria might interact more closely with the host plant, as first expected, and therefore could be efficient biological control agents in sustainable crop production (Sturz et al. 2000). Most information regarding the occurrence of endophytic bacteria and their community structure has been obtained by using cultivation-dependent approaches. Isolation of endophytes is appropriate for functional analysis; however, as a high percentage of naturally occurring bacteria cannot be cultivated, cultivation-independent methods generally provide less biased information on the diversity and community structure. Garbeva et al. (2001) published differences in the potato-associated bacterial community from stem peel and roots using denaturing gradient gel electrophoresis of PCR based on 16S rDNA, and following cloning and sequencing, unknown bacteria were detected. Furthermore, it has been demonstrated by techniques based on 16S rRNA that abiotic as well as biotic stress affect potato endophytic communities (Reiter et al. 2002; Sessitsch et al. 2002). Microenvironment specificity for potato-associated bacteria was shown using terminal restriction fragment length polymorphism (T-RFLP) analysis by Krechel et al. (2002). Some studies indicate that the plant-growth-promoting potential of endophytes is high in comparison to rhizosphere microbes (van Buren et al. 1993; Reiter et al. 2002); however, the role of bacterial endophytes on plant performance is not yet fully understood.

To examine whether the growth performance of field-grown plants is correlated with the associated endophyte microflora, particularly with important functional groups, such as pathogen antagonists or those possessing plant-growth-improving properties, endophytes of plants with different heights and robustness were analyzed by a polyphasic approach. On the one hand, a cultivation-independent approach using T-RFLP analysis of 16S rRNA genes was applied. In addition, endophytes were isolated on 10% tryptic soy agar, identified by

partial 16S rRNA gene analysis, and further characterized regarding their biocontrol activity and their in vitro production of active compounds related to plant growth promotion.

## Materials and methods

### Sampling and isolation of bacteria

Plants were sampled from a organically cultivated potato (*Solanum tuberosum* L. 'Bionta') field in Neustift, Lower Austria. They had fully developed tubers and were ready for harvest and belonged to growth stages 91–97, according to Hack et al. (1993). Plants were classified as well grown (strong) or poorly grown (weak), whereby high plants with a large number of stems or particularly thick stems were characterized as well grown. All plants had more or less severe symptoms of scab disease caused by *Streptomyces scabies*. Fourteen strong (plants 1–14) and fourteen weak plants (plants 15–28) were selected for further analysis. Stems between 5 and 15 cm above ground were used for the isolation of endophytes and endophytic DNA.

Stems were soaked in 5% bleach for 10 min, rinsed four times with sterile, distilled water, rinsed with ethanol, and finally flamed. Subsequently, stems were aseptically peeled and tested for their sterility on 1/10 strength tryptic soy agar plates. No growth was observed. To 0.2–0.5 g of plant material, 3 mL of tryptic soy broth (Merck, Darmstadt, Germany) was added and the tissue was macerated. Aliquots of 50, 100, and 200 µL were plated on 1/10 strength tryptic soy agar. Plates were incubated for 24 h at 28 °C. Colonies of each plate that could be distinguished based on their colony morphology were picked, resulting in a total of 67 isolates, which were further analyzed.

### DNA isolation

To avoid the isolation of surface bacterial DNA, stems were soaked in 5% bleach for 10 min, rinsed four times with sterile, distilled water, rinsed with ethanol, and finally flamed. Subsequently, stems were aseptically peeled. Stem sections (0.2–0.5 g) were amended with 0.8 mL of TN150 buffer (10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl), frozen in liquid nitrogen, and ground in a mixer mill (Type MM2000, 220V, 50 Hz, Retsch GmbH & Co. KG, Haam, Germany) in the presence of two sterilized steel beads (5 mm) at thawing. Then, 0.3 g of 0.1 mm acid-washed glass beads (Sigma Chemical Co., St. Louis, Mo.) was added and bead-beating was performed twice for 1 min at full speed with an interval of 30 s. After extraction with phenol and chloroform, the DNA was precipitated with 0.1 volumes of 3 mol/L sodium acetate solution (pH 5.2) and 0.7 volumes of iso-propanol for 20 min at –20 °C. The DNA was centrifuged for 10 min at 14 000 r/min (1 r = 2π rad; 10 000g),

washed, and dried. Finally, the DNA was resuspended in 60  $\mu\text{L}$  of Tris–EDTA containing RNase (0.1 mg/mL).

