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Phenotypic and genotypic characterization of antagonistic bacteria associated with roots of transgenic and non-transgenic potato plants

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Abstract

Rhizobacteria obtained during a risk assessment study from parental and transgenic T4 lysozyme-expressing potato plants were investigated to determine whether or not the strains could be grouped based on the source of isolation, transgenic or non-transgenic plants, respectively. A total of 68 representative bacterial strains of the group of enterics and pseudomonads were investigated by phenotypic profiling (the antagonistic activity towards bacterial and fungal plant pathogens, the production of the plant growth hormone indole-3-acetic acid [auxin], and the sensitivity to T4 lysozyme *in vitro*) and genotypic profiling by PCR fingerprints using BOX primers. All isolates were identified by fatty acid methyl ester (FAME) analysis. Computer-based analysis of the phenotypic characteristics showed that both, enterics and *Pseudomonas* strains clustered into six to seven groups at an Euclidian distance of 10. According to their BOX-PCR-generated fingerprints the *Pseudomonas* strains clustered into seven groups and the enterobacteria into two groups at the same genetic distance level of 10. The majority of groups were heterogeneous and contained isolates from all plant lines. In conclusion, cluster analysis of the phenotypic and genotypic features did not reveal correlations between bacterial isolates and transgenic character of plants.

Key words: transgenic plants – T4 lysozyme – antagonistic bacteria – fingerprinting

Introduction

The composition and diversity of the bacterial community associated with plant roots is influenced by a variety of plant factors such as plant species, cultivar or exudation of nutrients (Bachmann and Kinzel 1992; Grayston *et al.* 1998; Siciliano *et al.* 1998). Siciliano and Germida (1999) found differences in the composition of the root-associated microbial community between transgenic and non-transgenic canola (*Brassica napus L.*) cultivars. Di Giovani *et al.* (1999) investigated the bacterial communities of parental and two transgenic alfalfa cultivars by metabolic fingerprinting and PCR fingerprinting using enterobacterial repetitive intergenic consensus (ERIC) sequence polymorphism. They showed that the transgenic plant genotype may affect rhizosphere microorganisms. Recently, Saxena *et al.* (1999) showed that *Bt* (*Bacillus thuringiensis*) toxin is released into the rhizosphere from *Bt* corn, but the authors had no indication of how soil communities might be affected by *Bt* toxin in root exudates in the field.

Fluorescent pseudomonads are important members of the root-associated microbial community due to their aggressive colonization of the root surface, importance in plant disease interactions and plant growth promoting abilities (O'Sullivan and O'Gara 1992; Patten and Glick 1996; Lugtenberg and Dekkers 1999). Thus, pseudomonad populations may be appropriate for analyzing microbial shifts in the rhizosphere. Enterobacterial rhizobacteria with beneficial properties were also described (Kalbe *et al.* 1996; Lottmann *et al.* 1999) and evaluated to control plant pathogens (Hinton and Bacon 1995). Genotypic fingerprints of rhizobacteria are suitable for detecting plant or cultivar specificity of rhizobacteria on subspecies level (Lemanceau *et al.* 1995; Sikorski *et al.* 1999). A molecular technique useful for this purpose is PCR using BOX primers (Martin *et al.* 1992; Louws *et al.* 1994; Selenska-Pobell *et al.* 1995). The BOX-PCR technique is based on amplification

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of repetitive sequences of the bacteria genome (Martin *et al.* 1992).

The transgenic T4 lysozyme expressing potato plants investigated in this study were developed to enhance the resistance against the bacterial pathogen *Erwinia carotovora* (Düring *et al.* 1993). The release of T4 lysozyme from growing roots of transgenic potato plants and the ability of T4 lysozyme to lyse various soil bacteria has been demonstrated (De Vries *et al.* 1999). In a previous study, no influence of T4 lysozyme produced by transgenic potato plants on abundance and function of potentially beneficial bacteria could be observed (Lottmann *et al.* 1999). On the other hand, exudation of transgenic T4 lysozyme might in the long run cause the enrichment of lysozyme-tolerant bacteria or specific genotypes in the rhizosphere.

