

Biocontrol Strain *Pseudomonas* sp. W34: Specific Detection and Quantification in the Rhizosphere of *Cucumis sativus* with a DNA Probe and Genotypic Characterization by DNA Fingerprinting

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A DNA probe specific for biocontrol strains of *Pseudomonas* was produced by screening randomly amplified polymorphic DNA (RAPD) PCR fragments. Specificity of the probe was assessed by dot blot and colony hybridization. It was used to specifically determine the population of these strains on roots of *Cucumis sativus* cv. Delikatess. Two polymorphic RAPD fragments of 750 bp, and 550 bp showed identical specificity. The biocontrol strain *Pseudomonas* sp. W34 was shown to be competitive in the rhizosphere of cucumber and to maintain a stable population for at least 10 days when inoculated on the seed.

The phylogenetic relationships between the biocontrol and reference strains were analyzed at the strain level by means of RAPD and repetitive sequence-based PCR genomic fingerprinting (rep-PCR), and at higher taxonomic levels by means of amplified 16S ribosomal DNA restriction analysis ARDRA. It was shown that the antagonistic strains are closely related, forming a separate cluster from other non-antagonistic and reference *Pseudomonas* strains, their taxonomic placement remaining uncertain.

Introduction

Biological control of plant pathogens by disease-suppressive microorganisms has been the subject of many studies. Several possible mechanisms are considered to be involved in this phenomenon, including production of antibiotics and siderophores Hammer *et al.*, 1997; Weller *et al.*, 1997), competition for micronutrients and parasitism or induced resistance (Handelsman and Stabb, 1996; De Leij and Lynch 1997; Lopper *et al.*, 1997; Thomashow and Mavrodi, 1997; Weisbeck and Gerrits, 1997; Werner *et al.*, 1997).

Certain strains of fluorescent pseudomonads are among the most promising candidates for control of soil-borne pathogens (Schroth and Hancock,

1982; Weller and Cook, 1983; Maurhofer *et al.*, 1992; Pierson and Weller, 1994). However, in the rhizosphere the interactions between plant, soil-borne pathogen, biocontrol agent and other microbiota are complex and the variability of physical, chemical and biological factors in this system often renders it difficult to obtain reliable biocontrol effects (Weller, 1988).

Prerequisites for successful disease control are effective colonization of the rhizosphere by the antagonistic organism (Bull *et al.*, 1991; Kloepper and Beauchamp, 1992) and the persistence of its population throughout plant growth. It is therefore important to study the populations of biocontrol agents after coinoculation with other microorganisms. Nucleic acid probes or marker genes (de Weger *et al.*, 1991) allow specific detection and quantification of effective biocontrol strains. With these molecular tools the population dynamics of these bacteria in the rhizosphere can be studied and the time optimum for the application of the agent can be determined.

Several approaches are possible to obtain specific probes. Some authors used sequences of ribosomal RNA genes (Amann *et al.*, 1995; Boye *et al.*, 1995) or subtractive hybridization (Bjour-

Abbreviations: ARDRA, Amplified ribosomal DNA restriction analysis; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism.

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son *et al.*, 1992) to isolate strain-specific sequences. rDNA-derived probes bear the advantage that their target is present in a high copy number in metabolically active cells, but usually hold less promise in distinguishing closely related organisms at or below the species level (Fox *et al.*, 1992).

With a variety of organisms the screening of Random Amplified DNA Polymorphisms (RAPDs, Williams *et al.*, 1990) has been shown to be a promising method to generate DNA probes (Bazzicalupo and Fani, 1995). This method proved to be suitable for antagonistic pseudomonads in our study. At the same time, this method allows insights into the phylogenetic relationships of the organisms studied on the strain and isolate level (Harrison *et al.*, 1992). In our study, RAPD results were extended and supported by repetitive sequence-based PCR (rep-PCR, Versalovic *et al.*, 1994) using BOX and enterobacterial repetitive intergeneric consensus (ERIC) primers, a technique that also yields strain-specific genomic fingerprints (de Bruijn, 1992; Rademaker *et al.*, 1997). To determine the phylogenetic position of the antagonistic strains, a RFLP analysis was performed on nearly full-length 16S rDNA sequences of the biocontrol and some reference strains, amplifying this gene-region with universal PCR-primers (Weisburg *et al.*, 1991), and using four tetrameric endonucleases for the restriction analysis (Moyer *et al.*, 1996; Vinuesa *et al.*, 1998).

The DNA probe generated by RAPD was used to study the population of biocontrol strain W34 in the rhizosphere of cucumber. The antagonistic effects of the biocontrol strains of *Pseudomonas* we used in our study (W34, W24, WB1, WB11, WB15, WB52) had been reported before by other authors (Vogt and Buchenauer, personal communication).

Materials and Methods

Bacteria

Table I shows the bacterial strains used and their sources.

Pseudomonas strains were cultivated on King B agar or NB medium (Difco, Detroit, MI, USA).

The other bacteria were grown on standard growth media.

