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ORIGINAL PAPER

Structural characteristics and plant-beneficial effects of bacteria colonizing the shoots of field grown conventional and genetically modified T4-lysozyme producing potatoes

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Abstract Genetically modified potatoes expressing antibacterial protein T4 lysozyme may offer effective control strategies for bacterial pathogens causing severe potato diseases. Apart from this beneficial effect, it is very important to investigate such engineered potatoes carefully for potential adverse effects on potato-associated bacteria which frequently exhibit plant beneficial functions such as plant growth promotion and antagonism towards pathogens invading the plant. Two field experiments were carried out in Spain to analyze the potential effects of conventional and genetically modified T4-lysozyme producing potatoes on shoot-associated bacteria. The first baseline field trial 2002 was performed in Meliana in which three conventional potato lines, Achirana Inta, Desirée, and Merkur, were cultivated and sampled at flowering. The second field trial was conducted in Cella in 2003 in order to compare the effects of a senescent transgenic, T4 lysozyme expressing potato trait, Desirée DL 12, with its isogenic, non-transformed parental line Desirée. Structural characteristics of potato shoot-associated bacteria was assayed by 16S rRNA-based terminal restriction fragment length polymorphism (T-RFLP) analysis and dominant community members within T-RFLP profiles were identified by sequence analysis of generated 16S rRNA gene libraries. Cultivable bacteria isolated from shoots of potatoes grown in the Meliana field trial were monitored for antibiosis against *Ralstonia solanacearum*, whereas isolates derived from shoots of potatoes cultivated in the Cella trial were screened for antagonism against *Ralstonia solanacearum* and *Rhizoctonia solani*, and for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production. Determined antagonists were identified by 16S rRNA gene analysis. All potato traits hosted a cultivar-specific community of bacteria with antagonism against the pathogens and/or potential to produce

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ACC deaminase. Several antagonists obtained from the Cella field potatoes were also observed as ACC deaminase producers. Community profiling revealed a greater diversity differentiation between the senescent T4 lysozyme expressing and parental Desirée lines grown in the Cella field as compared to the variations between the three flowering conventional lines cultivated in the Meliana field trial. Effects of the two varying field sites and different vegetation stages were greater than those of T4 lysozyme when investigating the community composition of bacteria colonizing the shoots of the Desirée line cultivated in both field trials.

Keywords T4 lysozyme expressing potatoes · Shoot-associated bacteria · Community composition · Functional abilities

Introduction

The production of potato (*Solanum tuberosum* L.) suffers from severe diseases caused by a broad range of bacterial (e.g. *Ralstonia solanacearum* and *Erwinia carotovora* ssp.) and fungal phytopathogens (e.g. *Rhizoctonia solani*) (Oerke et al. 1994). Resistance traits are difficult to achieve by conventional breeding, and the application of antibiotics as a chemical control strategy is inappropriate due to rapid development of resistance to antibiotics in bacteria. The genetic modification of potatoes with antibacterial T4 lysozyme (Düring et al. 1993) could be a promising approach to limit the detrimental impact of potato pathogens. T4 lysozyme exhibits a strong lytic activity against bacterial cell walls (Düring et al. 1999; Tsugita et al. 1968), and might be therefore effective in combating pathogenic bacteria invading the potato (de Vries et al. 1999). Düring et al. (1993) have shown that T4 lysozyme expressing potatoes exhibit enhanced resistance against the potato pathogen *Erwinia carotovora*.

Plant-associated bacteria may confer beneficial effects on plant growth and exhibit antagonistic activities towards phytopathogens (Sessitsch et al. 2004; Lodewyckx et al. 2002; Sturz et al. 2000; Lugtenberg et al. 1991). An additional important function of plant-associated bacteria is their ability to produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Penrose and Glick 2003). This enzyme cleaves the plant ethylene precursor ACC (Yang and Hoffman 1984), and thereby lowers the ethylene level in a stressed plant. Mayak et al. (2004) and Grichko and Glick (2001) have shown that ACC deaminase producing bacteria have beneficial effects on plants exposed to abiotic stress. Plant-associated bacteria may further act as biocontrol agents by producing antibacterial or antifungal agents (Chernin and Chet 2002), siderophores (Kloepper et al. 1980), or they may induce indirectly systemic acquired host resistance or immunity (Chen et al. 1995).

It is therefore appropriate to assay genetically modified potatoes producing antibacterial T4 lysozyme for their potential adverse effects on plant-associated bacteria and to relate these to effects of conventional, unmodified cultivars. T4 lysozyme diffuses from the plant cell into the intercellular space where it subsequently contacts with bacteria (de Vries et al. 1999). Thus, it can be anticipated that this release of T4 lysozyme could alter the affected bacterial community composition which may be then different from that of the corresponding unmodified, isogenic parental line. Consequently, such a diversity modification might lead to alterations of relevant plant-beneficial functions of affected shoot-associated bacteria. Most studies assaying T4 lysozyme expressing potatoes have focused on potential effects on rhizosphere soil microbiota (e.g. Rasche et al. 2006a; Heuer et al. 2002; Ahrenholtz et al. 2000), but little attention has been paid to shoot-associated bacteria which colonize the endosphere and phyllosphere of plants (Rasche et al. 2006b; Heuer and Smalla 1999).

Field experiments are suitable for assaying the effects of different crops on plant-associated, potentially beneficial bacteria under natural growth conditions. In this study, field experiments were conducted in Spain to assess the effects of conventional and transgenic, T4 lysozyme expressing potato cultivars on the diversity and function of potato shoot-associated bacteria. The first experimental baseline field trial 2002 was performed in Meliana with three conventional potato cultivars, Achirana Inta, Desirée, and Merkur. A second field experiment was

conducted in Cella in 2003 to compare the effects of a transgenic, T4 lysozyme expressing potato trait, Desirée DL 12, with the corresponding isogenic, non-transformed wild-type line Desirée. Community fingerprinting of potato shoot-associated bacteria was performed by 16S rRNA-based terminal restriction fragment length polymorphism (T-RFLP) analysis and dominant community members within T-RFLP fingerprint profiles were identified by sequence analysis of generated 16S rRNA gene libraries. In addition, cultivable shoot-associated bacteria were isolated, and monitored for ACC deaminase production and antagonism against potato pathogens *Ralstonia solanecearum* and *Rhizoctonia solani*. Bacterial antagonists were then identified by 16S rRNA gene analysis.

Materials and methods

Field experiments and sampling

Two field experiments were performed in the years 2002 (Meliana, Valencia Province, Spain) and 2003 (Cella, Teruel Province, Spain). The field sites differed mainly in soil properties (Meliana: sand (50.4%), silt (20.4%), clay (29.2%), organic carbon (1.0%), pH value (8.4)); Cella: sand (34.6%), silt (30.0%), clay (35.4%), organic carbon (1.4%), pH value (8.7)), and climatic characteristics (presented climate data are monthly average values from May to August) (Meliana: temperature (22.7°C), relative humidity (66%), precipitation (21 mm), sun hours (282 h); Cella: temperature (18.6°C), relative humidity (57%), precipitation (42 mm), sun hours (291 h). Both experiments were set up in a randomized block design. In detail, the Meliana trial was performed with 12 plots (plot size, 5.0 by 3.0 m). For each of the three conventional *Solanum tuberosum* L. cultivars, Achirana Inta (AIC), Desirée (DC), and Merkur (MC), four replicate plots were arranged. In July, 60 tubers of each cultivar were planted per plot and distributed in four rows of 15 plants each. The Cella trial was assayed with eight plots (plot size, 3.5 by 1.5 m) with four replicate plots for each of the two Desirée cultivars, DC and the T4 lysozyme expressing line DL 12 (DL). Twenty tubers of each cultivar were planted in May in every plot and distributed in four rows of five plants each.

The T4 lysozyme expressing potato line DL 12 harbored the T-DNA of the binary vector pSR8-30 (Düring 1994) containing the *nptII* marker gene and one T4 lysozyme gene in a small polylinker site (Düring, 1996). The GM potatoes constitutively expressed T4 lysozyme under control of the cauliflower mosaic virus 35S promoter (Düring et al. 1993). The lysozyme gene was fused to the barley α-amilase signal peptide gene. This leading sequence caused the secretion of the lysozyme into the intercellular spaces (Düring et al. 1993; Hippe et al. 1989). T4 lysozyme expression in DL 12 cultivars was verified by real-time PCR as well as by Northern-blot hybridizations using RNA isolated from roots and shoots in other experiments (data not shown).