In this study, antagonistic isolates of two important groups of rhizobacteria (pseudomonads, enterics) obtained during a risk assessment study from transgenic and non-transgenic potato plants were analysed phenotypically by (i) fatty acid methyl ester (FAME) analysis, (ii) *in vitro* assays to determine antagonistic activity towards the phytopathogens *Erwinia carotovora* and *Verticillium dahliae,* (iii) the production of the plant growth hormone indole-3-acetic acid, and (iv) the sensitivity to T4 lysozyme in order to find a possible influence of the transgenic plant genotype on root-associated bacteria. For genotypic characterization BOX-PCR fingerprinting was used.

Materials and methods

Potato plants. The transgenic potato lines DL4 and DL5, which constitutively expressed and secreted T4 lysozyme (Düring *et al.* 1993), a transgenic control line DC1 without the T4 lysozyme gene and the parental potato line (*Solanum tuberosum* L. cv. Désirée = DESI) were provided by Dr. K. Düring (Federal Centre for Breeding Research on Cultivated Plants, Quedlinburg, Germany; now: Molecular Plant & Protein Biotechnology, Köln, Germany).

Bacterial isolates. All bacterial isolates used in this study are listed in Tables 1 and 2. They were obtained from the rhizosphere of non-transgenic and transgenic potato plants during the field experiments in 1996 and 1997. The numbers contain the abbreviation of the field site (L = Lüsewitz), the plot from which the isolate was obtained (first number), the sampling times over a period of two years (1 to 6 in 1996 and 1997), and the strain number. The plot number corresponds to the four plant lines (DESI: plots 4, 7, 9, 16, 19, 21, 26, 30; DC1: plots 3, 5, 12, 15, 18, 22, 25, 32; DL4: plots 2, 6, 11, 13, 17, 24, 28, 31, and DL5: plots 1, 8, 10, 14, 20, 23, 27, 29). Stock cultures of all isolates were grown in nutrient broth II (NBII, SIFIN, Berlin, Germany), and aliquots of 1 ml were kept frozen at –70 °C in 15 % glycerol. The isolates were identified by fatty acid methyl ester (FAME) analysis, by using the MIDI system (Microbial Identification System, Inc. Newark, USA). Strains that were not present in the MIDI library were identified with the BIOLOG microbial identification system according to the manufacturer's recommendations (BIOLOG, Hayward, USA).

In vitro antagonism towards plant pathogens. The *in vitro* inhibition of *Erwinia carotovora* was determined in a dual culture assay on Luria-Bertani-agar (LB, DIFCO, Detroit, USA). The test strain *E. carotovora* ssp. *atroseptica* (Strain No. 549, National Collection of Plant Pathogenic Bacteria, Harpenden, UK) was used. 100 µl of an overnight culture of *E. carotovora* ssp. *atroseptica* was plated on LB agar and bacteria were streaked as broad bands. Zones of inhibition were measured after incubation at 20 °C for 24 and 48 h. The *in vitro* inhibition of *Verticillium dahliae* was determined in a dual culture assay on Waksman agar, according to Berg and

Table 1. Phenotypic characterization of *Pseudomonas* isolates obtained from the rhizosphere of transgenic T4-lysozyme producing potato plants (DL4, DL5), transgenic control plants without the T4-lysozyme gene (DC1) and from the parental line (DESI).

No.	strain	species[a]	SIM[b]	potato line[c]	antagonistic activity against[d]		IAA-production[g]	Lysozyme sensitivity[h]	Cluster group
					V. dahliae[e]	*E. carotovora*[f]			
P5	L12-5-7	*Pp*	0.800	DC1	II	0	0	0	1
P20	L7-2-9	*Pf*	0.927	DESI	II	0	0	0	1
P22	L7-2-9	*Pf*	0.516	DESI	II	0	0	0	1
P24	L9-5-9	*Pf*	0.231	DC1	I	0	0	0	1
P25	L12-6-1	*Pf*	0.763	DC1	I	0	0	0	1
P26	L2-4-4	*Pf*	0.731	DL4	I	0	0	0	1
P27	L2-4-12	*Pf*	0.887	DL4	I	0	0	0	1
P31	L9-1-12	*Pc*	0.581	DESI	I	0	0	0	1
P36	L12-2-2	*Pc*	0.75	DC1	I	0	0	0	1