### T-RFLP analysis

Partial 16S rRNA gene sequences were amplified with a thermocycler (PTC-100™, MJ Research, Inc., Massachusetts, USA) using an initial denaturing step of 5 min at 95 °C, followed by 30 cycles (each) of 30 s at 95 °C, 1 min annealing at 54 °C, and 2 min extension at 72 °C. PCR reaction mixtures (50  $\mu\text{L}$ ) contained 0.5  $\mu\text{L}$  of extracted DNA; 1 $\times$  reaction buffer (Invitrogen, Carlsbad, Calif., USA); 200  $\mu\text{mol/L}$  (each) dATP, dCTP, dGTP, and dTTP; 2 mmol/L  $\text{MgCl}_2$ ; and 2.5 U *Taq* DNA polymerase (Gibco, BRL); and 0.2  $\mu\text{mol/L}$  of the primers 8f (5'-AGAGTTTGA-TCTGGCTCAG-3') labelled with 6-carboxyfluorescein (6-Fam; MWG) at the 5' end and 926r (5'-CCGTCAATTCCT-TT(AG)AGTTT-3'). Three independent PCR reactions of each sample was pooled and used for subsequent T-RFLP analysis. Approximately 200 ng of fluorescently labelled PCR amplification products was digested with the restriction enzymes *AluI* (Gibco, BRL) as well as with a combination of *HhaI* and *HaeIII* (Gibco, BRL). Aliquots of 0.75  $\mu\text{L}$  were mixed with 1  $\mu\text{L}$  of loading buffer (diluted 5 $\times$  in deionized formamide, Fluka Chemika, Buchs, Switzerland) and 0.3  $\mu\text{L}$  of the DNA fragment length standard (Rox 500; PE Applied Biosystems Inc., Foster City, Calif., USA). Mixtures were denatured for 2 min at 92 °C and immediately chilled on ice prior to electrophoretic separation on 5% polyacrylamide gels. Fluorescently labelled terminal restriction fragments were detected using an ABI 373A automated DNA sequencer (PE Applied Biosystems Inc.) in the GeneScan mode. Lengths of labelled fragments were determined by comparison with the internal standard, using the GeneScan 2.5 software package (PE Applied Biosystems Inc.).

Terminal restriction fragments (T-RFs) between 35 and 500 bp long with heights of  $\geq 50$  fluorescence units were included in the analysis. T-RFs derived from chloroplast and mitochondrial small subunit rRNA genes were excluded from the analysis. Profiles were normalized by adding peak heights (total DNA) in each profile and adjusting the fluorescence intensities to the smallest value. To determine similarities between T-RFLP profiles, a binary matrix recording the absence or presence of T-RFs was established. Cluster analysis was performed based on similarities, as described by Nei and Li (1979), by using the unweighted pair group with mathematical average (UPGMA) method. Tree generation was performed by using the TREECON software package (van de Peer and de Wachter 1994) with 100 bootstrap replications.

### Identification of plant-associated bacteria

For the isolation of genomic DNA from isolates, bacteria were grown overnight in 5 mL of tryptic soy broth in a rotatory shaker at 28 °C. Cells were harvested by centrifugation for 10 min and 6000 r/min (5000g) at 4 °C. After decanting the supernatant, 300 mg of glass beads was added and DNA was isolated as described above.

RFLP analysis of the 16S rRNA gene was used to group isolates at the species level, whereas characterization of the 16S–23S rRNA intergenic spacer (IGS) region was applied

to distinguish different strains of the same species. Small subunit rRNA gene sequences were amplified with a thermocycler (PTC-100™, MJ Research, Inc.) using an initial denaturing step of 5 min at 95 °C, followed by 30 cycles (each) of 30 s at 95 °C, 1 min annealing at 52 °C, and 2 min extension at 72 °C. PCR reaction mixtures (50  $\mu\text{L}$ ) contained 0.5  $\mu\text{L}$  of extracted DNA; 1 $\times$  reaction buffer (Gibco, BRL); 200  $\mu\text{mol/L}$  (each) dATP, dCTP, dGTP, and dTTP; 2 mmol/L  $\text{MgCl}_2$ ; and 2.5 U of *Taq* DNA polymerase (Gibco, BRL); and 0.2  $\mu\text{mol/L}$  of the primers 8f (5'-AGA-GTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTG-ATCCAGCCGCA-3'). The primers pHr (5'-TGCGGC-TGGATCACCTCCTT-3') and P23SR01 (5'-GGCTGCTTC-TAAGCCAAC-3') were used for the amplification of the 16S–23S IGS region. Aliquots of PCR product containing 200 ng of amplified DNA were digested with 5 U of the endonucleases *HhaI* (Gibco, BRL) and *AluI* (Gibco, BRL) individually for 3 h at 37 °C. The resulting DNA fragments were analyzed by gel electrophoresis in 2.5% agarose gels.

A representative isolate of each IGS type was identified by partial 16S rRNA gene sequence analysis. For sequence analysis, 16S rDNA genes were PCR amplified using the primers 8f and pH and the conditions described above. PCR products were purified using the NucleoTraPCR kit (Macherey-Nagel), according to the manufacturer's instructions, and were used as templates in sequencing reactions. Partial DNA sequencing was performed applying either the 16S rRNA gene primer 8f, 518r (5'-ATTACCGCGGCT-GCTGG-3'), or occasionally uni 360s (5'-GGAATCTTC-CACAATGGGCG-3') by the dideoxy chain termination method using an ABI 373A automated DNA sequencer and the ABI PRISM Big Dye terminator cycle sequencing kit (PE Applied Biosystems Inc.). Sequences were subjected to BLAST analysis (Altschul et al. 1997) with the National Center for Biotechnology Information database.