DNA probes

Genomic DNA was purified from liquid cultures using QIAGEN tip20 columns (QIAGEN, Hilden, Germany) according to the instructions provided by the manufacturer. RAPD PCR (Williams *et al.*, 1990) was performed with a reagent mix containing 1 μ M primer, 3 mM MgCl₂, 200 μ M of each nucleotide, 1 U/25 μ l Taq polymerase (Amplitaq, Perkin Elmer Cetus, Weiterstadt, Germany) and the reaction buffer supplied with the enzyme. Cycling parameters were: initial denaturation 1 min 30 sec at 94 °C; 35 cycles of 10 sec at 94 °C, 20 sec at 36 °C and 2 min at 72 °C; final elongation 2 min at 72 °C.

Products were run on a 1.5% (w/v) agarose gel in Tris-boric acid-EDTA (TBE) buffer (Sambrook *et al.*, 1989) at 4.5 V/cm for 2 h. Gels were stained with ethidium bromide and the image was digitalized with a CCD camera system (Intas, Göttingen, Germany) and stored to disk in Tagged Image File Format (TIFF). Polymorphic bands were picked with the tip of a Pasteur pipette into a new PCR reaction mix and re-amplified. Re-amplification used the same parameters as described above, except for the facts that only 25 PCR cycles at 50 °C annealing temperature were performed and 1.5 mM MgCl₂ was used. The DNA sequence of primer 1290 was: GTGGATGCGA.

For hybridizations, the Digoxigenin system (Boehringer, Mannheim, Germany) with colorimetric detection was used according to the instructions by the manufacturer. Probes were labeled with digoxigenin by PCR using the Boehringer Probe Synthesis Kit. The nucleotide labeling mixture contained 70 μ M digoxigenin-dUTP, 0.33 mM dTTP and 0.4 mM of each other nucleotide. PCR conditions were as described above for re-amplification.

As non-specific control probe, a 1500 bp fragment from the 16S rDNA of *Bradyrhizobium japonicum* USDA110spc4 was used (Vinuesa *et al.*, 1998) that was digoxigenin-labeled as described above.

For dot blots, 5 μ g of whole genomic DNA of bacterial strains isolated by standard methods were denatured and immobilized on nylon membrane filters (Boehringer Mannheim, Germany).

Table I. Origin of bacteria used in this study. (Abbreviations: Hohenheim: AG Buchenauer, Universität Hohenheim, Germany; BBA: Biologische Bundesanstalt; DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig; Germany).

Strain	Origin
<i>Pseudomonas</i> sp. W 34	Hohenheim
<i>Pseudomonas</i> sp. W 24	Hohenheim
<i>Pseudomonas</i> sp. WB1	Hohenheim
<i>Pseudomonas</i> sp. WB11	Hohenheim
<i>Pseudomonas</i> sp. WB15	Hohenheim
<i>Pseudomonas</i> sp. WB52	Hohenheim
<i>Pseudomonas</i> sp. W3	Hohenheim
<i>Pseudomonas</i> sp. W5	Hohenheim
<i>Pseudomonas</i> sp. W10	Hohenheim
<i>P. fluorescens</i> 2–79 (NRRL B15 132)	AG Werner, Marburg
<i>Pseudomonas</i> sp. B1/3	BBA, Kleinmachnow
<i>Pseudomonas</i> sp. G9	Hohenheim
<i>P. fluorescens</i> A12	Höflich, Müncheberg
<i>Pseudomonas</i> sp. I112	BBA, Darmstadt
<i>Pseudomonas</i> sp. I0026	BBA, Darmstadt
<i>Pseudomonas</i> sp. T58	AG Sikora, Bonn
<i>Pseudomonas</i> sp. G6	Hohenheim
<i>Pseudomonas</i> sp. G51	Hohenheim
<i>Pseudomonas</i> sp. G307	Hohenheim
<i>P. fluorescens</i> USDA NRRL B10	AG Werner, Marburg
<i>P. fluorescens</i> USDA NRRL B14 678	AG Werner, Marburg
<i>P. fluorescens</i> ATTC 12983	DSM
<i>P. fluorescens</i> DSM 6290	DSM
<i>P. stutzeri</i> DSM 50227 ^T	DSM
<i>P. putida</i> DSM 549	DSM
<i>P. putida</i> DSM 291 ^T	DSM
<i>P. syringae</i> DSM 1241	DSM
<i>P. putida</i> DSM 50202	DSM
<i>P. pseudoalcaligenes</i> DSM 50186	DSM
<i>Bacillus subtilis</i> FZB26	FZB Biotechnik GmbH, Berlin
<i>Bacillus subtilis</i> JH642	AG Bremer, Marburg
<i>Agrobacterium tumefaciens</i> (Stamm 38)	AG Werner, Marburg
<i>Agrobacterium rhizogenes</i> DSM 30148	DSM
<i>Azospirillum brasilense</i> DSM 1843 / ATCC 29710	DSM
<i>Acinetobacter calcoaceticus</i> DSM 30006	DSM
<i>Enterobacter agglomerans</i>	AG Werner, Marburg
<i>Escherichia coli</i> k12K	AG Werner, Marburg
<i>Erwinia herbicola</i>	AG Werner, Marburg
<i>Klebsiella planticola</i>	AG Werner, Marburg

Colony lift and colony hybridization were conducted as described in the DIG System User Guide for Filter Hybridization (Boehringer Mannheim, 1996).