Table 1. (continued)

No.	strain	species[a]	SIM[b]	potato line[c]	antagonistic activity against[d]		IAA-production[g]	Lyso-zyme	Cluster group
					V. dahliae[e]	*E. carotovora*[f]		sensitivity[h]	
P37	L12-4-4	*Pc*	0.624	DC1	I	0	0	0	1
P38	L15-4-12	*Pc*	0.686	DL1	I	0	0	0	1
P39	L17-1-12	*Pc*	0.618	DL4	II	0	0	0	1
P40	L11-4-8	*Pc*	0.339	DL4	I	0	0	0	1
P43	L10-2-1	*Pc*	0.811	DL5	II	0	0	0	1
P45	L20-6-9	*Pc*	0.329	DL5	I	0	0	0	1
P46	L26-4-11	*Ps*	0.806	DESI	I	0	0	0	1
P47	L4-5-12	*Ps*	0.741	DESI	I	0	0	0	1
P48	L5-5-9	*Ps*	0.862	DC1	I	0	0	0	1
P51	L27-4-9	*Ps*	0.248	DL5	I	I	0	0	1
P1	L7-2-3	*Pp*	0.893	DESI	III	0	0	0	2
P4	L12-5-4	*Pp*	0.116	DC1	III	I	0	0	2
P8	L22-6-9	*Pp*	0.797	DC1	III	0	0	0	2
P9	L32-6-8	*Pp*	0.704	DC1	III	II	0	0	2
P10	L11-1-3	*Pp*	0.86	DL4	III	0	I	0	2
P13	L8-6-2	*Pp*	0.812	DL5	III	0	I	0	2
P14	L8-6-9	*Pp*	0.822	DL5	III	0	I	0	2
P32	L9-2-5	*Pc*	0.767	DESI	III	0	0	0	2
P33	L30-3-6	*Pc*	0.663	DESI	III	0	0	0	2
P34	L30-3-8	*Pc*	0.582	DESI	III	0	0	0	2
P35	L30-3-9	*Pc*	0.621	DESI	III	0	0	0	2
P6	L3-6-5	*Pp*	0.762	DC1	II	0	I	0	3
P11	L17-4-10	*Pp*	0.862	DL4	II	I	I	0	3
P12	L13-6-12	*Pp*	0.785	DL4	II	0	I	0	3
P19	L21-1-4	*Pf*	0.895	DESI	II	0	I	0	3
P21	L9-5-1	*Pf*	0.503	DESI	II	0	I	0	3
P23	L3-2-1	*Pf*	0.885	DC1	I	0	I	0	3
P41	L10-1-6	*Pc*	0.78	DL5	II	0	I	0	3
P42	L29-1-8	*Pc*	0.656	DL5	I	0	I	0	3
P44	L23-4-11	*Pc*	0.104	DL5	I	0	I	0	3
P49	L5-6-11	*Ps*	0.542	DC1	II	0	I	0	3
P50	L24-5-7	*Ps*	0.618	DL4	I	0	I	0	3
P3	L18-4-2	*Pp*	0.838	DC1	I	II	I	0	4
P28	L31-6-9	*Pf*	0.300	DL4	0	II	0	0	4
P30	L1-6-11	*Pf*	0.660	DL5	I	II	I	0	4
P15	L10-6-1	*Pp*	0.659	DL5	0	II	0	I	5
P16	L10-6-5	*Pp*	0.593	DL5	0	II	0	I	5
P17	L10-6-6	*Pp*	0.864	DL5	0	III	I	I	5
P2	L22-1-6	*Pp*	0.757	DC1	II	I	0	I	6
P29	L31-6-11	*Pf*	0.824	DL4	II	II	I	I	6
P7	L12-6-9	*Pp*	0.828	DC1	II	II	I	I	7
P18	L27-6-10	*Pp*	0.814	DL5	II	III	I	I	7