### Screening of bacteria for antagonism towards plant pathogens

Bacterial isolates were screened for their activity towards plant pathogenic microorganisms by a dual culture in vitro assay on Waksman agar (WA) containing 5 g of proteose-peptone (Merck), 10 g of glucose (Merck), 3 g of meat extract (Chemex, München, Germany), 5 g of NaCl (Merck), 20 g of agar (Difco), and distilled water (to 1 L), pH 6.8. Zones of inhibition were measured after 5 days of incubation at 20 °C, according to Berg et al. (2002). All strains were tested in three independent replicates with (i) *Verticillium dahliae* V16 (isolated from *Solanum tuberosum* L.), (ii) *Rhizoctonia solani* Kühn (culture collection of the University of Rostock, Microbiology), (iii) *Sclerotinia sclerotiorum* Lib., and (iv) *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. PF8 (culture collection of the Federal Biological Research Centre for Agriculture and Forestry, Darmstadt, Germany). These fungi were routinely grown on Sabouraud medium (Gibco) and stored at –70 °C in broth containing 15% glycerol. The in vitro inhibition of *Erwinia carotovora* subsp. *atroseptica* DSM 30168, *Streptomyces scabies* DSM 41658, and *Xanthomonas campestris* DZM 3586 was determined in dual culture assay in Luria–Bertani agar (LB, Difco) in microtiter plates. Ten microlitres of an

overnight culture of the bacterial pathogens was mixed with LB agar, and the isolates to be tested were spotted on the solidified agar surface. Zones of inhibition were measured after incubation at 20 °C for 24 and 48 h.

### Bacterial characterization based on production of hydrolytic enzymes and secondary metabolites

Production of cell-wall-degrading enzymes and secondary metabolites is a common mechanism used by bacteria to inhibit growth of other microorganisms. For a better characterization of the antagonistic bacterial isolates, their potential to produce hydrolytic enzymes and secondary metabolites was studied. Chitinase activity ( $\beta$ -1,4-glucosamine polymer degradation) and pectinase activity were tested in minimal medium, according to Chernin et al. (1995). Clearing zones were detected 5 days after incubation at 20 °C.  $\beta$ -Glucanase was tested using chromogenic azurine-dyed cross-linked (AZCL) and reamazolbrilliant blue R substrates (Megazyme, Bray, Ireland), respectively. Formation of blue haloes was recorded until 5 days after incubation. Protease activity indicated by casein degradation was determined from clearing zones in skim milk agar (50 mL sterilized skimmed milk mixed at 55 °C with 50 mL of 1/5 tryptic soy agar and 4% agar) after 5 days of incubation at 20 °C.

The ability of bacterial isolates to produce indole-3-acetic acid (IAA) was determined using the microplate method developed by Sawar and Kremer (1995). Antibiosis against *Rhizoctonia* sp. by the bacterial strains was assayed on WA plates (15 mL) containing 5 mL sterile culture filtrate (64 h culture, nutrient broth II (Sifin, Berlin, Germany)). The pH was adjusted to between 7 and 8. A 5-mm plug from a *Verticillium dahliae* agar plate was placed in the center of a WA plate. As a control, WA plates (20 mL) were similarly inoculated with mycelial plugs. Colony diameters were measured daily for 10 days, and the reduction (%) in linear growth of the fungi was calculated. Siderophore production was assayed according to the method of Schwyn and Neilands (1987).

### Screening of isolated bacteria for biocontrol activity in vivo

Isolated endophytes were tested for their ability to promote plant growth, using *Solanum tuberosum* L. 'Merkur'. Plantlets grown in tissue cultures were dipped in bacterial endophyte cultures ( $1 \times 10^8$  cells) and subsequently transplanted on MS agar (Murashige and Skoog 1962). After incubation for 3 weeks in a growth chamber at 22 °C with a light period of 14 h/day, plant growth performance was scored. Control plants were treated with 0.9% sterile NaCl solution.

### Assessment of in vitro screening

All the tested parameters were used to efficiently select potential biological control agents from the endophytic strains, according to Berg et al. (2001).

### Nucleotide sequence numbers

The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AY336530–AY336564.

## Results

### Molecular fingerprinting of potato-associated bacterial communities

The number of T-RFs derived from bacterial endophytes was obtained and ranged from 9 (plants 12, 14, 16, and 19) to 17 (plants 5 and 23) (Fig. 1). In general, the better-growing plants contained a higher richness (on average 13 T-RFs) than the weak plants (on average 11 T-RFs). All plants showed different endophytic communities, but a certain degree of correlation between bacteria associated with strong plants and those associated with weak plants was observed (Fig. 1).