For Southern hybridization, agarose gels were blotted onto a nylon membrane by vacuum transfer (20 min 1 M NaCl, 0.5 M NaOH; 50 min 1 M ammonium acetate) and hybridized as described for the colony lifts.

Phylogenetic analyses

Rep-PCR with the BOXA1R and ERIC1R/ERIC2 primer was performed in 25 µl reaction volumes as described elsewhere (Versalovic *et al.*, 1994), except that 50 ng of purified DNA and 2 units of Taq polymerase (USB-Amersham, Little Chalfont, UK) were used with the reaction buffer supplied by the manufacturer. Six microliter aliquots of the amplified reaction mixtures were fractionated on 18 cm long 1.5% (w/v) agarose gels, run at room temperature in Tris-acetic acid-

EDTA (TAE) buffer (Sambrook *et al.*, 1989) for 4.2 h at 4 V/cm. For normalization, a 1 kb ladder (GIBCO BRL, Eggenstein, Germany) was run at both sides and the central lane of each gel.

Nearly full-length 16S rDNA regions were amplified with the universal primers fD1 and rD1 (Weisburg *et al.*, 1991) in 50 µl reaction volumes using 50 ng of purified genomic DNA as described previously (Vinuesa *et al.* 1998). PCR products were extracted once with phenol:chloroform 1:1 (v/v), ethanol precipitated, and redissolved in 21 µl of H₂O. Five microliters of this solution were digested in 20 µl reaction volumes with the restriction enzymes *Cfo* I, *Dde* I, *Mbo* I and *Msp* I (Boehringer, Mannheim, Germany) and resolved on 2.5% NuSieve 3:1 agarose gels (Biozym, Hess. Oldendorf, Germany) in TBE at 5 V/cm. 16S rDNA-RFLP patterns were normalized with a 100 bp ladder (GIBCO BRL), as described above.

Electrophoretic banding patterns were visualized, digitalized and stored to disk in TIFF format as described above.

For computer-assisted pattern analysis the digitalized gel images were converted, normalized using the above mentioned molecular size markers, and analyzed with GelCompar (version 4.0, Applied Maths, Kortrijk, Belgium) as previously described (Vinuesa *et al.*, 1998). The "rolling disk" background subtraction method was applied. Similarity matrices of whole densitometric curves of gel tracks were calculated using the pair-wise Pearson's product-moment correlation coefficient (*r*-value) for RAPD- and rep PCR-patterns (Häne *et al.*, 1993). For ARDRA, a band-matching approach was selected instead (Heyndrickx *et al.*, 1996), using the Dice coefficient for calculating a pairwise similarity matrix of the combined restriction patterns yielded by the four enzymes. Cluster analysis of similarity matrices was performed using the UPGMA method (unweighted pair group method using arithmetic averages, Sokal and Michener, 1958) in both cases.

Plant experiments

Cucumber seeds were sterilized for 2×5 min in 30% H₂O₂ and washed several times with sterile tap water. Sterility was controlled by placing them on NB agar for three days. They were inoculated by incrustation with a suspension of 2.3×10^9 col-

ony forming units (cfu)/ml containing 3.3% gum arabic (Roth, Karlsruhe, Germany) for 45 min and air-dried for 1 h in a laminar flow hood.

Plants were either grown in petri dishes on water agar or in pots in a mixture of soil (Floragard, Oldenburg, Germany) and washed sand (2:1, v/v). Water content was adjusted to 60%. This substrate was filled into pots (70 g each), covered and stored for 3 days before use. During the experiments, pots were covered with transparent plastic wrap and irrigated to keep constant weight.

In some experiments, bacterial strains were inoculated into the soil before planting. For this purpose, overnight cultures of these bacteria were washed in 0.85% NaCl and resuspended in the tap water that was used to adjust humidity. The resulting bacterial titer was 3.7×10^8 cfu/g soil for W10 and 4.6×10^8 cfu/g for 2-79.

To reisolate bacteria, roots were cut off, placed in 10 ml ice-cold 0.85% (w/v) NaCl solution and sonicated for 30 min. Samples were diluted in the same solution, plated onto King B or NB agar and incubated for 24 h at 28 °C. Numbers of cfu were determined and colony lifts performed as described above. In soil experiments, tightly adhering soil that could not be removed by careful shaking was included into the analysis.

To identify colonies of the biocontrol strain W34, colony hybridizations were performed. In some experiments it was possible to use colony fluorescence on King B agar as method of identification as verified by colony hybridization. Data were statistically evaluated by the pairwise t-test.