[a] Species abbreviations: *Pp* (*Pseudomonas putida*), *Pf* (*Pseudomonas fluorescens*), *Pc* (*Pseudomonas chlororaphis*), *Ps* (*Pseudomonas syringae*)

[b] SIM (similarity index of identification) FAME analysis

[c] Potato lines: DESI = parental line, derived from *Solanum tuberosum* cv. Désirée, DC1 = transgenic control line without T4 lysozyme gene (carries the *npt*II gene), DL4, DL5 = transgenic T4 lysozyme expressing lines

[d] *in vitro* assay according to Berg and Ballin (1994); 0 = no inhibition of the phytopathogens, I = low inhibition , II = medium inhibition , III = good inhibition,

[e] *Verticillium dahliae* V16 (Culture collection of the University of Rostock, Germany) was originally isolated from *Solanum tuberosum* L.

[f] *Erwinia carotovora* (Strain No. 549, National Collection of Plant Pathogenic Bacteria, Harpenden, UK).

[g] Indole-3-acetic acid (IAA), determined in a colorimetric assay as previously described by Lottmann *et al.* (1999)

[h] determined in an agar diffusion assay with three different concentrations of T4 lysozyme: 0 = not sensitive, I = sensitive

Table 2. Phenotypic characterization of enterobacterial isolates obtained from the rhizosphere of transgenic T4-lysozyme producing potato plants (DL4, DL5), transgenic control plants without the T4-lysozyme gene (DC1) and from the parental line (DESI).

No.	strain	species[a]	SIM[b]	potato line[c]	antagonistic activity against[d]		IAA-production[g]	Lyso-zyme	Cluster group
					V. dahliae[e]	*E. carotovora*[f]		sensitivity[h]	
E4	L5-3-11	*Sg*	0.808	DC1	II	0	I	0	1
E6	L1-3-5	*Spr*	0.914	DL5	II	0	I	0	1
E9	L24-5-10	*Ea*	0.412	DL4	II	0	I	0	1
E12	L5-3-10	*Pa*	0.862	DC1	II	0	I	0	1
E14	L11-1-12	*Pa*	0.847	DL4	I	0	I	0	1
E1	L16-3-3	*Sg*	0.930	DESI	III	0	I	0	2
E2	L21-3-10	*Sg*	0.924	DESI	III	0	I	0	2
E15	L14-5-2	*Pa*	0.922	DL5	III	0	0	0	2
E16	L14-3-11	*Pa*	0.803	DL5	III	0	I	0	2
E10	L27-6-4	*Ea*	0.653	DL5	II	I	0	0	3
E11	L29-6-3	*Ea*	0.708	DL5	II	I	0	0	3
E17	L6-4-2	*Pv*	0.827	DL4	II	I	0	0	3
E5	L9-6-11	*Spl*	0.819	DESI	II	0	0	0	4
E7	L5-2-6	*Ea*	0.784	DC1	I	0	0	0	4
E8	L12-6-12	*Ea*	0.658	DC1	III	II	I	0	5
E13	L13-5-8	*Pa*	0.659	DL4	III	I	I	0	5
E3	L19-6-1	*Sg*	0.847	DESI	II	III	I	0	6

[a] Species abbreviations: *Sg* (*Serratia grimesii*), *Spr* (*Serratia proteamaculans*), *Ea* (*Enterobacter agglomerans*), *Pa* (*Pantoea agglomerans*), *Pv* (*Proteus vulgaris*), *Spl* (*Serratia plymuthica*)

[b] SIM (similarity index of identification) FAME analysis

[c] Potato lines: DESI = parental line, derived from *Solanum tuberosum* cv. Désirée, DC1 = transgenic control line without T4 lysozyme gene (carries the *npt*II gene), DL4, DL5 = transgenic T4 lysozyme expressing lines

[d] *in vitro* assay according to Berg and Ballin (1994); 0 = no inhibition of the phytopathogens I = low inhibition , II = medium inhibition , III = good inhibition,

[e] *Verticillium dahliae* V16 (Culture collection of the University of Rostock, Germany) was originally isolated from *Solanum tuberosum* L.