### Identification of potato-associated bacteria

A total of 67 bacterial isolates were characterized by 16S rRNA RFLP and proved to belong to 21 16S rRNA types. To differentiate isolates at the strain level, bacteria with identical 16S rRNA patterns were further analyzed by 16S–23S rRNA IGS RFLP analysis. In total, 35 strains showing different IGS regions were found. For identification, the 16S rRNA genes of representative isolates of each IGS type were partially sequenced and sequences were compared with entries available in public databases (Table 1). Endophytic 16S rRNA genes showed high homology to known sequences belonging to the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -*Proteobacteria*, to low and high G+C Gram positives, as well as to the *Cytophaga/Flavobacterium/Bacteroides* phylum. Fifteen different genera were found; however, only five genera could be detected on both plant groups: *Clavibacter*, *Frigoribacterium*, *Pantoea*, *Pseudomonas*, and *Sphingomonas*. At the species level, only *Clavibacter michiganensis* was able to colonize weak and strong plants. In better-growing plants, high G+C Gram positives and  $\gamma$ -*Proteobacteria* were highly prominent, whereas from other plants mainly low G+C Gram positives were isolated.  $\gamma$ -*Proteobacteria* were equally abundant in strong and weak plants (Table 1).

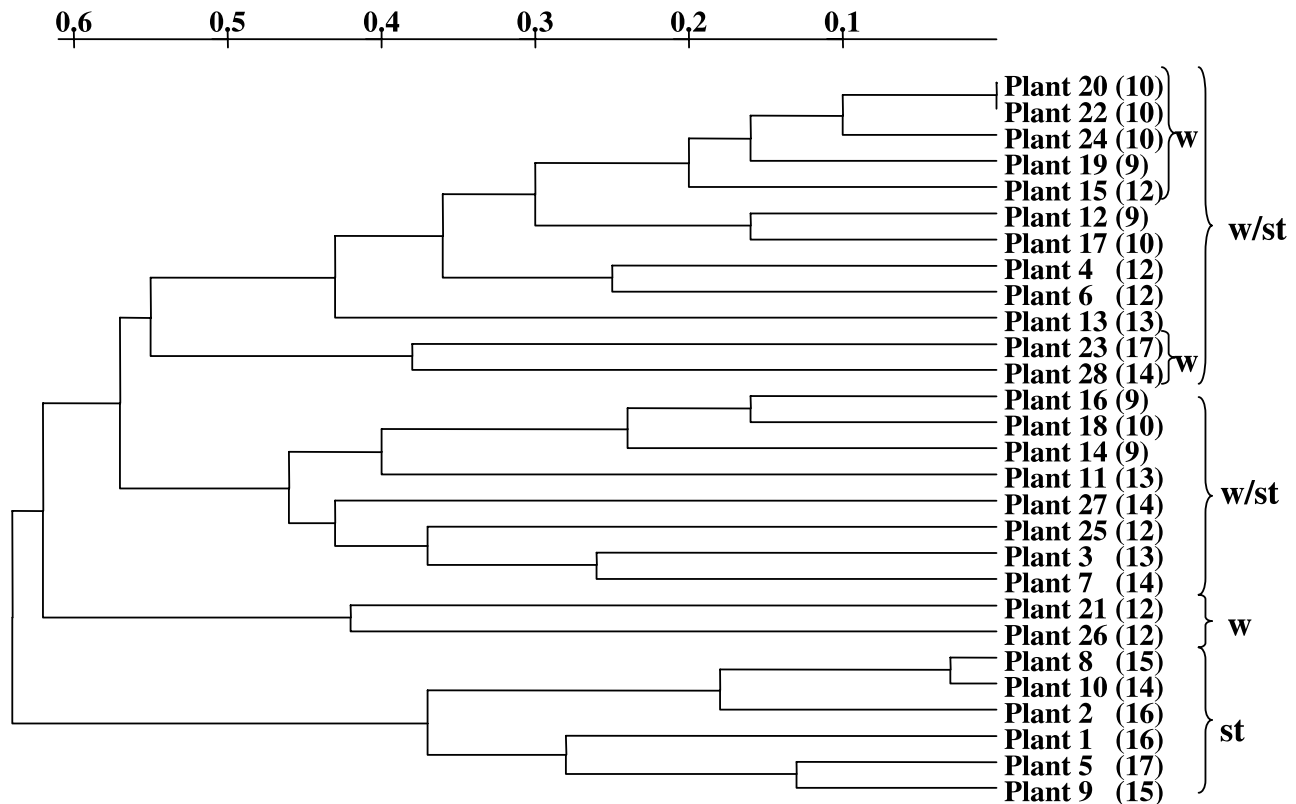
### Screening of bacteria for plant growth promotion

The isolates were tested for their ability to promote growth of potato plants. Altogether 14 isolates (= 40%) caused a promotion in plant growth as compared with the control (Table 1). The highest effect was found for the following strains: *Arthrobacter* sp. pFB2, *Arthrobacter* sp. pFB3, *Pseudomonas* sp. pFB8, *Brevundimonas* sp. pFB9, and *Arthrobacter* sp. pFB10. These isolates were obtained from strong plants.