Results

Generation and specificity testing of probes

For RAPD PCR analysis of biocontrol strains of *Pseudomonas* (Fig. 1), primers were selected that had proved before to be useful in RAPD analysis of other bacteria (Bazzicalupo and Fani, 1995) and produced a sufficient number of bands with *Pseudomonas* strains. Several polymorphic bands were picked from the gel, re-amplified and tested for specificity as probes. This screening process led to the selection of DNA fragments of 750 and 550 bp apparent length, respectively.

In dot blot hybridizations (Fig. 2) the reamplified bands of 750 bp and 550 bp were specific for the biocontrol strains W34, W24, WB1, WB11 and

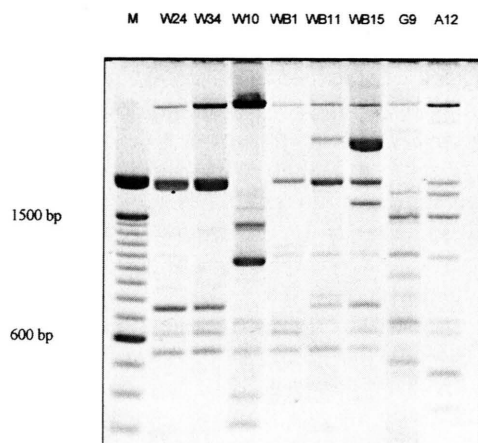


Fig. 1. RAPD pattern produced from *Pseudomonas* strains with primer 1290 (negative image of agarose gel). M = molecular weight marker (100 base pair ladder).

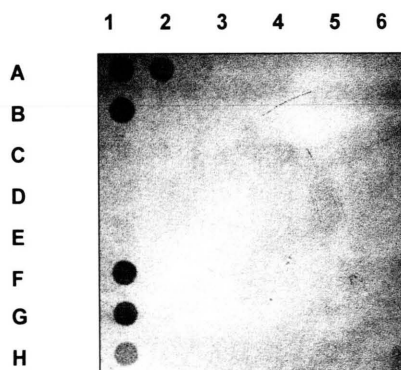


Fig. 2. Specificity of the digoxigenin-labeled 750 bp and 550 bp probes in a dot blot assay. Each dot contains 5 μ g of genomic DNA of the following strains:

A1: *Pseudomonas* sp. W24, B1: *Pseudomonas* sp. W34, C1: *Pseudomonas* sp. W3, D1: *Pseudomonas* sp. W5, E1: *Pseudomonas* sp. W10, F1: *Pseudomonas* sp. WB1, G1: *Pseudomonas* sp. WB11, H1: *Pseudomonas* sp. WB15.

A2: *Pseudomonas* sp. WB52, B2: *Pseudomonas* sp. G6, C2: *Pseudomonas* sp. G9, D2: *Pseudomonas* sp. G51, E2: *Pseudomonas* sp. G307.

A3: *Pseudomonas* sp. T58, E3: *Pseudomonas* sp. B1/3, F3: *Pseudomonas* sp. I112, G3: *Pseudomonas* sp. I0026.

A4: *P. fluorescens* A12, B4: *P. fluorescens* USDA NRRL B10, C4: *P. fluorescens* USDA NRRL B14 678, D4: *P. fluorescens* 2-79, E4: *P. fluorescens* DSM 6290, F4: *P. fluorescens* ATTC 12983.

A5: *P. denitrificans*, B5: *P. pseudoalcaligenes*, C5: *P. stutzeri*, D5: *P. syringae*, G5: *Bacillus subtilis*.

A6: *Agrobacterium tumefaciens*, B6: *Agrobacterium rhizones*, C6: *Azospirillum brasilense*, D6: *Acinetobacter calcoaceticus*, E6: *Enterobacter agglomerans*, F6: *Escherichia coli*, G6: *Erwinia herbicola*, H6: *Klebsiella planticola*.

WB15, which also show a high degree of similarity in their RAPD banding patterns. The probes did not hybridize to a number of other strains of *Pseudomonas* and other genera tested. A minimum of 1 μ g genomic DNA bound to the filter was required to be detected with the digoxigenin system. Control hybridizations conducted with the cloned 750 bp and 550 bp probes showed that they were identical in specificity. For population studies, the cloned 750 bp fragment was used.

To prove its value as a molecular tool, the probe was tested in colony hybridization assays. Colonies were lifted from agar plates (Fig. 3b) on a nylon membrane, their DNA was immobilized and hybridized with the probes. Strains W34, W24, WB1, WB11, WB15 and WB52 could be easily distinguished from reference pseudomonads by their positive reaction with the specific probe (Fig. 3c). The presence of DNA of the non-hybridizing reference strains on the filter was verified by hybridization with a non-specific probe (Fig. 3d). The 550 bp fragment presented the same specificity when labeled and used as probe (data not shown).