[f] *Erwinia carotovora* (Strain No. 549, National Collection of Plant Pathogenic Bacteria, Harpenden, UK).

[g] Indole-3-acetic acid (IAA), determined in a colorimetric assay as previously described by Lottmann *et al.* (1999)

[h] determined in an agar diffusion assay with three different concentrations of T4 lysozyme: 0 = not sensitive, I = sensitive

Ballin (1994). Zones of inhibition were determined after 5 days of incubation at 20 °C. The used *Verticillium dahliae* strain V16 (Culture collection of the University of Rostock, Germany) was originally isolated from *Solanum tuberosum* L.

In vitro production of indole-3-acetic acid (IAA). The applied microplate method was a modification of the method developed by Sawar and Kremer (1995), and was previously described by Lottmann *et al.* (1999).

In vitro sensitivity to T4 lysozyme. The sensitivity against T4 lysozyme was tested on nutrient agar II (NAII, SIFIN) by using an agar diffusion test. The strains were precultured in 2 ml of NBII. 200 µl of these cell suspensions and a protease inhibitor (200 µmol phenylmethylsulfonylfluoride, = PMSF, [Fluka, Buchs, Switzerland]) were mixed with 4.5 ml of NAII (0.8%) tempered at 35 °C. This mixture was directly poured into Petri dishes containing 20 ml of NAII. After drying 10-µl aliquots containing different amounts of T4 lysozyme (10, 50 and 100 µg) were dropped on to the agar surface. The plates were incubated up to 24 hours and the appearance of clear zones was determined visually. The T4 lysozyme was purified by affinity chomatography and provided by Dr. Klaus Düring (MPB Cologne, Germany). The enzyme was dissolved in PBS and aliquots of 200 µl were stored at –20 °C.

DNA extraction. Total genomic DNA was prepared using the Qiagen Genomic-tip 20/G-kit (QIAGEN, Hilden, Germany) following the instructions of the manufacturer.

BOX-PCR genomic fingerprints. The primer sequence corresponding to BOX A (BOXA1R, 5'-CTA CGG CAA GGC GAC GCT GAC G-3') was provided by MWG-Biotech (Ebersberg, Germany). PCR amplification was performed with a Peltier Thermal Cycler PTC-200 (Biozym Diagnostic, Hess. Oldendorf, Germany)

using the following cycles: 1 initial cycle at 95 °C for 6 min; 1 cycle of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and extension at 65 °C for 8 min with a single final extension cycle at 65 °C for 16 min and a final soak at 4 °C. 10 µl of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5xTBE buffer for 6 h, stained with ethidium bromide, and photographed under U.V. transillumination. The reproducibility of the results was verified in three independent experiments.

Computer-assisted cluster analysis. The analysis of the phenotypic characterization was performed by using the STATISTICA program (StatSoft, Hamburg, Germany). Data were converted to a binary code, and interisolate relationships were measured by Ward's algorithm at an Euclidian distance of 10. BOX-PCR generated fingerprints were evaluated computer-assisted by using the GelCompar® program of Applied Maths (Version 4.1, Kortrijk, Belgium). To perform the cluster analysis Ward's algorithm was used.

Results

Phenotypic characterization

Most of the isolates were considered positively identified by FAME analysis because of similarity index (SIM) of 0.3 or greater (Tables 1 and 2). Strains with a SIM of less than 0.3 were additionally tested by the BIOLOG system. Two isolates, P26 and E5 were not present in the MIDI library; these isolates were identified using the BIOLOG system as strains of *Pseudomonas fluorescens* and *Serratia grimesii,* respectively.