### Screening of bacteria for antagonism towards fungal and bacterial pathogens

All the selected endophytic isolates were tested in vitro for their activity against the fungal plant pathogens *Verticillium dahliae* (ascomycete with a chitin–glucan-containing cell wall), *Rhizoctonia solani* (basidiomycete with a chitin–glucan-containing cell wall), *Sclerotinia sclerotiorum* (ascomycete with a chitin–glucan-containing cell wall), and *Phytophthora cactorum* (oomycete with a cellulose-containing cell wall) (Table 2). A rather small proportion of endophytes showed antifungal activity (0%–11%). Only two isolates (*Paenibacillus* sp. pFB19, *Methylobacterium* sp. pFB20) showed antagonistic activity against the soilborne *Verti-*

**Fig. 1.** Unweighted pair group with mathematical average dendrogram generated from all terminal restriction fragment length polymorphism profiles. The scale indicates the distance level. The number of terminal restriction fragments in each individual sample is given in parentheses. s, strain was isolated from a strong plant; w, strain was isolated from a weak plant; w/st, strain was isolated from a weak or strong plant.



*cillium dahliae*. The same isolates, and additionally pFB26 (*Clavibacter michiganensis*), were able to antagonize *Rhizoctonia solani*, which is also an important soilborne pathogen of potato. Four isolates showed antagonistic activity against *Sclerotinia sclerotiorum*. Against the widely distributed potato pathogen *Phytophthora cactorum*, no isolate with antagonistic activity was found. Additionally, isolates were tested for their antibacterial activity. While no antagonists against *Erwinia* sp. were found, a high proportion of isolates was able to antagonize *Streptomyces* sp. (15 isolates = 43%) as well as *Xanthomonas* sp. (10 isolates = 29%). Altogether 15 isolates showed activity against *Streptomyces* sp. and six of them showed also activity against *Xanthomonas* sp. Almost all  $\gamma$ -Proteobacteria showed biocontrol activities against *Streptomyces scabies*. Generally, selective antagonistic activity was widely distributed among endophytic isolates. Only isolates of the genera *Paenibacillus*, *Clavibacter*, and *Pseudomonas* showed antifungal as well as antibacterial activity. The highest antagonistic spectrum was found for pFB26 (*Clavibacter michiganensis*), which was antagonistic towards the genera *Rhizoctonia*, *Sclerotinia*, *Streptomyces*, and *Xanthomonas*. *Pseudomonas rhodesiae* pFB13 and *Paenibacillus* sp. pFB33 were active against three different pathogens.

#### Production of hydrolytic enzymes and secondary metabolites

Representative isolates were characterized regarding their production of hydrolytic enzymes and secondary metabolites

(Tables 1 and 2). In general, endophytic bacteria produced a high spectrum of hydrolytic enzymes (Table 2).  $\beta$ -1,3-Glucanase production was found in many isolates (43%), but only 5 out of 35 isolates (14%) showed chitinolytic activity. Proteolytic activity was detected for 12 isolates (34%) and pectinolytic activity for 11 isolates (31%). Two isolates, *Sphingomonas* sp. pFB1 and *Paenibacillus* sp. pFB33, produced all four enzymes tested.

Siderophores were produced by 77% of the endophytic isolates (Table 1). Sixteen isolates obtained from strong plants were able to produce siderophores, whereas only 10 strains from weak plants had this ability. Additionally, near half of the isolates were able to produce the plant growth hormone IAA in vitro, whereby nine of them were obtained from better-growing plants and seven from weak plants (Table 1).

#### Assessing the biotechnological capacities of potato-associated endophytic bacteria

In an attempt to better select bacterial isolates with high antagonistic and biotechnological potential, points were given for each bacterial trait determined within this study. Up to seven points were given for antagonistic activity towards plant pathogens and seven points for hydrolytic enzymes (glucanases, chitinases, proteinases, and pectinases), siderophores, antibiotic production, and auxin production. A total number of 14 points were possible. For the 35 endophytes tested, the number of points varied between 2 and 10. The isolate with the highest number of points was