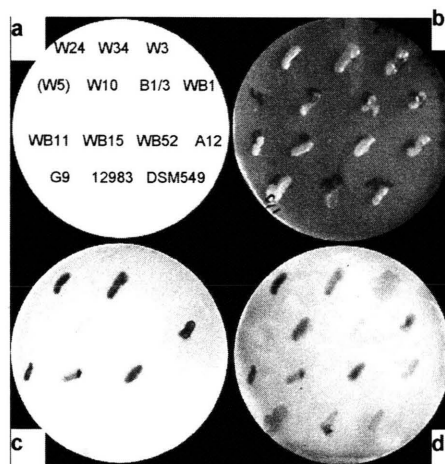


Fig. 3. Specificity of the 750 bp probe in a colony hybridization assay.

a) bacterial strains used in this assay and their relative position on the membrane. See Table 1 for complete names;

b) bacterial colonies on agar plate;

c) colony hybridization with 750 bp probe;

d) colony hybridization of same membrane with universal probe.

Population studies of *Pseudomonas* sp. W34 in the rhizosphere

The 750 bp probe was used to determine the population of *Pseudomonas* sp. W34 on the roots of *Cucumis sativus*. Analyses were conducted in an *in vitro* system on water agar and in non-sterilized soil, respectively. In the latter experiments, bacteria that were attached to the surface of the whole root and tightly adhering soil ("rhizosphere soil") were included in the analysis.

After inoculation of seeds with single bacterial strains (W34, 2-79 or W10) in the *in vitro* system, similar numbers of bacteria could be reisolated from the roots, indicating that all these strains were able to grow on root systems (Fig. 4). When inoculated together with W10, W34 also colonized the root effectively and the population of W10 was suppressed nearly to the limit of detection. Although W10 was able to colonize the root *in vitro*, it was not clear if this was the original habitat of this strain, since it was originally isolated from leaves of wheat. Therefore, the experiment was also conducted with W34 and *Ps. fluorescens* 2-79, a strain that is well-established as rhizosphere colonizer and biocontrol agent (e.g. Bull *et al.*, 1991; Mazzola and Cook, 1991). W34 was equally

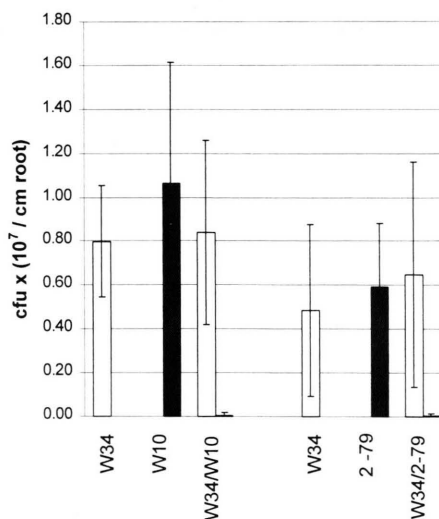


Fig. 4. Populations of *Pseudomonads* isolated from the rhizosphere of *Cucumis sativus*. In two separate experiments, *Pseudomonas* sp. W34 was inoculated on the seeds alone and in combination with *Pseudomonas* sp. W10 or *P. fluorescens* 2-79. Plants were grown on water agar for 3 days. Error bars represent standard deviation.

competitive in this combination (Fig. 4). The differences between W34 and its competitors were statistically significant (t-test, $p=0.05$). Each of the two strains inoculated without competitor reached a similar level of colonization.

To make sure that W34 did not inhibit the growth of the other strains *in vitro*, strains were plated together from separately grown liquid cultures. No antagonistic effect of W34 on 2-79 or W10 could be detected.

The persistence of seed-inoculated W34 on the root during a period of 10 days was monitored in non-sterilized soil (Fig. 5). It was found that colony fluorescence on King B agar was a reliable character to identify W34 in this experiment, because 100% of fluorescent colonies also showed positive reaction with the DNA probe. Colonies in the control treatments harvested after 7 and 10 days did not fluoresce. Together with the total bacterial count per cm root, the number of cfu of W34 measured per root length slowly decreased with time but after 10 days W34 still constituted 27.5% of the bacterial population detected on the roots.

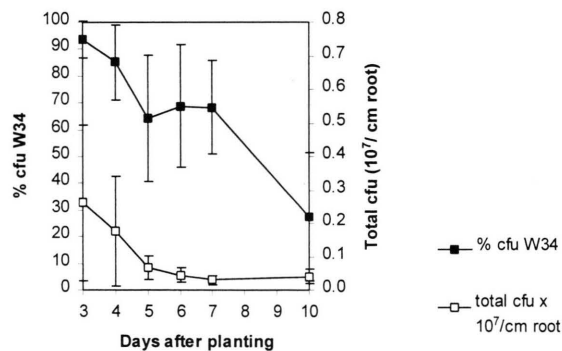


Fig. 5. Total cfu isolated from the rhizosphere of *Cucumis sativus* in non-sterilized soil and proportion of total cfu of seed-inoculated *Pseudomonas* sp. W34. Plants were harvested at 3, 4, 5, 6, 7 and 10 days. Error bars represent standard deviation.