Sampling of potatoes of the 2002 field trial was performed at flowering (vegetation stage 6 as defined by Hack et al. 1993), whereas plants of the second field trial 2003 were sampled at senescent stage shortly before harvest of tubers (vegetation stage 9 as defined by Hack et al. 1993). Four composite stem samples per cultivar were obtained at each sampling date. Shoot sections (0–5 cm above ground) were cut into pieces of 0.5–1.0 cm length. These pieces of each cultivar were mixed and divided into two portions ranging in weight of approximately 1.0 g. Both shoot portions were surface-sterilized with 70% ethanol followed by rinsing in sterile distilled water. One portion was dedicated to plate count and isolation of bacteria, whereas the other part was frozen and stored at –20°C until further cultivation-independent analysis.

Isolation of shoot-associated bacteria

For the isolation of shoot-associated bacteria, sterilized shoot material was homogenized using mortar and pestle in 3 ml of sodium phosphate buffer (19.9 g $Na_2HPO_4 \times H_2O$, 1.27 g $NaH_2PO_4 \times 2H_2O$, 1 l H_2O, pH 8.0). Plate counts were determined on R2A medium (Difco) supplemented with 100 mg cycloheximide l^{-1} to inhibit fungal growth. Dishes were incubated at 25°C and colony forming units (cfu) were counted after four days incubation. Representative colonies of

different morphology were selected, isolated, purified on King's B agar (King et al. 1954), and stored at –80°C with 30% glycerol.

Screening of isolated bacteria for antagonistic activity against *Ralstonia solanacearum* and *Rhizoctonia solani*

Isolated bacteria from both field trials were screened for their antagonistic activity towards the bacterial potato pathogen *Ralstonia solanacearum* (*R. solanacearum*). For antagonism test, a culture of the *R. solanacearum* strain 1602.1 from the strain collection of Instituto Valenciano de Investigaciones Agrarias (Valencia, Spain), or strain 1609 from the strain collection of Plant Research International (PRI, Wageningen, The Netherlands) were used for antagonism tests of trials 2002 and 2003, respectively. Cultures of strains were propagated by resuspension from tryptic soy broth (TSB) plus agar or directly from TSB medium. Tests were performed on 10% TSB agar dishes supplemented with 0.1% sucrose and mixed with the *R. solanacearum* culture at a ratio of 1:10 to reach bacterial cell final concentrations of 10^6–10^8 cfu ml^{-1}. After solidification of agar, isolates to be assayed were point-inoculated and plates were incubated at 25–29°C. Inhibition zones were observed after one to four days of incubation. Antibiosis against the fungal pathogen *Rhizoctonia solani* (*R. solani*) was tested on R2A (Difco) plates. A mycelium of a fresh culture of *R. solani* Ag3 (strain 42, PRI strain collection), which was cultivated on R2A medium, was placed in the center of each test plate. Bacterial isolates obtained from field trial 2003 were point-inoculated around the plug at the margin of the plates. Inhibition zones around the *R. solani* colony were checked daily. Only isolates showing antagonism against at least one of the two pathogens were subjected to 16S rRNA gene sequence analysis.

Phylogenetic characterization of bacterial antagonists

Genomic DNA of bacterial isolates exhibiting antagonistic activity against *R. solanecearum* and *R. solani* was isolated using a bead beating protocol (Sessitsch et al. 2002). The 16S rRNA gene of bacterial isolates was PCR-amplified using the primers 8F (Weisburg et al. 1991) and 1520R (Edwards et al. 1989). PCR-reaction cocktails of 50 μl contained 0.5 μl of undiluted DNA, 1 x PCR reaction buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.15 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate, and 2 U *Taq* polymerase (Invitrogen). PCR amplifications were performed with an initial denaturation for 5 min at 95°C, 30 cycles consisting of denaturation for 30 s at 95°C, primer annealing for 1 min at 52°C, polymerization for 2 min at 72°C, and completed by a final extension for 10 min at 72°C. PCR products (5 μl) were checked by electrophoresis in 1% (w/v) agarose gels (Biozym). Digestion of 10 μl PCR products was performed with 5 U of restriction endonuclease *Alu*I (Invitrogen) at 37°C for 4 h. Resulting DNA fragments were analyzed by gel electrophoresis in 3% (w/v) agarose gels (Biozym). Isolates were identified by partial 16S rRNA gene sequence analysis applying the BigDye V3.1 Terminator-Kit (Applied Biosystems, Warrington, UK) and the reverse primer 518r (Liu et al. 1997) resulting in sequence information of approximately 500 bp. Sequences were subjected to BLAST analysis with the National Center for Biotechnology Information (NCBI) database.

ACC deaminase activity of bacterial antagonists

Activity of ACC deaminase production of bacterial antagonists was tested on Brown & Dilworth (BD) minimal medium (Brown and Dilworth 1975) containing 0.7 g l^{-1} ACC as a sole nitrogen source (Rasche et al. 2006b). BD plates containing 0.7 g l^{-1} NH_4Cl were used as positive controls, BD plates containing no nitrogen source were used as negative controls. An ACC deaminase producing (*Burkholderia phytofirmans* strain PsJN) and a non-producing strain (*Methylobacterium* sp. iEII1) were used as positive and negative controls, respectively. ACC deaminase production was monitored after 7 days incubation at 30°C.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

Genomic DNA from plant shoots was isolated according to the protocol described by Rasche

et al. (2006b). Briefly, DNA from 250 to 300 mg frozen shoot material was isolated using a bead beating protocol. After extraction with phenol and chloroform, DNA was precipitated, washed with 70% ethanol, and air-dried. DNA was dissolved in TE buffer (pH 8.0) containing 0.1 mg ml^{-1} RNase (Concert RNase A, Invitrogen). DNA was finally purified with Sephadex™ G-50 (Amersham). It can be assumed that this DNA derives mainly from endophytes, but may also contain nucleic acids from phylloplane bacteria. DNA extracts were stored at –20°C.

Bacterial 16S rRNA genes were PCR-amplified using the primers 799F labeled with 6-carboxyfluorescein at the 5′ end (Chelius and Triplett 2001) and 1520R (Edwards et al. 1989). PCR-reaction cocktails of 50 μl contained 1–4 μl of undiluted DNA, 1x PCR reaction buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.15 μM of each primer, 0.2 mM of each dNTP, and 2 U *Taq* polymerase (Invitrogen). PCR amplifications were performed with an initial denaturation for 5 min at 95°C, 30 cycles of denaturation for 30 s at 95°C, primer annealing for 1 min at 53°C, polymerization for 2 min at 72°C, and a final extension for 10 min at 72°C. PCR products (5 μl) were checked by electrophoresis in 1% (w/v) agarose gels (Biozym). Four PCR products of each sample were pooled and precipitated with a 0.1 volume of 3 M sodium acetate solution (pH 5.2) and a 0.7 volume of isopropanol at –20°C for at least 30 min. DNA was centrifuged at 10.000*g* for 20 min, air-dried, and pellet was dissolved in 40 μl of TE buffer (pH 8.0). PCR products were subjected to electrophoresis in 2% (w/v) agarose gels (Biozym). The band of interest containing the PCR-product of bacterial 16S rDNA (~720 bp) was excised and purified using the QIAquick Gel Extraction Kit (Qiagen).

Digestion of 10 μl PCR product was performed with a combination of *Hae*III/*Hha*I (5 U each, Invitrogen) at 37°C for 4 h. Prior to the T-RFLP analysis, digests were purified with Sephadex™ G-50 (Amersham). Labeled terminal-restriction fragments (T-RFs) were detected on an ABI 3100 automatic DNA sequencer. Ten microliter digested PCR products were mixed with 15 μl HiDi formamide (Applied Biosystems) and 0.3 μl internal size standard (500 ROX™ Size Standard, Applied Biosystems). Samples were denatured at 92°C for 2 min and chilled on ice. The GeneScan® analysis software packet (Version 3.7, Applied Biosystems) was used for data collection. The GenoTyper 3.7 NT software (Applied Biosystems) was used to transform the electropherograms of each sample into numeric data. Both fragment length and peak height were used as parameters for profile comparison. Normalization of T-RFLP profiles was performed according to Dunbar et al. (2001). Finally, the values of peak heights of ≥30 fluorescence units of 115 normalized T-RFs with different fragment lengths were used for analysis of community patterns. Analysis of variance combined with post-hoc Bonferroni tests (SPSS for Windows, version 11.7, SPSS Inc., Chicago, IL, USA) was used to determine significant cultivar effects on peak height of T-RFs. Comparison of T-RFLP community profiles was done by cluster analysis using the Ward method (Statistica 6.0, StatSoft. Inc., Tulsa, OK, USA).