**Table 1.** Identification of isolates by partial 16S rDNA sequence analysis and plant-growth-promoting activities.

Isolate	Origin	Closest database match (accession No.)	% homology	Potato plant-growth-promoting activity*	Siderophore production	Indole-3-acetic acid production
<b><math>\alpha</math>-Proteobacteria</b>						
pfB1	st	<i>Sphingomonas aurantiaca</i> (AJ429237)	99	nd	+	-
pfB9	st	<i>Brevundimonas</i> sp. (AY177781)	98	++	+	-
pfB20	w	<i>Methylobacterium</i> sp. II (AY177351)	99	nd	+	-
pfB21	w	<i>Sphingomonas</i> sp. M3C203B-B (AF395031)	100	-	-	+
pfB27	w	<i>Sphingomonas aurantiaca</i> (AJ429237)	99	-	-	-
<b><math>\beta</math>-Proteobacteria</b>						
pfB24	w	Uncultured eubacterium WD270 (AJ292636)	99	-	+	+
pfB29	w	<i>Variovorax paradoxus</i> (AF451851)	99	+	+	-
<b><math>\gamma</math>-Proteobacteria</b>						
pfB8	st	<i>Pseudomonas</i> sp. WBC-3 (AY040872)	98	++	+	+
pfB12	st	<i>Pseudomonas</i> sp. NZ011 (AY014803)	99	-	++	+
pfB13	st	<i>Pseudomonas rhodesiae</i> (AF064459)	99	-	++	-
pfB18	st	<i>Pseudomonas oryzihabitans</i> (AF501349)	99	-	+	-
pfB35	w	<i>Pseudomonas</i> sp. NZ062 (AY014814)	99	-	nd	nd
pfB16	st	<i>Pantoea ananatis</i> (AF364847)	99	-	+	+
pfB25	w	<i>Pantoea agglomerans</i> (AF373197)	98	-	+	+
<b>Low G+C Gram positives</b>						
pfB19	w	<i>Paenibacillus</i> sp. V2 (AF324200)	99	nd	+	-
pfB33	w	<i>Paenibacillus</i> sp. V2 (AF324200)	99	+	++	+
pfB23	w	<i>Staphylococcus</i> sp. (X86636)	99	+	-	-
<b>High G+C Gram positives</b>						
pfB2	st	<i>Arthrobacter</i> sp. IBN110 (AY247743)	99	++	+	-
pfB3	st	<i>Arthrobacter</i> sp. SMCC ZAT262 (AF197055)	99	++	+	+
pfB10	st	<i>Arthrobacter</i> sp. 12E (AY191847)	100	++	+	-
pfB5	st	<i>Frigoribacterium</i> sp. 312 (AF157480)	98	-	+	+
pfB6	st	<i>Frigoribacterium</i> sp. 312 (AF157480)	99	-	-	-
pfB17	st	<i>Frigoribacterium</i> sp. 312 (AF157480)	99	-	+	+
pfB31	w	<i>Frigoribacterium</i> sp. 301 (AF157479)	99	+	+	-
pfB7	st	<i>Microbacterium testaceum</i> (AF474327)	97	+	+	+
pfB11	st	<i>Microbacterium</i> sp. LB030 (AF474326)	99	-	+	+
pfB4	st	<i>Clavibacter michiganensis</i> (AF406667)	99	+	+	-
pfB14	st	<i>Clavibacter michiganensis</i> (AJ310415)	98	-	+	+
pfB22	w	<i>Clavibacter michiganensis</i> (AJ310415)	99	-	-	+
pfB26	w	<i>Clavibacter michiganensis</i> (AF406667)	99	-	+++	+
pfB28	w	<i>Clavibacter michiganensis</i> (AJ310415)	98	+	-	-
pfB15	st	Uncultured hydrocarbon seep bacterium (AF154098)	99	-	-	+
pfB30	w	<i>Rhodococcus</i> sp. P_wp0233 (AY188941)	99	-	+	-
pfB34	w	<i>Streptomyces bottropensis</i> (AF397893)	100	nd	++	-
<b>Cytophaga/Flavobacterium/Bacteroides</b>						
pfB32	w	<i>Flavobacterium</i> sp. 36 (AF388029)	98	-	++	+

Note: s, strain was isolated from a strong plant; w, strain was isolated from a weak plant; nd, not determined; -, no activity; +, moderate activity; ++, high activity; +++, very high activity.

\*Growth promotion of potato tissue cultures.

*Paenibacillus* sp. pfB33, isolated from the endosphere of a poorly performing potato plant. *Methylobacterium* sp. pfB20 and *Clavibacter michiganensis* pfB26 reached eight points. A high biotechnological potential was also found for strains pfB13, pfB14, pfB19, and pfB24. On average, the highest antagonistic potential was achieved for bacterial isolates be-

longing to the low G+C Gram positives, with 6.3 points on average, followed by isolates belonging to the  $\alpha$ - and  $\gamma$ -*Proteobacteria*, with 5.0 points on average.  $\beta$ -*Proteobacteria*, which were only represented by two isolates (pfB24, pfB29), reached six points. Only 3.6 points were found for high G+C Gram positives, the largest group. No differences

in the biotechnological potential of strains isolated from plants with different growth performances could be detected.

## Discussion

Various reports demonstrated that bacterial endophytes contribute to the growth and health of a variety of plants (reviewed by Sturz et al. 2000). The mechanisms by which plant growth is improved may be similar to those exhibited by rhizosphere microorganisms and include the production of phytohormones, promotion through enhanced availability of nutrients, reduction of ethylene levels, production of antibiotics, induced systemic resistance, and outcompetition of pathogens. Sturz et al. (1999) suggested that functioning communities of microbial endophytes contribute to the resistance of potato plants to bacterial soft rot. In addition, it has been reported that the community structure of bacterial endophytes colonizing potato clearly correlates with the absence or presence of a phytopathogen (Reiter et al. 2002). It has also been shown that abiotic plant stress, such as light deficiency, leads to a decreased diversity of the potato endosphere microflora, probably due to stress metabolites and enzymes produced by the plant (Sessitsch et al. 2002). In accordance with these findings, our results showed that the bacterial community structure correlated to a certain extent with the growth of field-grown plants. In general, strong plants contained a higher richness of bacterial species (= number of T-RFs) than did weak plants. However, it is not clear from these results, whether the endophytic microflora is only affected by plant stress metabolites or whether specific microbial populations are also responsible for the good or bad performance of a plant. The observed variability between populations in individual plants may be due to the analysis of field-grown plants. It has been suggested that the majority of endophytes derive from the rhizosphere soil (Sturz et al. 2000; McInroy and Kloepper 1995), which may host more variable populations in a field than in a greenhouse experiment.