The analysis of the phenotypic characterization of the *Pseudomonas* isolates including antagonistic activity, the ability to produce indole-3-acetic acid (IAA) and the sensitivity towards T4 lysozyme *in vitro* resulted in seven groups at a Euclidian distance of 10 (Tab. 1). The four *Pseudomonas* species (*Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas chlororaphis, Pseudomonas syringae*) were distributed relatively consistent over all cluster groups. Six of these groups were heterogeneous and contained isolates from T4 lysozyme-expressing plants (DL4, DL5) and control plants (DESI, DC1). Group 5 was homogeneous, the three isolates were all obtained from transgenic (DL5) plants. All group 5 isolates proved sensitive towards T4 lysozyme *in vitro.* Five of the seven T4 lysozyme-sensitive isolates were obtained from T4 lysozyme-expressing plants (DL4, DL5). In addition, six of the seven T4 lysozyme-sensitive strains exhibited antagonism to *E. carotovora*. The grouping of the *Pseudomonas* isolates was independent of the plant genotype.

Cluster analysis of the phenotypic characterization of the enterobacterial isolates resulted in six groups at a Euclidian distance of 10 (Tab. 2). Three of these groups were heterogeneous and included 68% of the investigated isolates; three groups were homogeneous. Group 3 contained only isolates from T4 lysozyme-expressing plants (DL4, DL5). In contrast, group 4 contained only isolates from the parental line (DESI) and transgenic control line (DC1). Group 6 contained only a single isolate from the parental line that was phenotypically very similar to both group 5 isolates. The investigated enterobacterial isolates did not exhibit sensitivity towards T4 lysozyme in the applied assay. The grouping of the investigated enterobacterial isolates was essentially independent of the plant genotype.

Genotypic characterization

PCR amplification with BOX primers was used to compare the isolates at the molecular level. The electrophoretic profiles of the *Pseudomonas* isolates are shown in Fig. 1. The BOX-PCR yielded 8 to more than 20 distinct PCR products, ranging in size from approximately 250 bp to over 3000 bp and allowed differentiation of

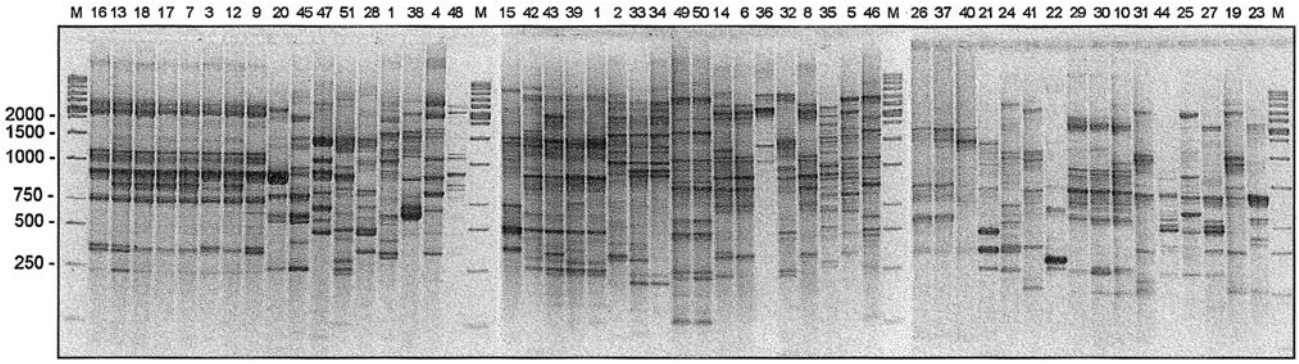

Fig. 1. BOX-PCR fingerprinting patterns from genomic DNA of *Pseudomonas* isolates. The numbers labelled 1 to 51 correlate with the numbers P1-P51 of Table 1. M shows the DNA molecular size marker (1 kb ladder, MBI Fermentas, Vilnius, Lithuania); the sizes are indicated in base pairs.

strains. The BOX-PCR profiles were compared by numerical methods and the resultant dendrogram showed a high degree of genetic diversity (data not shown). Cluster analysis of the pseudomonads resulted in seven groups based on a genetic distance of 10 (Tab. 3). All isolates clustered at a genetic distance of 64.5. Six groups were heterogeneous and contained isolates from T4 lysozyme-expressing plants and control plants. Group 5 was homogeneous and included only isolates from parental plants (DESI) and control plants without T4 lysozyme gene (DC1). Group 1 contained five of the seven T4 lysozyme-sensitive isolates. Nine of the 14 antagonists to *E. carotovora* belonged also to cluster group 1. However, the grouping of the isolates based on BOX-PCR profiles was independent of the origin of strains, transgenic or non-transgenic plants, respectively. The distribution of the *Pseudomonas* strains within and between each plant line is shown in Table 3.