The rhizosphere population of W34 was assessed in competition with other fluorescent *pseudomonads* that were inoculated into soil 24 h before *Cucumis sativus* was planted (Fig. 6). W34 colonies were discerned by colony hybridization with the specific 750 bp probe from other fluorescing-colonies that grew on the agar plates. Only 1 to 2% of colonies from non-inoculated treatments were fluorescing. In the treatments where W10 had been inoculated into the soil prior to W34, all

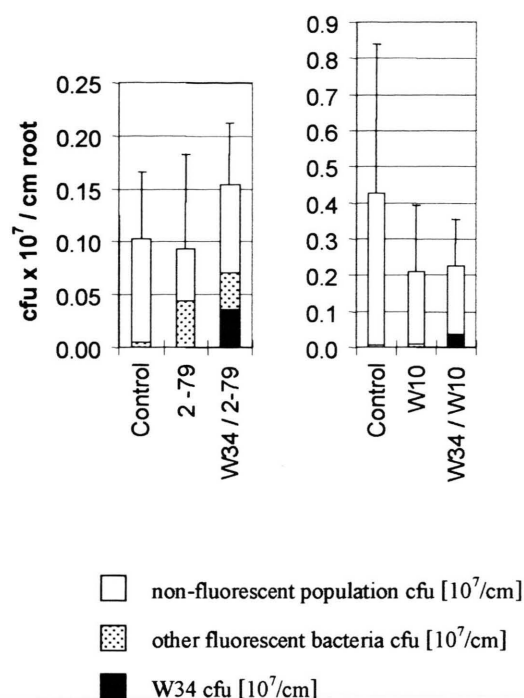


Fig. 6. Populations of *Pseudomonads* isolated from the rhizosphere of *Cucumis sativus*. *Pseudomonas* sp. W34 was inoculated on seeds alone (controls) and in combination with *Pseudomonas* sp. W10 or *Ps. fluorescens* 2-79 (inoculated into the soil). Plants were grown in non-sterilized soil for 3 (W34/2-79) and 4 days (W34/W10), respectively. Error bars represent standard deviation.

fluorescent colonies showed positive reaction with the DNA probe, indicating that W10 was not detectable when competing with W34 under these conditions. Strain 2-79, however, was able to co-exist with W34 and accounted for 35 to 75% of the fluorescent colonies. In general, the percentage of fluorescent colonies was lower than in the experiment presented in Fig. 5.

Phylogenetic analysis of biocontrol strains

Several biocontrol and reference strains used in this study were analyzed for their phylogenetic relationships by DNA-based methods. Although the original intention to perform RAPDs was the generation of DNA fragments to be screened as specific probes, the fragment patterns can also be used to estimate the relatedness of the strains under study. In the case of the biocontrol strains W24, W34, WB1, WB11, WB15 and WB52 a strik-

ing similarity of RAPD patterns could be detected with three different random primers. Fig. 1 shows the patterns produced with primer 1290. Strains W34 and W24 even presented identical patterns with all of the three primers used. However, branching orders of the dendrograms calculated from RAPD patterns varied with the primers used and several repetitions of the same reactions could produce slightly different banding patterns.

Therefore rep-PCR genomic fingerprinting with BOX and ERIC primers was chosen as an independent test for the results gained by RAPD fingerprinting. Rep-PCR is known to produce highly complex and reproducible genomic fingerprints useful to compare closely related bacterial at the strain level (Versalovic *et al.*, 1994; Vinuesa *et al.*, 1998). In our hands, the reproducibility of the patterns yielded by this technique is significantly higher than those generated by RAPD-PCR, as found also by other groups (Tyler *et al.*, 1997). BOX- and ERIC-PCR fingerprinting confirmed the high degree of genetic relatedness of biocontrol strains W34, W24 and WB1 (Fig. 7a), as suggested by RAPD fingerprinting. Furthermore, cluster analysis of both rep-PCR patterns resulted in identical groupings, illustrating the consistency of the results obtained by this fingerprinting technique. Strain 2-79, a well-known biocontrol strain, was not related to the W34 group. The two non-antagonistic strains W3 and W10 constitute another group of closely related strains, as indicated by the high similarity (*r*-values) of their rep-PCR patterns.

To analyze the phylogenetic relationships between some of the biocontrol and reference strains used in this study, PCR/RFLP analysis was performed on nearly full-length 16S rDNA sequences amplified with the universal primer pair fD1/rD1 (Weisburg *et al.*, 1991). As expected, all strains included in the ARDRA yielded a single 1500 bp PCR product, indicating that no insertions are present in these 16S rDNA sequences, as has been reported for some *Rhizobium tropici* strains (Laguerre *et al.*, 1994; Vinuesa, unpubl. results). Cluster analysis on the combined *CfoI*-, *DdeI*-, *MboI*- and *MspI*-restriction patterns of the PCR products resulted in three groupings within the *Pseudomonas* strains studied (Fig. 7b). The first cluster (P1) comprised the two *P. fluorescens* strains NRRL B14678 and