Cultivation-independent analysis generally does not give any information on the function of individual community members. Therefore, endophytes were isolated from potato stems, identified by partial 16S rDNA sequencing, and characterized regarding potential plant-growth-promoting abilities. In agreement with cultivation-independent analysis a slightly higher richness of bacterial species was found in strong plants (18 different IGS rRNA types) than in weak plants (16 different IGS rRNA types). However, this effect may not be significant. Nevertheless, the culturable endophytic bacterial populations found in weak and strong plants were highly different. Striking was the high abundance of high G+C Gram positives and  $\gamma$ -*Proteobacteria* in better-growing plants. Among them various *Arthrobacter* species as well as several isolates possessing different 16S rRNA genes showing highest homology to *Frigoribacterium* sp. 312 were found. Evenmore, all three *Arthrobacter* isolates showed high plant-growth-promoting ability, although members of this genus are rather known for their capacity to degrade a variety of toxic organic substances than for plant beneficial effects. Several isolates were identified as *Clavibacter michiganensis*, the bacterium that causes potato ring rot disease. One of these strains showed high

siderophore and IAA production and was even able to antagonize *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Streptomyces scabies*. Similarly, Reiter et al. (2002) isolated an endophytic *Clavibacter michiganensis* strain from potato with biocontrol activities against *Erwinia carotovora*. These antagonistic *Clavibacter* strains may belong to the group of "low pathogens" which belong to a pathogenic species but show a beneficial interaction with plants. Similar phenomena are known from apathogenic *Pseudomonas syringae* or *Fusarium oxysporum* strains which are already used as biocontrol strains. However, a clear molecular evidence for apathogenicity should be the basis for such applications. Other bacterial groups including  $\beta$ -*Proteobacteria*, low G+C Gram positives, and members of the *Cytophaga/Flavobacterium/Bacteroides* were only detected in weak plants.

Five isolates strongly promoted growth of in vitro potato plants. They all derived from better growing plants, however also weak plants hosted bacteria with growth-promoting abilities. About 50% of the isolates had the ability to produce IAA, which is known to stimulate both rapid and long-term responses in plants (Cleland 1990; Hagen 1990). The percentage of IAA-producing endophytes was lower than the 80% reported for rhizosphere bacteria on average (Patten and Glick 1996) but more than 33% found in the rhizosphere of field-grown potato plants found by Lottmann et al. (1999). Better-growing plants hosted slightly more IAA producers than weak plants (10 vs. 7). Almost all isolated endophytes were able to synthesize siderophores. Usually, these low molecular weight compounds are produced by various soil microbes to bind  $Fe^{3+}$ , transport it back to the microbial cell, and make it available for growth (Leong 1986; Neilands and Leong 1986). Microbial siderophores may also be utilized by plants as Fe source (Wang et al. 1993; Bar-Ness 1991). Endophytes have to compete with plant cells for Fe supply, and therefore siderophore production may be highly important for endophytic growth. Additionally, the production of siderophores has been reported to be one mechanism to outcompete pathogens (O'Sullivan and O'Gara 1992; Schippers et al. 1987) and may have the same function in endophytes.

A variety of endophytes showing antagonistic activities against bacterial and fungal pathogens have been reported (see reviews by Lodewyckx et al. 2002 and Sturz et al. 2000). Furthermore, the proportion of endophytes able to suppress disease symptoms has been found to be high in comparison to that observed for rhizosphere bacteria (Reiter et al. 2002; Chen et al. 1995; van Buren et al. 1993). In agreement with these reports, in this study a high percentage of the isolated potato endophytes showed antagonistic activities towards *Streptomyces scabies* (43%) and *Xanthomonas campestris* (29%). As the majority of plants had more or less severe scab disease symptoms (caused by *Streptomyces scabies*), we concluded that the presence of the phytopathogen may have induced the colonization of appropriate antagonists. The proportion of strains showing biocontrol activities towards other pathogens was comparably low. However, only direct effects, e.g., antibiosis, were tested, and isolates may exhibit other mechanisms, such as outcompetition or induction of systemic resistance, to fight off pathogens. Biocontrol activities were found in phylogenetically different bacteria, although this characteristic was particularly