Table 3. Distribution of *Pseudomonas* isolates obtained from parental (DESI), transgenic T4 lysozyme expressing (DL4, DL5) and transgenic control plants (DC1) according to their BOX-PCR generated fingerprints.

Cluster group	Plant lines	*Pseudomonas isolates*
1	Desi	–
	DC1	P3; P6; P7; P8; P9; P48
	DL4	P10; P12; P29
	DL5	P13; P14; P16; P17; P18; P30
2	Desi	P19; P31; P32
	DC1	–
	DL4	P11; P39
	DL5	P41; P42; P 43; P51
3	Desi	P20; P21; P47
	DC1	P23; P38
	DL4	P27
	DL5	P44; P45
4	Desi	P46
	DC1	P4; P5; P25; P36; P37
	DL4	P26; P40
	DL5	–
5	Desi	P1; P33; P34; P35
	DC1	P2
	DL4	–
	DL5	–
6	Desi	–
	DC1	P24
	DL4	P28
	DL5	P15
7	Desi	P22
	DC1	P49
	DL4	P50
	DL5	–

Table 4. Distribution of enterobacterial isolates obtained from parental (DESI), transgenic T4 lysozyme expressing (DL4, DL5) and transgenic control plants (DC1) according to their genotypic characteristics at a Euclidian distance of 10.

Cluster group	Plant lines	Isolates
1	DESI	E2; E3; E5
	DC1	E8; E12
	DL4	E9; E13; E17
	DL5	E9; E13; E17
2	DESI	E1
	DC1	E4; E7
	DL4	E14
	DL5	E15; E16

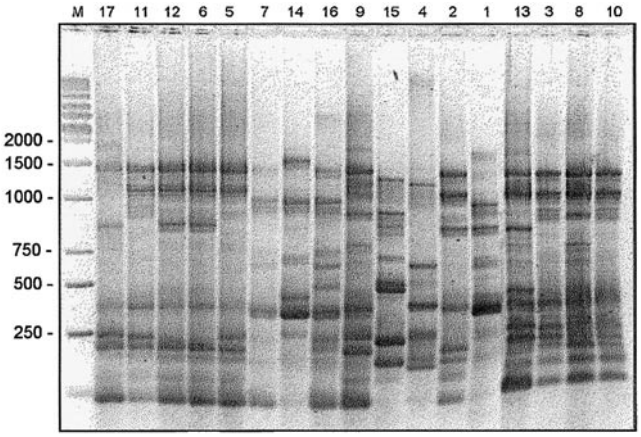

Fig. 2. BOX-PCR fingerprinting patterns from genomic DNA of enterobacterial isolates. The numbers labelled 1 to 17 correlate with the numbers E1-E17 of Table 2. M shows the DNA molecular size marker (1 kb ladder, MBI Fermentas); the sizes are indicated in base pairs.

For the enterobacterial isolates the BOX-PCR yielded between 8 and 14 distinct bands, ranging in size from 500 bp to 5 kbp. A total of 14 different profiles was obtained. The PCR profiles were compared at a genetic distance of 10 and two groups were defined (Fig. 2). Both groups were heterogeneous, and each contained isolates from all four plant lines. All of the isolates clustered at a Euclidian distance of 86.1. All of the six strains which were antagonists to *E. carotovora* belonged to cluster group 1. The distribution of strains according to their BOX-PCR profiles within and between each plant line is shown in Table 4. In summary, the grouping of enterobacterial isolates was not correlated with the plant genotype.