Cloning and sequence analysis

16S rRNA gene libraries were generated from all Desirée cultivars. PCR products of the four treatment replicates were pooled and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified amplicons were ligated into the TpCR 4-TOPO vector (Invitrogen). *Escherichia coli* strain DH5α-TI^R (Invitrogen) was then transformed with the ligation products. Sixty colonies per line were randomly picked and transferred to a new medium for 24 h incubation. Colonies were suspended in a reaction tube containing 50 μl TE buffer (pH 8), boiled for 10 min, chilled on ice, centrifuged for 10 min at 13.000*g*, and the supernatant was used for PCR. Clones were PCR-amplified by using the primers M13f and M13r. Amplicons were purified with Sephadex™ G-50 (Amersham) and used as templates for sequencing analysis. Partial sequencing of 16S rDNA was performed by applying the BigDye V3.1 Terminator-Kit (Applied Biosystems) and the reverse primer 1385r (Lane 1991) resulting in sequence information of about 500 bp. Clones were finally checked for chimaeric artifacts by using CHECK_CHIMERA of the Ribosomal Database

Project and chimaeric sequences were discarded. Sequences were subjected to BLAST analysis with the National Center for Biotechnology Information (NCBI) database. In order to identify the 16S rRNA gene clones with the T-RFs in the corresponding T-RFLP fingerprint, clones were subjected to T-RFLP analysis as described above.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the NCBI database under accession numbers DQ122190–DQ122319, DQ122332–DQ122259, and DQ124724–DQ124860.

Results

Colony forming units of cultivable bacteria

Differences in cfu among the three potato cultivars Achirana Inta (AIC, 1.9×10^6 cfu g^{-1} fresh plant material), Desirée (DC, 3.6×10^6 cfu g^{-1}), and Merkur (MC, 4.3×10^6 cfu g^{-1}) cultivated in the Meliana field trial 2002 and sampled at flowering were not significant ($P < 0.05$). Similarly, there was no significant difference observed between the senescent conventional Desirée (DC; 5.3×10^5 cfu g^{-1}) and transgenic Desirée DL 12 (DL; 6.5×10^5 cfu g^{-1}) cultivars assayed in the Cella field experiment 2003 ($P < 0.05$).

Identification of potato shoot-associated bacteria with antagonistic abilities

Bacteria isolated from the potato shoots were screened for their antagonistic potential against the potato pathogens *Ralstonia solanacearum* (*R. solanacearum*) and *Rhizoctonia solani* (*R. solani*). Phylogenetic affiliation of bacterial antagonists was performed by partial sequence analysis of 16S rRNA genes. Sequences covered approximately 500 bp each, and most sequences showed at least 97% similarity to known sequences in the NCBI database, whereas three isolates were only distantly (91–96%) related to known 16S rRNA genes (Tables 1 and 2).

A total of 210 strains, obtained from conventional Achirana Inta (AIC, 58 isolates), Desirée (DC, 97 isolates) and Merkur (MC, 55 isolates) cultivated in the Meliana field trial 2002, was screened for their antagonistic potential towards *R. solanacearum* (Table 1). Twenty-two isolates of line AIC (37%) exhibited antibiosis, whereas 20 and 18 isolates were detected for lines DC (21%) and MC (31%), respectively. In detail, the majority of AIC isolates belonged to α-proteobacteria (50%) and high-G+C gram positives (32%). The α-proteobacteria were dominated by *Agrobacterium tumefaciens* and *Ochrobactrum* sp. Three highly antagonistic isolates with low homology to known sequences (*Brachypodium fresconis* (95%) and *Microbacterium* sp. (96%) fell into the division of high-G+C gram positives. Only 9% of isolates corresponded with γ-proteobacteria (*Enterobacter* sp., *Shigella flexneri*), whereas 5% and 2% were affiliated with Firmicutes (*Bacillus* sp.) and β-proteobacteria (*Delftia* sp.), respectively. For line DC, 50% of antagonists were affiliated with the γ-proteobacteria in which three strains, *Erwinia persicina*, *Pseudomonas* sp. (91% homology) and *Xanthomonas campestris*, showed a high antagonistic activity against *R. solanacearum*. The other ten isolates, exhibiting a lower activity against *R. solanacearum*, were identified as Bacteroidetes (30%), Firmicutes (10%), α-proteobacteria (5%), and high-G+C gram positives (5%). In line MC, most isolates with antagonistic activity were dominated by *Rhizobium* sp. and *Ochrobactrum intermedium* (α-proteobacteria, 67%). Four isolates could be assigned to *Klebsiella granulomatis* (γ-proteobacteria, 22%), from which one isolate was highly antagonistic. In the high-G+C gram positive division (11%), there was one *Arthrobacter oxydans* strain showing strong antagonism.

A total of 72 bacterial isolates obtained from the senescent conventional Desirée (DC, 27 isolates) and its transgenic derivative DL 12 (DL, 45 isolates) were monitored for antagonism against *R. solanacearum* and *R. solani* (Table 2). Twenty-eight of 45 antagonistic bacteria were obtained from line DL. These antagonists were dominated by 24 strains (86%) corresponding to γ-proteobacteria. In detail, isolates related to *Klebsiella* sp., *Pantoea* sp. and *Erwinia persicina* were

Table 1 Sequence analysis of partial 16S rDNA (approximately 500 bp) of shoot-associated bacteria with antagonistic activity against *Ralstonia solanacearum* (*R. solanacearum*) isolated from conventional Achirana Inta, Desirée and Merkur potato cultivars grown in the Meliana field experiment 2002 at flowering stage

Potato line and isolate number	*R. solanacearum* inhibition zone [mm]	Closest NCBI match (accession number)/% homology	Phylogenetic group
Conventional Achirana Inta			
75	1	*Agrobacterium tumefaciens* (D14500)/100	α-proteobacteria
31; 34; 39; 47; 48; 52	1; 0.5; 1.5; 2; 1; 1	*Agrobacterium tumefaciens* (M11223)/99	α-proteobacteria
13; 21; 91	1; 0.5; 0.5	*Ochrobactrum antropi* (AB120120)/100	α-proteobacteria
93	1	*Ochrobactrum tritici* (AJ242584)/100	α-proteobacteria
92	1.5	*Delftia* sp. AN3 (AY052781)/100	*β*-proteobacteria
98	0.5	*Enterobacter* sp. 2457T (AE016991)/99	γ-proteobacteria
88	<0.2	*Shigella flexneri* (AE015415)/99	γ-proteobacteria
27	1.5	*Bacillus* sp. TKSP21 (AB017591)/100	Firmicutes
84	0.5	*Arthrobacter ilicis* (X83407)/98	high-G+C gram positives
38	10	*Brachypodium fresconis* (AJ415378)/95	high-G+C gram positives
99	1	*Microbacterium imperiale* (AF526906)/100	high-G+C gram positives
42	0.5	*Microbacterium* sp. IFO16060 (AB004713)/97	high-G+C gram positives
76	1	*Microbacterium imperiale* (AB007414)/97	high-G+C gram positives
68; 94	13; 11	*Microbacterium* sp. GWS-SE-H100 (AB332149)/96	high-G+C gram positives
Conventional Desirée			
75	1	*Chryseobacterium* sp. LDVH 42/00 (AY468475)/98	Bacteroidetes
1; 4; 9; 16; 22	0.5; 0.5; 0.5; 1.5; 0.5	*Flavobacterium* sp. PF7 (AF500323)/98	Bacteroidetes
50, 74	<0.2, <0.2	*Enterococcus casseliflavus* (AF039903)/99	Firmicutes
30	0.5	*Ochrobactrum anthropi* (AB120120)/99	α-proteobacteria
44	0.5	*Acinetobacter calcoaceticus* ssp. *anitratus* (U10874)/100	γ-proteobacteria
93	<0.2	*Enterobacter cancerogenus* (AY310730)/100	γ-proteobacteria
8	<0.2	*Enterobacter cancerogenus* (AY310730)/99	γ-proteobacteria
79	2	*Erwinia persicina* (AJ001190)/99	γ-proteobacteria
10; 25	1; 1	*Klebsiella* sp. rennanqilfy18 (AY363386)/99	γ-proteobacteria
78	0.5	*Klebsiella oxytoca* (AY292870)/96	γ-proteobacteria
13	1	*Pseudomonas* sp. pfB35 (AY336564)/98	γ-proteobacteria
77	4	*Pseudomonas* sp. PILH1 (AY456708)/91	γ-proteobacteria
11	2	*Xanthomonas campestris* (X99299)/99	γ-proteobacteria
57	1.5	*Arthrobacter oxydans* (AJ243423)/99	high-G+C gram positives
Conventional Merkur			
5	0.5	*Agrobacterium* sp. JS71 (AY174112)/100	α-proteobacteria
73; 77; 78	0.5; 1.5; 0.5	*Ochrobacterum intermedium* (AJ242583)/98	α-proteobacteria
6; 11; 12; 14; 17; 19; 22; 72	0.5; 1; 0.5; 0.5; 1; 1; 2; 1	*Rhizobium* sp. GH-2001 (AY029336)/100	α-proteobacteria
31; 32; 33; 37	<0.2; 0.5; 0.5; 10	*Klebsiella granulomatis* (AF010251)/100	γ-proteobacteria
3	4	*Arthrobacter oxydans* (AJ243423)/99	high-G+C gram positives
20	1	*Microbacterium paraoxydans* (AJ491806)/98	high-G+C gram positives