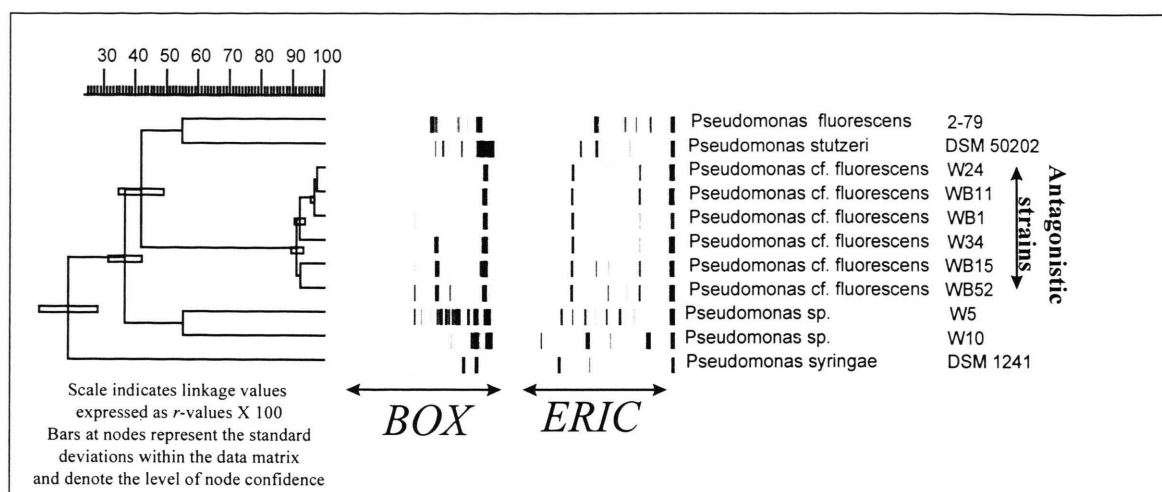


Fig. 7a. Rep-PCR genomic fingerprints of antagonistic and reference *Pseudomonas* strains: UPGMA dendrogram constructed from Pearson product-moment correlation coefficients of whole densitometric patterns of ERIC and BOX-PCR genomic fingerprints. Scale: r -values $\times 100$.

ATTC 12983. The second one (P2) clustered two of the non-antagonistic isolates from Hohenheim together with *P. putida* strain 549. All four enzymes consistently grouped the selected antagonistic strains together, conforming cluster P3, and displaying a 16S rDNA genotype that is clearly different from that of the strains found in clusters P1 and P2.

The estimated sizes of the restriction fragments obtained for each of the three types of RFLP patterns (P1 to P3) are presented in Table II, together with the exact sizes of the corresponding restriction fragments derived from type strain 16S rRNA gene sequences retrieved from sequence data bases. The accuracy of the size estimation made on normalized gels with GelCompar is well-illustrated by the values presented in this table, as previously shown for *Bradyrhizobium* strains (Vinuesa *et al.*, 1998). These data also suggest that the strains in clusters P2 are members of the "*P. putida* 16S rDNA lineage", whereas those of cluster P3 are not members of "*P. fluorescens* (rDNA) intragenetic group" (Moore *et al.*, 1996). This last conclusion is in contradiction with that obtained by phenotypic characterization of P3 strains with the Biolog system, which ascribed them to *P. fluorescens* biovar C (data not shown).

Discussion

The 750 bp DNA probe generated by RAPD was shown to be a reliable and useful tool to detect and quantify biocontrol strain W34 in the rhizosphere both on agar and in soil. The level of naturally occurring bacteria found in the soils tested that reacted with the probe was negligible. The probe was specific for a group of closely related strains with biocontrol properties. Further efforts to find a probe to differentiate within this group of strains therefore do not seem promising and worthwhile.

As no genetically engineered organisms are involved, this technique is easy to conduct under greenhouse conditions to further elucidate the role of W34 as biocontrol agent, especially in the presence of pathogens.

With the methods described, organisms that do not grow on complex media are not detectable. This is a general problem of microbial ecology (Amann *et al.*, 1995) and will have to be assessed in the future by *in situ* hybridization with DNA probes.

W34 was clearly shown to belong to a phylogenetic group that contained several strains with biocontrol properties. Their close genetic relatedness at the strain level of taxonomic refinement, as revealed by genomic fingerprinting with BOX and