**Table 2.** Biocontrol activities of isolates.

Isolate	Origin	Antagonistic activities against:				
		<i>Verticillium dahliae</i>	<i>Rhizoctonia solani</i>	<i>Sclerotinia sclerotiorum</i>	<i>Phytophthora catorum</i>	<i>Erwinia carotovora</i> ssp. <i>atroseptica</i>
<b><i>α-Proteobacteria</i></b>						
pfB1	st	–	–	–	–	–
pfB9	st	–	–	–	–	–
pfB20	w	+	+	–	–	–
pfB21	w	–	–	–	–	–
pfB27	w	–	–	–	–	–
<b><i>β-Proteobacteria</i></b>						
pfB24	w	–	–	–	–	–
pfB29	w	–	–	–	–	–
<b><i>γ-Proteobacteria</i></b>						
pfB8	st	–	–	–	–	–
pfB12	st	–	–	+	–	–
pfB13	st	–	–	+	–	–
pfB18	st	–	–	–	–	–
pfB35	w	nd	nd	nd	nd	nd
pfB16	st	–	–	–	–	–
pfB25	w	–	–	–	–	–
<b>Low G+C Gram positives</b>						
pfB19	w	+	+	–	–	–
pfB33	w	–	–	+	–	–
pfB23	w	–	–	–	–	–
<b>High G+C Gram positives</b>						
pfB2	st	–	–	–	–	–
pfB3	st	–	–	–	–	–
pfB10	st	–	–	–	–	–
pfB5	st	–	–	–	–	–
pfB6	st	–	–	–	–	–
pfB17	st	–	–	–	–	–
pfB31	w	–	–	–	–	–
pfB7	st	–	–	–	–	–
pfB11	st	–	–	–	–	–
pfB4	st	–	–	–	–	–
pfB14	st	–	–	–	–	–
pfB22	w	–	–	–	–	–
pfB26	w	–	+	+	–	–
pfB28	w	–	–	–	–	–
pfB15	st	–	–	–	–	–
pfB30	w	–	–	–	–	–
pfB34	w	–	–	–	–	–
<b><i>Cytophaga/Flavobacterium/Bacteroides</i></b>						
pfB32	w	–	–	–	–	–

**Note:** s, strain was isolated from a strong plant; w, strain was isolated from a weak plant; nd, not determined; –, no activity; +, moderate activity; ++, high activity; +++, very high activity; +++++, extremely high activity.

\*% inhibition of *Rhizoctonia solani* after 3 days.

prominent among *γ-Proteobacteria*. Our results indicate that the hydrolytic enzyme activities did not necessarily correlate with pathogen antagonism. Nevertheless, some of the hydrolytic enzyme activity may be important for endophytes to gain entry into the plant, as it is well-known that bacteria (pathogens and endophytes) can penetrate plant cells by cell-

wall-degrading enzymes (Quadt-Hallmann et al. 1997; Baras et al. 1994; Huang 1986).

Endophytes are promising candidates for use in agriculture for biocontrol and fortification of plants. They colonize the same niche as plant pathogens and may therefore be better suited than rhizosphere bacteria to either outcompete



<i>Streptomyces scabies</i>	<i>Xanthomonas campestris</i> ssp. <i>carotae</i>	Hydrolytic enzyme production					Antibiotic*
		Glucanase	Chitinase	Protease	Pectinase		
-	-	++	+	+	+	49	
-	-	++	-	++	+	54	
-	+	++	++	-	+	58	
-	-	++	-	-	-	59	
-	+	-	-	-	-	53	
+	-	+	++	-	+	54	
+	-	++	-	-	+	55	
+	+	-	-	-	-	54	
+	-	-	-	+++	-	56	
+	+++	-	-	+++	+	48	
-	+++	-	-	++	-	49	
nd	nd	nd	nd	nd	nd	nd	
+	-	-	-	-	-	48	
+	-	-	-	-	-	53	
-	-	++	++	-	+	56	
+	++	++++	++	++++	+	53	
-	+	-	-	-	-	55	
-	-	-	-	+	-	49	
-	-	++	-	++	-	55	
-	-	-	-	+	+	55	
-	-	++	-	-	-	62	
-	-	++	-	-	-	58	
+	+	-	-	-	-	56	
-	-	-	-	-	-	53	
-	-	-	-	-	-	55	
-	-	-	-	-	-	56	
-	-	+	-	++	-	56	
+	+	+++	-	-	+	51	
-	-	+	-	-	-	60	
+	+++	-	-	+++	-	53	
+	-	-	-	-	-	50	
+	-	-	-	-	-	55	
-	-	-	-	-	-	54	
+	-	-	-	+++	-	49	
+	-	++	-	-	+	55	

or directly antagonize pathogens. Because of the intimate contact between endophytes and plant cells, induced systemic resistance may be an important mechanism of biocontrol (Nejad and Johnson 2000; Hallmann et al. 1997; Benhamou et al. 1996). Our results clearly show that endophytes belonging to different phylogenetic groups may

exhibit various properties that are highly interesting for biotechnological application. However, appropriate inoculation strategies have to be developed that guarantee the entry of the inoculant strain into the plant and subsequent successful colonization. This study also showed that differently growing plants are colonized by distinct bacterial endophytes,

which may vary in their beneficial effects. A better understanding of the functioning of endophytes and plant growth will lead to the identification of methods to support and improve endophytic bacterial populations.

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