Table 2 Sequence analysis of partial 16S rDNA (approximately 500 bp) of shoot-associated bacteria with antagonistic activity against *Ralstonia solanacearum* (*R. solanacearum*) and *Rhizoctonia solani* (*R. solani*) and ACC deaminase activity isolated from senescent transgenic Desirée DL 12 and conventional Desirée potatoes cultivated in the Cella field experiment 2003

Potato line and isolate number	*R. solanacearum* inhibition zone [mm]	*R. solani* inhibition zone [mm]	ACC deaminase activity[a]	Closest NCBI match (accession number)/% homology	Phylogenetic group
Transgenic Desirée DL 12					
621	2	1	xx	*Staphylococcus epidermis* (AE016751)/99	Firmicutes
637	–	2	x	*Rhodococcus* sp. 871-AN053 (AF420422)/100	high-G+C gram positives
629	–	7	–	*Sanguibacter suarezii* (AY275516)/93	high-G+C gram positives
630	4	3	–	*Comamonas* sp. D1 (AF532869)/100	β-proteobacteria
628	1	2	x	*Acinetobacter calcoaceticus* (AF458218)/100	γ-proteobacteria
622	3	–	xx	*Erwinia persicina* (AJ001190)/98	γ-proteobacteria
623; 625	4; –	–; 2	xx; x	*Klebsiella oxytoca* (AF440521)/99	γ-proteobacteria
604; 606; 607	7; 6; 5	3; –; –	xx; xxx; xxx	*Klebsiella pneumoniae* (AY310729)/98	γ-proteobacteria
608; 620; 633	4; 4; 1	–; 3; –	xxx; xxx; x	*Klebsiella pneumoniae* (AY310729)/98	γ-proteobacteria
598; 600; 612	2; 6; 2	–; –; –	x; xx, x	*Pantoea agglomerans* (AY691545)/99	γ-proteobacteria
613	2	–	–	*Pantoea agglomerans* (AF157694)/98	γ-proteobacteria
592	0.5	–	xx	*Pantoea* sp. pfB16 (AY336545)/99	γ-proteobacteria
626	–	3	x	*Pantoea* sp. pfB25 (AY336554)/97	γ-proteobacteria
627	–	3	x	*Pseudomonas congelans* (AJ492828)/98	γ-proteobacteria
605	1	–	x	*Pseudomonas fulva* (AB046997)/99	γ-proteobacteria
596; 599; 616	–; –; 4	7; 3; –	xxx; xxx; xx	*Pseudomonas fluorescens* (AF375844)/99	γ-proteobacteria
590	4	3	–	*Pseudomonas* sp. AEBL3 (AY247063)/100	γ-proteobacteria
610	5	5	–	*Stenotrophomonas maltophilia* (AY445079)/99	γ-proteobacteria
632	1	3	x	*Stenotrophomonas maltophilia* (AJ293464)/99	γ-proteobacteria
Conventional Desirée					
666	2	3	–	*Bacillus* sp. BacB1 (AF497247)/99	Firmicutes
669	4	–	x	*Bacillus pumilus* (AY373359)/99	Firmicutes
645	–	2	x	*Paenibacillus pabuli* (AB045104)/99	Firmicutes
652	6	2	x	*Microbacterium oleivorans* (AJ698725)/99	high-G+C gram positives
648	6	–	–	*Agrobacterium tumefaciens* (AF501343)/99	α-proteobacteria
677	5	–	xx	*Acinetobacter calcoaceticus* (AF458218)/99	γ-proteobacteria
663	3	3	x	*Enterobacter asburiae* (AY297785)/98	γ-proteobacteria
676	6	–	x	*Enterobacter asburiae* (AY297785)/97	γ-proteobacteria
661	2	2	x	*Enterobacter cancerogenus* (AY310730)/99	γ-proteobacteria
653; 659	–; –	5; 2	x; –	*Klebsiella pneumoniae* (AY310729)/99	γ-proteobacteria
646; 657; 660	–; 5; –	3; 3; 2	x; x; x	*Pantoea agglomerans* (AF157694)/99	γ-proteobacteria
644	–	3	x	*Pantoea* sp. pfB25 (AY336554)/100	γ-proteobacteria
649; 662; 667	3; 3; 2	3; –; 3	xx; –; –	*Pantoea* sp. 44 (AY579155)/99	γ-proteobacteria
640	–	2	–	*Pseudomonas corrugata* (AF348508)/98	γ-proteobacteria
639	3	–	–	*Pseudomonas fulva* (AB046997)/99	γ-proteobacteria

–: no antagonistic and ACC deaminase activity

[a] Levels of ACC deaminase activity: x, moderate; xx, high; xxx, very high

antagonistic against *R. solanacearum*, whereas their antibiosis against *R. solani* was less intense. Isolates affiliated with *Pseudomonas* sp. and *Stenotrophomonas maltophila* showed antagonism towards both pathogens. Several isolates identified as Firmicutes, high-G+C gram positive bacteria, and β-proteobacteria showed antibiosis against both pathogens. From line DC, 20 of 27 antagonistic isolates (74%) were obtained from which 15 belonged to γ-proteobacteria (75%). In detail, isolates affiliated with *Acinetobacter calcoaceticus* and *Pseudomonas fulva* showed antagonism towards *R. solanacearum*, and *Pseudomonas corrugata* was antagonistic against *R. solani*. Strains assigned as *Enterobacter* sp., *Klebsiella* sp. and *Pantoea* sp. were antagonistic against both pathogens. Firmicutes and high-G+C gram positives with antagonism against both pathogens were represented by *Bacillus* sp. (10%) and *Microbacterium oleivorans* (5%), respectively. One *Agrobacterium tumefaciens* strain (α-proteobacteria, 5%) was found to antagonize *R. solanacearum*, and one isolate *Paenibacillus pabuli* strain (Firmicutes, 5%) revealed antibiosis against *R. solani*.

ACC deaminase activity of bacterial antagonists

Bacterial antagonists obtained from field trial 2003 were monitored for their production of the enzyme ACC deaminase (Table 2). A large number of different ACC deaminase producing strains associated with cultivar DL were affiliated with the γ-proteobacteria. In detail, various isolates assigned to *Klebsiella* sp., *Pseudomonas fluorescens*, *Pantoea* sp., and *Erwinia persicina* showed a moderate to very high ACC deaminase activity. Within the Firmicutes and high-G+C gram positives, two isolates proved to exhibit a moderate and high ACC deaminase activity. Five strains did not show any ACC deaminase production.

For line DC, one *Acinetobacter calcoaceticus* and one *Pantoea* sp. strain, both γ-proteobacteria, showed high activity, however, most isolates of this division, e.g. belonging to *Enterobacter* sp., showed a moderate ACC deaminase production. A moderate ACC deaminase activity was determined for *Bacillus pumilus* and *Paenibacillus pabuli* strains (Firmicutes). A *Microbacterium oleivorans* strain (high-G+C gram positives) was screened as a moderate ACC deaminase producer. Seven isolates identified were not able to produce ACC deaminase.

Community structure of potato-associated bacteria

Analysis of variance with Bonferroni post-hoc tests determined 28 terminal restriction fragments (T-RFs) which significantly differentiated the shoot-associated bacterial community composition of the conventional flowering and senescent Desirée cultivars cultivated in the Meliana and Cella field trial, respectively. Four T-RFs revealed a significant difference between the two conventional and transgenic Desirée lines cultivated in the Cella field experiment ($P < 0.05$) (Table 3). Comparison of the bacterial community structures of cultivars Achirana Inta (AIC), Merkur (MC), conventional Desirée (DC), cultivated in the Meliana field trial revealed no significant difference ($P < 0.05$).