Discussion

Due to the non-selective effects of T4 lysozyme, it is necessary to assess the risks of the released enzyme on the root-associated antagonistic bacteria. If this group of bacteria is disturbed due to the influence of T4 lysozyme, the

positive effect of the constructed plant could be impaired (Lottmann *et al.* 1999). Differences in composition of the root-associated microbial community between transgenic and non-transgenic plants have been described (Donegan *et al.* 1995; Siciliano and Germida 1999; Di Giovani *et al.* 1999). Garcia de Salomone *et al.* (1996) suggested that populations of *Azospirillum* spp. in maize depend on the plant genotype. Although, only a small part of the rhizosphere bacteria was investigated, both groups of rhizobacteria (fluorescent pseudomonads, enterics) include important members of plant-associated, antagonistic bacteria. In a previous risk assessment study, the percentage of antagonistic bacteria from the rhizosphere of transgenic T4 lysozyme-producing plants and non-transgenic parental plants was determined (Lottmann *et al.* 1999). In the present study, antagonistic bacteria evaluated during this risk assessment were included. Our study of fluorescent *Pseudomonas* isolates and enterobacterial isolates originally isolated from the rhizosphere of transgenic and non-transgenic potato plants included phenotypic characterization, identification, and the analysis of genetic variability among the strains in relationship to their origin.

The phenotypic analysis of *Pseudomonas* isolates resulted in six heterogeneous groups (isolates from transgenic and non-transgenic plant genotypes) and one homogeneous (isolates from one plant genotype) group. The heterogeneous groups contained isolates from all four plant lines and included 94% of the investigated isolates, suggesting that the transgenic plant genotype did not affect the distribution of fluorescent pseudomonads in the rhizosphere of the four investigated plant lines. The phenotypic analysis of the enterobacterial isolates resulted in three heterogeneous groups and three homogeneous groups. The heterogeneous groups contained with 68% also the majority of the investigated strains. Although the microbial diversity often was analysed by methods revealing phenotypic features, this approach has several limitations, such as precultivation in the laboratory or the fact that only a restricted part of the genetic information is revealed through phenotypic testing. A combination of phenotypic and genetic methods is known to provide a good estimate of soil bacteria diversity (Torsvik *et al.* 1990). The BOX-PCR generated fingerprints obtained with DNA from the pseudomonads suggested a high degree of DNA heterogeneity over all 51 strains. Cluster analysis revealed six heterogeneous groups and one homogeneous group. The heterogeneous groups contained most of the isolates (90%). For these isolates no correlation between their genotypes and sources of isolation (plant line) was observed. The enterobacterial isolates were genetically less heterogeneous than the *Pseudomonas* strains. On the basis of genetic distance of 10 only two clusters were obtained. Both groups were heterogeneous; they contained isolates from all four plant lines. Taken together, there was no correlation between the transgenic plant genotype and the distribution of single phenotypes and genotypes of root-associated bacteria.

The release of T4 lysozyme from growing roots of transgenic potato plants and the ability of T4 lysozyme to lyse various soil bacteria has been demonstrated (de Vries *et al.* 1999). We supposed that strains which were sensitive to T4 lysozyme *in vitro,* colonize with priority the parental or transgenic control plants without T4 lysozyme gene, but surprisingly most of the sensitive isolates were obtained from the transgenic plant lines (DL4, DL5). All investigated enterobacterial isolates were not sensitive to T4 lysozyme in the agar diffusion assay. De Vries *et al.* (1999) observed different levels of sensitivity among different species, e.g. *Xanthomonas campestris* was highly sensitive even to low concentrations of T4 lysozyme in a survival assay, whereas cells of different strains of *Ralstonia solanacearum* were nearly tolerant towards T4 lysozyme in the same assay.

In this study, antagonistic isolates of two important groups of rhizobacteria (pseudomonads, enterics) obtained from transgenic and non-transgenic potato plants were analysed phenotypically and genotypically to evaluate a possible influence of plant-derived T4 lysozyme on the composition of root-associated bacterial antagonists. It was shown that the distribution of isolates was not affected by the plant genotype. This supports the results of our previous investigations of the functions of rhizobacterial antagonists obtained from transgenic and non-transgenic potatoes (Lottmann *et al*. 1999; 2000), which were also not influenced by the expression of T4 lysozyme.

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