Community profile comparison was based on cluster analysis using the Ward method. In order to facilitate a better discrimination of the shoot-associated bacterial community structures characterized by the four different potato lines, Achirana Inta (AIC), Merkur (MC), conventional Desirée (DC), and transgenic Desirée DL 12 (DL) as well as the two field sites, Meliana and Cella, analysis of the T-RFLP data set was based on data derived from the flowering potatoes of the Meliana field experiment 2002 and the senescent potatoes of the Cella field trial 2003. At these two vegetation stages, the cfu numbers were mostly similar for all plants of both field trials which guaranteed a more representative analysis of impact level of all treatments potato line and field site. Cluster analysis revealed that the shoot-associated population structures between the potatoes cultivated either in Meliana or Cella were highly different (Fig. 1). Comparing each field trial separately, a greater population difference was determined between the senescent conventional and transgenic Desirée cultivars, DC and DL, cultivated in the Cella field experi-

Table 3 Terminal restriction fragments (T-RFs) which were significantly affected by the Desirée cultivars as determined by analysis of variance

T-RF size[a] [bp]	Significance level[b]	
	DC 2002 vs. DC 2003[c]	DC 2003 vs. DL 2003[c]
42	**	–
55	**	–
58	***	–
59	*	–
72	*	–
73	*	–
75	*	–
84	**	–
99	***	–
101	**	–
103	*	–
104	*	–
107	**	*
112	**	–
113	*	–
116	**	–
121	**	–
142	–	**
188	**	–
191	**	–
201	*	–
224	**	–
247	–	*
249	*	–
272	***	–
274	*	–
326	***	–
331	*	*
335	**	–
350	*	–

[a] T-RFs which were significantly influenced by the two Desirée potato cultivars

[b] Significance levels: –: $P > 0.05$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[c] Potato lines: DC 2002, flowering conventional Desirée derived from field trial 2002; DC 2003, flowering conventional Desirée derived from field trial 2003; DL 2003, flowering transgenic Desirée DL 12 derived from field trial 2003

ment as compared to the smaller community differences between the three flowering conventional cultivars, AIC, DC, and MC, grown in the Meliana field trial.

Analysis of 16S rRNA gene libraries

In order to identify dominant community members with T-RFLP fingerprint profiles, 16S rRNA gene libraries were generated and partial insert sequences were determined. These gene libraries consisted of sequences derived from all Desirée cultivars. In total, 180 sequences were determined; however, for 43 sequences the presence of chimaeric sequences could not be unambiguously excluded. Most of the clearly non-chimaeric sequences showed at least 97% similarities to known sequences in the NCBI database, whereas seven clones were only distantly (94–96%) related to known 16S rRNA genes (Tables 4–6).

The 37 non-chimaeric clones obtained from line DC cultivated in field trial 2002 belonged to γ-proteobacteria (62%), and Firmicutes (22%), whereas 16% of sequenced clones were determined as α-proteobacteria (Table 4). The γ-proteobacteria were dominated by *Pseudomonas* sp., *Enterobacter* sp., *Serratia marcescens*, and *Leminorella grimotii*, whereas clones matching with Firmicutes and the α-proteobacteria were represented by *Enterococcus* sp. and *Agrobacterium tumefaciens*, respectively.

A higher diversity was found in the gene libraries of lines DC and DL cultivated in the field trial 2003 as compared to that of line DC of field trial 2002 (Tables 5 and 6). For both lines of field trial 2003, 50 non-chimaeric sequences were determined. For line DC, most clones fell into the α-, β- and γ-proteobacteria. In detail, the α-proteobacteria (4%) were represented by a *Paracoccus* sp. and *Brevundimonas mediterrana* clones. Most clones of the β-proteobacteria (36%) were assigned as uncultured bacterial clones, however, four clones matched with *Acidovorax* sp., *Comamonadaceae* sp., *Oxalobacter* sp., and *Paucimonas* sp. All clones representing γ-proteobacteria (18%) could be affiliated with known species, e.g. *Pantoea agglomerans* and *Pseudomonas* sp. The Cytophaga/Flexibacter/Bacteroidetes (CFB, 6%) were dominated by *Hymenobacter* sp. and an uncultured *Taxeobacter* sp., whereas the Firmicutes (28%) were represented by *Exiguobacterium* sp. and *Paenibacillus* sp. The remaining clones belonged to the high-G+C gram positives (8%) and were represented by *Arthrobacter* sp., *Rhodococcus fascians* and *Kinecoccus aurantiacus*.

The majority of clones obtained from the gene library of line DL matched with the β-proteo-

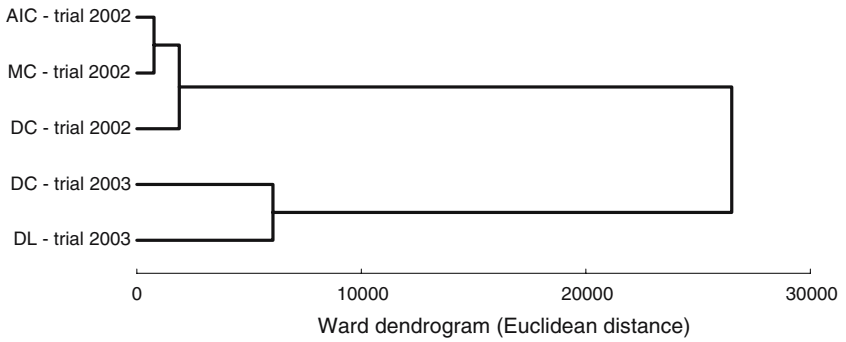

Fig. 1 Cluster analysis based on the Ward method of T-RFLP fingerprints obtained from bacterial communities colonizing the shoots of the different potato cultivars. Potato lines are: conventional Achirana Inta (AIC), conventional Merkur (MC), conventional Desirée (DC), transgenic Desirée DL 12 (DL); field experiments: Meliana (trial 2002), Cella (trial 2003)

bacteria (34%) and the γ-proteobacteria (42%). These divisions were dominated by uncultured clones, as well as by *Pantoea agglomerans* and *Erwinia herbicola*, respectively. The Firmicutes (10%) were represented by *Exiguobacterium* sp. and *Paenibacillus* sp., whereas *Arthrobacter* sp. and *Curtobacterium* sp. belonged to the high-G+C gram positives (14%).

Clones have been subjected to T-RFLP analysis in order to determine their position in the

Table 4 Phylogenetic assignment of clone libraries of amplified bacterial 16S rRNA genes (380–590 bp) derived from the shoots of the flowering conventional potato cultivar Desirée (DC) of the Meliana field experiment 2002

Theoretical T-RF size [bp]	Actual T-RF size [bp]	Corresponding clone	Closest NCBI match (accession number)/% homology
Firmicutes			
150	149	cloEDC6	*Enterococcus gallinarum* (AJ420805)/100
151	149	cloEDC21/41/49	Uncultured *Enterococcus* sp. Mc-9.2e-28 (AB18970)/100
151	149	cloEDC7/8/24/36	Uncultured *Enterococcus* sp. Mc-9.2e-28 (AB18970)/99
α-proteobacteria			
77	78	cloEDC14/48	*Agrobacterium tumefaciens* (AY504963)/100
77	78	cloEDC17	*Agrobacterium tumefaciens* (AY504963)/99
78	78	cloEDC30/39	*Agrobacterium tumefaciens* (AY850392)/100
78	78	cloEDC50	*Agrobacterium tumefaciens* (AY850392)/99
γ-proteobacteria			
78	78	cloEDC44/46	*Pseudomonas* sp. K94.08 (AY456703)/100
78	78	cloEDC18	*Pseudomonas* sp. K94.08 (AY456703)/99
115	113	cloEDC19/25	*Enterobacter cloacae* (AY787819)/100
115	113	cloEDC26	*Enterobacter* sp. XW110 (AY941832)/100
115	113	cloEDC13	*Serratia marcescens* (AY043386)/100
115	113	cloEDC43	*Serratia marcescens* (AY566180)/100
115	113	cloEDC32/34	*Serratia marcescens* (AY566180)/99
115	113	cloEDC40	*Serratia marcescens* (AY514434)/99
115	113	cloEDC38	*Shigella boydii* (AY696681)/99
149	149	cloEDC5/15/42	Uncultured bacterial clone LKC3_125.60 (AY510238)/99
149	149	cloEDC35	*Leminorella grimontii* (AJ233421)/100
149	149	cloEDC10/12/23/29/33/37	*Leminorella grimontii* (AJ233421)/99
149	149	cloEDC11	*Citrobacter werkmanii* (AF025373)/100

corresponding community profiles. This was necessary as the actual T-RF lengths may differ from the theoretical, sequence-determined T-RF lengths. The drift ranged from 0 to 3 bases (Tables 4–6). Results obtained by sequence analysis and by T-RFLP analysis were in good agreement. Bacteria belonging to proteobacteria, high-G+C gram-positives and Firmicutes were highly represented in T-RFLP patterns by various fragments indicating high abundance (Fig. 2). 16S rRNA gene libraries indicated community shifts between the line DC and DL cultivated in field trial 2003. Gene libraries indicated further population differentiations between the conventional

Table 5 Phylogenetic assignment of clone libraries of amplified bacterial 16S rRNA genes (380–590 bp) derived from the shoots of the senescent conventional potato line Desirée (DC) cultivated in the Cella field experiment 2003

Theoretical T-RF size [bp]	Actual T-RF size [bp]	Corresponding clone	Closest NCBI match (accession number)/% homology
Cytophaga/Flexibacter/Bacteroidetes			
54	54	cloENT27	*Hymenobacter roseosalivarius* (Y18834)/97
146	147	cloENT45	Uncultured *Taxeobacter* sp. KL-59-7-7 (AF408279)/98
149	147	cloENT89	*Hymenobacter* sp. NS/2 (AJ549284)/97
Firmicutes			
116	116	cloENT90	*Exiguobacterium* sp. 5138 (AY831656)/100
116	116	cloENT25/28/88/93	*Exiguobacterium* sp. 5138 (AY831656)/99
116	116	cloENT59/70	*Exiguobacterium* sp. HHS31 (AJ846291)/100
116	116	cloENT26/48/53	*Exiguobacterium* sp. HHS31 (AJ846291)/99
116	116	cloENT16	*Exiguobacterium* sp. HHS31 (AJ846291)/97
329	326	cloENT21	*Paenibacillus daejeonensis* (AF290916)/96
329	326	cloENT71	*Paenibacillus polymyxa* (AY359632)/96
329	326	cloENT72	*Paenibacillus* sp. 2301065 (AY323608)/94
High-G+C gram positives			
45	45	cloENT20	*Rhodococcus fascians* (AY730713)/99
45	45	cloENT92	*Kineococcus aurantiacus* (X77958)/97
76	75	cloENT66	*Arthrobacter* sp. SMCC G966 (AF197031)/99
77	78	cloENT10	*Arthrobacter* sp. Fa21 (AY131225)/100
α-proteobacteria			
148	147	cloENT19	*Paracoccus* sp. SAFR-029 (AY167832)/100
148	147	cloENT38	*Brevundimonas mediterranea* (AJ244709)/99
β-proteobacteria			
114	113	cloENT54	Uncultured bacterial clone TLM05 (AF534429)/99
114	113	cloENT56	Uncultured bacterial clone cEV44 (AY357983)/99
115	116	cloENT12	*Acidovorax* sp. IMI 357678 (AF078763)/99
118	116	cloENT30	*Comamonadaceae* sp. SB1 (AJ606333)/98
226	224	cloENT49/50	Uncultured bacterial clone HJ12 (AY237409)/99
226	224	cloENT62	Uncultured bacterial clone FTL254 (AF529109)/99
226	224	cloENT67	Uncultured bacterial clone FTL217 (AF529102)/99
329	326	cloENT36	*Oxalobacter* sp. p8E (AJ496038)/99
329	326	cloENT34	*Paucimonas lemoignei* (X92554)/98
329	326	cloENT61	Uncultured bacterial clone Wuba26 (AF336351)/100
329	326	cloENT9/44/52/57/95	Uncultured bacterial clone Wuba26 (AF336351)/99
329	326	cloENT23	Uncultured bacterial clone OCS7 (AF001645)/99
329	326	cloENT8	Uncultured bacterial clone B-BH93 (AY622261)/97
γ-proteobacteria			
47	50	cloENT33	*Cellvibrio vulgaris* (AF448513)/97
75	75	cloENT29	Candidatus *Phlomobacter betae* (AY057392)/97
77	77	cloENT18	*Pseudomonas* sp. CG20106T (AY787208)/99
78	78	cloENT81	*Pseudomonas syringae* (AY574913)/98
115	116	cloENT13	*Pantoea agglomerans* (AF130948)/99
115	116	cloENT82/96	*Erwinia herbicola* (AF130954)/99
117	116	cloENT51	*Pantoea agglomerans* (AF130938)/99
120	121	cloENT84	*Pantoea agglomerans* (AJ583835)/96

Table 6 Phylogenetic assignment of clone libraries of amplified bacterial 16S rRNA genes (420–590 bp) derived from the shoots of the senescent transgenic potato line Desirée DL 12 (DL) cultivated in the Cella field experiment 2003

Theoretical T-RF size [bp]	Actual T-RF size [bp]	Corresponding clone	Closest NCBI match (accession number)/% homology
Firmicutes			
115	117	cloET90/92	*Exiguobacterium* sp. HHS31 (AJ846291)/99
329	329	cloET13/32	*Paenibacillus* sp. 2301065 (AY323608)/95
329	329	cloET39	*Paenibacillus* sp. 2301065 (AY323608)/94
High-G+C gram positives			
76	75	cloET1	*Arthrobacter* sp. Ellin159 (AF409001)/97
77	75	cloET17	*Arthrobacter pascens* (AJ576068)/99
77	79	cloET67/73/85	*Arthrobacter* sp. Fa21 (AY131225)/99
77	79	cloET9	*Arthrobacter* sp. Fa21 (AY131225)/97
119	122	cloET53	*Curtobacterium* sp. VKM Ac-2061 (AB042096)/99
β-proteobacteria			
114	113	cloET77	Uncultured bacterial clone HJ12 (AY237409)/99
114	113	cloET4	Uncultured bacterial clone B-E7 (AY622242)/99
226	224	cloET10/21/30	Uncultured bacterial clone FTL217 (AF529102)/99
229	225	cloET25	Uncultured bacterial clone FTL217 (AF529102)/98
329	329	cloET50	Uncultured bacterial clone HJ12 (AY237409)/100
329	329	cloET42/72	Uncultured bacterial clone Wuba26 (AF336351)/100
329	329	cloET26/27/31/36/49/70/96	Uncultured bacterial clone Wuba26 (AF336351)/99
330	331	cloET83	*Nitrosovibrio tenuis* (AY123803)/100
γ-proteobacteria			
77	75	cloET87	*Pseudomonas putida* (AY973266)/98
113	113	cloET33/34	*Pantoea agglomerans* (AF130928)/97
114	113	cloET12/69/93	*Erwinia herbicola* (AF130954)/99
114	113	cloET18/76/86	*Pantoea agglomerans* (AJ583835)/99
115	117	cloET2	*Pantoea agglomerans* (AJ583835)/100
115	117	cloET89	*Pantoea agglomerans* (AF130928)/99
115	117	cloET3	*Pantoea agglomerans* (AF130948)/99
116	117	cloET20/91	*Pantoea agglomerans* (AF130928)/98
117	117	cloET29	*Erwinia herbicola* (AF130954)/97
118	117	cloET15/45/65	*Pantoea agglomerans* (AF130948)/98
118	117	cloET52	*Pantoea agglomerans* (AF130948)/96
151	150	cloET5/19	*Pantoea agglomerans* (AJ583835)/98

Desirée cultivars cultivated in both field trials; although to a certain extent highly similar sequences were found in different cultivars. Nevertheless, the number of clones analyzed was too small to allow a statistical analysis.

Discussion

Shoot-associated bacteria live in a close association with their host plant as they colonize the intercellular spaces and vascular system (endosphere) as well as the surface (phyllosphere) of plants (Hallmann et al. 1997, Bell et al. 1995; Patriquin et al. 1978). Plants depend on these bacteria as they exhibit plant growth promotion and antagonize plant pathogens (Lodewyckx et al. 2002; Sturz et al. 2000). Plants such as potatoes are especially reliant upon tissue colonizing bacteria which possess the potential to combat potato pathogens such as *Ralstonia solanacearum* or *Rhizoctonia solani*. An alternative to this plant-microbe defense mechanism is the generation of genetically modified potatoes producing the antibacterial protein T4 lysozyme (Düring et al. 1993). Previous studies have shown that engineered potatoes expressing T4 lysozyme effectively enhanced plant resistance against the common potato pathogen *Erwinia carotovora* (Heuer and Smalla 1999). Contrastingly, the bactericidal properties of T4 lysozyme may have adverse effects on the diversity and functional

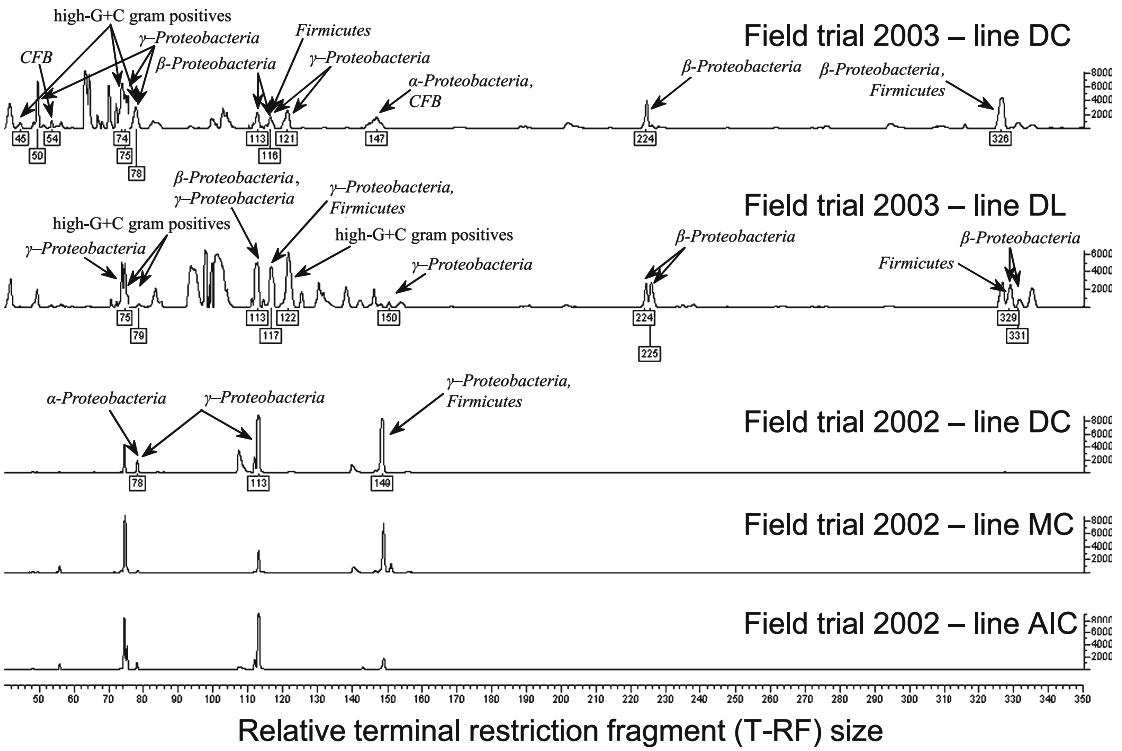

Fig. 2 Representative T-RFLP electropherograms of bacterial communities colonizing the shoots of the conventional lines Achirana Inta (AIC), Desirée (DC) and Merkur (MC) and transgenic line DL 12 (DL) grown in the Meliana field trial 2002 and the Cella field trial 2003. Fragments corresponding to dominant phylogenetic groups represented by 16S rRNA gene libraries from the same shoot samples of the conventional and transgenic Desirée lines are indicated and labeled with the respective fragment size

potential of the affected shoot-associated bacterial community. Previous studies confirmed that T4 lysozyme expressing potatoes can affect the diversity of the associated microflora (Rasche et al. 2006a, b; Heuer et al. 2002; Ahrenholtz et al. 2000; de Vries et al. 1999); however, this effect was no greater than those of environmental factors. It is also known that different plant genotypes or cultivars of a same species affect the diversity and functional potential of the bacterial community in the corresponding host (Berg et al. 2005, Sessitsch et al. 2004). The major goal of this study was therefore to assess structural characteristics of plant-associated bacteria colonizing different field-grown potato cultivars. In a first field experiment, the genetic diversity of shoot-colonizing bacterial communities of three flowering, conventional potato lines was assayed by 16S rRNA gene polymorphisms. In order to obtain information about the antagonistic potential of the plant-associated bacterial community, cultivable bacteria were isolated from potato shoots, and bacterial antagonists against *Ralstonia solanacearum* were further characterized by partial 16S rDNA sequencing. In a second field experiment, the potential effect of a senescent transgenic, T4 lysozyme expressing trait on structural characteristics of shoot-associated bacteria was compared with those of its isogenic, unmodified wild-type line. Cultivable bacteria showing antagonism against *Ralstonia solanacearum* and *Rhizoctonia solani* and ACC deaminase production were identified by partial 16S rDNA sequencing.

T-RFLP community fingerprinting revealed small bacterial population differences between the three flowering conventional potato cultivars, Achirana Inta, Desirée, and Merkur, cultivated in the Meliana field experiment 2002, whereas bacterial communities colonizing the senescent

conventional and transgenic Desirée lines of the Cella field trial 2003 showed greater diversity differentiations. This may suggest that the genetic modification had a more pronounced effect on shoot-associated bacteria than the genetic variation found in the conventional potato lines investigated. Adams and Kloepper (2002) as well as Elvira-Recuenco and van Vuurde (2000) have shown that diversity differences of plant-associated communities can be induced by plant species or varieties of the same species. Additionally, it has been reported that T4 lysozyme expression in planta can lead to shifts in plant-associated bacterial populations (Rasche et al. 2006b; Heuer and Smalla 1999). However, it has to be considered that plant genotype and transformation dependent variations were not determined in the same field trial and at the same vegetation stage. It was previously intended to perform both field experiments in Meliana as the experiment of 2002 ought to represent the baseline study using unmodified cultivars for the experiment in 2003, which should be then conducted with the T4 lysozyme trait. However, no permission from national authorities was obtained for the Meliana field site, whereas a release was approved for Cella. The conditions for bacterial growth were obviously distinct at both sites as shown by highly different colony counts and community structures found in Desirée. However, field site and vegetation stage exposed a greater effect in comparison to the genetic differences between the three conventional lines as well as between the T4 lysozyme trait and its unmodified parental line. This assumption is based on highly different bacterial colonization at different plant vegetation stages which is supported by McInroy and Kloepper (1995) who found fluctuations in endophytic bacterial colony counts occurring throughout the growing season. The two field locations differed in soil and climatic characteristics, thus representing different growth habitats. Different soil properties affect the community structure of the soil microflora, which represents a potential source of bacteria entering the plant through natural openings or wounds of plant roots (Girvan et al. 2003; Sessitsch et al. 2001; Dalmastri 1999; Hallmann et al. 1997). Thus, it can be assumed that such specific soil characteristics lead to differentiations in the bacterial diversity colonizing genetically similar plants (Surette et al. 2003; Hallmann et al. 1999). This fact explains the highly different endophyte communities colonizing the line Desirée cultivated in the contrasting fields which was supported by a high number of significant T-RFs and bacterial clones. Heuer et al. (2002) and Heuer and Smalla (1999) have shown that the effect of T4 lysozyme potatoes on the genetic characteristics of associated endosphere and rhizosphere bacterial communities is comparable to effects of field variations. Additionally, Rasche et al. (2006 a, b) recently determined a T4 lysozyme effect on rhizosphere and respective endosphere bacteria which was minor or comparable to the effect of soil type. Further, the climatic conditions differed between the two field locations. Such abiotic factors can influence the diversity of plant-associated bacteria (Malinowsky and Belesky 2000; Sessitsch et al. 2002). Therefore, we suggest that these climatic factors have attributed to a certain extent to the community variations between the conventional Desirée cultivar grown in both fields.

Analysis of bacterial isolates exposing antagonism against *Ralstonia solanacearum* and *Rhizoctonia solani* revealed only minor differences between the T4 lysozyme expressing and the corresponding unmodified Desirée lines analyzed in the Cella trial 2003. This finding is supported by Lottmann et al. (1999) who did not find a negative effect of T4 lysozyme producing potatoes on the associated rhizosphere bacterial diversity, and authors suggested that the community was able to tolerate or adapt to the presence of T4 lysozyme. Contrastingly, the three unmodified lines Achirana Inta, Desirée, and Merkur assayed in the Meliana field trial 2002, harbored different bacterial isolates indicating that cultivable endophytes are more influenced by the natural cultivar variability as compared to the modification effect. However, most of the detected antagonists which have been isolated from all four potato lines commonly occur within plant tissues (Sessitsch et al. 2004; Araújo et al. 2002; Berg et al. 2005; Kuklinsky-Sobral et al. 2004; Garbeva et al. 2001). Interestingly, a high number of ACC deaminase producing bacteria was determined which were also capable to antagonize at least one of the two potato patho-

gens *Ralstonia solanacearum* and *Rhizoctonia solani*, e.g. *Klebsiella pneumoniae*, *Pantoea* sp., *Pseudomonas fluorescens*. This finding was supported by Wang et al. (2000) who have demonstrated that plants which were treated with ACC deaminase-containing PGPR were more resistant to effects of stress ethylene induced by the presence of phytopathogens. Additionally, Chernin et al. (1995) have demonstrated the chitinolytic activity of *Pantoea agglomerans* against *Rhizoctonia solani* which was also capable to produce ACC deaminase. In this sense, *Pantoea agglomerans* was highly abundant in the 16S rRNA gene libraries and bacterial isolates. It is thus likely that ACC deaminase producing bacteria identified in this study, apart from directly antagonizing pathogens, support the plant's resistance against pathogen attack. However, this relationship of ACC production and antagonism merits further investigation.

In conclusion, it has been shown that different potato cultivars harbor genotype-specific bacteria which show antagonism against common potato pathogens such as *Ralstonia solanacearum* and *Rhizoctonia solani*. In detail, differences between the flowering conventional potato lines grown in the Meliana were slightly greater than those between the senescent conventional and T4 lysozyme producing potatoes of the Cella trial. Again, it has to be pointed out that the potatoes were analyzed in different fields and at different vegetation stages. Further, the impact of T4 lysozyme on structural characteristics of shoot-associated bacteria was minor or comparable in comparison to the field site variations and vegetation stages.

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References

Adams PD, Kloepper JW (2002) Effect of host genotype on indigenous bacterial endophytes of cotton (*Gossypium hirsutum* L.). Plant Soil 240:181–189

Ahrenholtz I, Harms K, de Vries J, Wackernagel W (2000) Increased killing of *Bacillus subtilis* on the hair roots of transgenic T4 lysozyme-producing potatoes. Appl Environ Microbiol 66:1862–1865

Araújo WL, Marcon J, Maccheroni W Jr, van Elsas JD, van Vuurde JWL, Azevedo JL (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl Environ Microbiol 68:4906–4914

Bell CR, Dickie GA, Harvey WLG, Chan JWYF (1995) Endophytic bacteria in grapevine. Can J Microbiol 41:46–53

Berg G, Krechel A, Ditz M, Sikora RA, Ulrich A, Hallmann J (2005) Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. FEMS Microbiol Ecol 51:215–229

Brown CM, Dilworth MJ (1975) Ammonia assimilation by *Rhizobium* cultures and bacteroids. J Gen Microbiol 86:39–48

Chelius MK, Triplett EW (2001) The diversity of archaea and bacteria in association with the roots of *Zea mays* L. Microbial Ecol 41:252–263

Chen C, Bauske EM, Musson G, Rodríguez-Kábana R, Kloepper JW (1995) Biological control of Fusarium wilt on cotton by use of endophytic bacteria. Biol Control 5:83–91

Chernin L, Chet I (2002) Microbial enzymes in biocontrol of plant pathogens and pests. In: Burns R, Dick R (eds) Enzymes in the environment: activity, ecology, and applications. Marcel Dekker Inc., New York, NY, pp 171–225

Chernin L, Ismailov Z, Haran S, Chet I (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Appl Environ Micobiol 61:1720–1726

Dalmastri C (1999) Soil type and maize cultivar affect the genetic diversity of maize root-associated *Burkholderia cepacia* populations. Microbial Ecol 38:273–284

de Vries J, Harms K, Broer I, Kriete G, Mahn A, Düring K, Wackernagel W (1999) The bacteriolytic activity in transgenic potatoes expressing a chimeric T4 lysozyme gene and the effect of T4 lysozyme on soil- and phytopathogenic bacteria. Syst Appl Microbiol 22:280–286

Dunbar J, Ticknor LO, Kuske CR (2001) Assessment of microbial diversity in four Southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl Environ Microbiol 66:2943–2950

Düring K (1996) Genetic engineering for resistance to bacteria in transgenic plants by introduction of foreign genes. Mol Breeding 2:297–305

Düring K (1994) Differential patterns of bacteriolytic activities in potato in comparison to bacteriophage T4 and hen egg white lysozymes. J Phytopathol 141:159–164

Düring K, Porsch P, Mahn A, Brinkmann O, Gieffers W (1999) The non-enzymatic microbicidal activity of lysozymes. FEBS Lett 449:93–100

Düring K, Porsch P, Fladung M, Lorz H (1993) Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. Plant J 3:587–598

Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (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, van Vuurde JWL (2000) Natural incidence of endophytic bacteria in pea cultivars under field conditions. Can J Microbiol 46:1036–1041

Garbeva P, van Overbeek LS, van Wuurde JWL, van Elsas JD (2001) Analysis of endophytic bacterial communities of potato by plating and denaturing gradient gel electrophoresis (DGGE) of 16S rDNA based PCR fragments. Microbial Ecol 41:369–383

Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS (2003) Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Appl Environ Microbiol 69:1800–1809

Grichko VP, Glick BR (2001) Amelioration of flooding stress by ACC deaminase-containing plant growth-promoting bacteria. Plant Physiol Bioch 39:11–17

Hack H, Gal H, Klemke T, Klose R, Meier U, Stauß R, Witzenberger A (1993) Phänologische Entwicklungsstadien der Kartoffel (*Solanum tuberosum* L.). Nachrichtenbl Deut Pflanzenschutzd 45:11–19

Hallmann J, Rodríguez-Kábana R, Kloepper JW (1999) Chitin-mediated changes in bacterial communities of the soil, rhizosphere and within roots of cotton in relation to nematode control. Soil Biol Biochem 31:551–560

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can J Microbiol 43:895–914

Heuer H, Kroppenstedt RM, Lottmann J, Berg G, Smalla K (2002) Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors. Appl Environ Microbiol 68:1325–1335

Heuer H, Smalla K (1999) Bacterial phyllosphere communities of *Solanum tuberosum* L. and T4-lysozyme-producing transgenic variants. FEMS Microbiol Ecol 28:357–371

Hippe S, Düring K, Kreuzaler F (1989) In situ localization of a foreign protein in transgenic plants by immunoelectron microscopy following high pressure freezing, freeze substitution and low temperature embedding. Eur J Cell Biol 50:230–234

King EO, Ward M, Raney DE (1954) Two simple media for the demonstration of pyocianin and fluorescein. J Lab Clin Med 44:401–407

Kloepper JW, Leong J, Teintze M, Schroth MN (1980) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885–886

Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL (2004) Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environ Microbiol 6:1244–1251

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. John Wiley and Sons, Inc., Chichester, UK, pp 115–175

Liu W-T, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M, van der Lelie D (2002) Endophytic bacteria and their potential applications. Crit Rev Plant Sci 21:583–606

Lottmann J, Heuer H, Smalla K, Berg G (1999) Influence of transgenic T4-lysozyme-producing potato plants on potentially beneficial plant-associated bacteria. FEMS Microbiol Ecol 29:365–377

Lugtenberg BJJ, de Weger LA, Bennett JW (1991) Microbial stimulation of plant growth and protection from disease. Curr Opin Microbiol 2:457–464

Malinowsky DP, Belesky DP (2000) Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci 40:923–940

Mayak S, Tirosh T, Glick BR (2004) Plant growth-promoting bacteria that confer resistance to water stress in tomato and peppers. Plant Sci 166:525–530

McInroy JA, Kloepper JW (1995) Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. Can J Microbiol 41:895–901

Oerke E-C, Dehne H-W, Schönbeck F, Weber A (1994) Crop production and crop protection. Elsevier, Amsterdam

Patriquin DG, Döbereiner J (1978) Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. Can J Microbiol 24:734–742

Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plantarum 18:10–15

Rasche F, Hödl V, Poll C, Kandeler E, Gerzabek MH, van Elsas JD, Sessitsch A (2006a) Rhizosphere bacteria affected by transgenic potatoes with antibacterial activities in comparison to effects of soil, wildtype potatoes, vegetation stage and pathogen exposure. FEMS Microbiol Ecol 56:219–235

Rasche F, Velvis H, Zachow C, Berg G, van Elsas JD, Sessitsch A (2006b) Impact of transgenic potatoes expressing antibacterial agents on bacterial endophytes is comparable to the effects of plant genotype, soil type and pathogen infection. J Appl Ecol 43:555–566

Sessitsch A, Reiter B, Berg G (2004) Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. Can J Microbiol 50:239–249

Sessitsch A, Reiter B, Pfeifer U, Wilhelm E (2002) Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubac-

terial and Actinomycetes-specific PCR of 16S rRNA genes. FEMS Microbiol Ecol 39:23–32

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

Sturz AV, Christie BR, Nowak J (2000) Bacterial endophytes: potential role in developing sustainable systems of crop production. Crit Rev Plant Sci 19:1–30

Surette MA, Sturz AV, Lada RR, Nowak J (2003) Bacterial endophytes in processing carrots (*Daucus carota* L. var. sativus): their localization, population density, biodiversity and their effects on plant growth. Plant Soil 253:381–390

Tsugita A, Inouye M, Terzaghi E, Streisinger G (1968) Purification of bacteriophage T4 lysozyme. J Biol Chem 243:391–397

Wang C, Knill E, Glick BR, Défago G (2000) Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its *gacA* derivative CHA96 on their grwoth-promoting and disease-suppressive capacities. Can J Microbiol 46:898–907

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 43:1691–1705

Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol 35:155–189