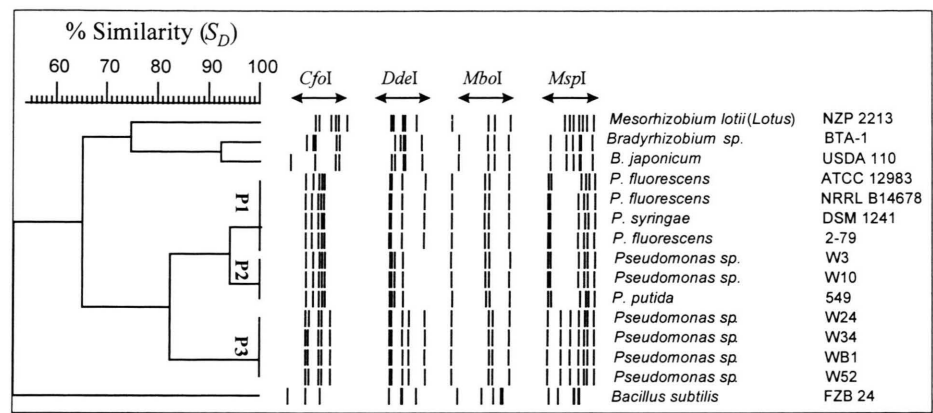


Fig. 7b. Phylogenetic analysis of antagonistic and reference *Pseudomonas* strains based on 16S rDNA PCR/RFLP analysis: UPGMA dendrogram using the Dice coefficient for calculating a pairwise similarity matrix of the combined restriction patterns of amplified 16S rDNA fragments generated with the restriction enzymes *CfoI*, *DdeI*, *MspI* and *MboI*. Cluster P3 contains the antagonistic strains.

Table II. Apparent sizes (in bp) of restriction fragments of PCR-amplified 16S rDNA of *Pseudomonas* strain clusters P1, P2 and P3 (See Fig. 8) in comparison to sizes of restriction fragments calculated from complete 16S rRNA gene sequences for some *Pseudomonas* spp. type strains (marked by “T”). Enzymes *CfoI*, *DdeI* and *MboI* were used. Only restriction fragments greater than 50 bp are shown. Restriction pattern of the type strains are based on the sequences with accession numbers Z76662, Z76667 and Z76653, respectively. These sequences were modified at their ends to include the primer sequences fD1/rD1, used for ARDRA, in order to make precise size comparisons between sequence and RFLP data possible.

	<i>CfoI</i>	<i>DdeI</i>
Cluster P1 (<i>P. fluorescens</i> ATTC 12983)	450, 355, 282, 253, 224	435, 407, 274, 94
Cluster P2 (<i>Pseudomonas</i> sp. W3)	453, 361, 284, 253, 224	445, 417, 377, 274
Cluster P3 (<i>Pseudomonas</i> sp. W34)	456, 410, 280, 253, 172	438, 407, 272, 213, 95
<i>P. fluorescens</i> DSM 50227 ^T	450, 357, 279, 250, 225	461, 408, 271, 266, 94
<i>P. putida</i> DSM 291 ^T	451, 357, 279, 250, 225	434, 409, 364, 266
<i>P. alcaligenes</i> LMG 1224 ^T	450, 357, 279, 250, 225	408, 324, 266, 150, 110, 93, 83
	<i>MboI</i>	<i>MspI</i>
Cluster P1 (<i>P. fluorescens</i> ATTC 12983)	926, 267, 230, 83	550, 509, 172, 130, 111, 79
Cluster P2 (<i>Pseudomonas</i> sp. W3)	926, 267, 235, 87	550, 504, 179, 133, 107, 82
Cluster P3 (<i>Pseudomonas</i> sp. W34)	926, 228, 193, 86	559, 346, 248, 172, 132, 111, 82
<i>P. fluorescens</i> DSM 50227 ^T	906, 260, 225, 83	549, 508, 170, 130, 110, 81
<i>P. putida</i> DSM 291 ^T	906, 260, 226, 83	549, 508, 170, 130, 110, 81
<i>P. alcaligenes</i> LMG 1224 ^T	449, 448, 260, 225, 83	549, 508, 170, 130, 110, 81

ERIC primers, explains why they are all recognized with the same DNA probe. The genus *Pseudomonas* contains a vast variety of metabolically diverse organisms. Therefore, its taxonomy is very

complex (Kerstens *et al.*, 1996), especially in the *P. fluorescens* intrageneric cluster (Moore *et al.*, 1996). Some studies with similar DNA-based characterization methods have been performed re-

cently with pseudomonads by other authors (Laguerre *et al.*, 1994; Lemanceau *et al.*, 1995; Frey *et al.*, 1997). The close relatedness of *P. putida* and *P. syringae* isolates to *P. fluorescens* strains, as depicted in Fig. 7b, has also been reported by some of these authors. Based on ARDRA with four enzymes, strains in cluster P3 can not be ascribed to the *P. fluorescens* intrageneric cluster, as the Biolog test suggests. Strains of this species have been shown to cluster in different phenons when analyzed with the Biolog system (Grimont *et al.*, 1996). This may lead to groupings that are inconsistent with those based on 16S rDNA analysis, as we show in our study. The genotypic typing approach used in this study will facilitate the accurate identification of related biocontrol isolates in the future, both at the species and at the strain levels of taxonomic refinement.

Our data indicate that inoculated strains replace the indigenous bacterial population on the root rather than add to it. As found for strain W10, the capability to grow on the root surface under sterile conditions and the competitiveness for rhizosphere colonization in nonsterile soil are traits that are not necessarily coupled. This agrees well with findings by other authors (Acea *et al.*, 1988; Campeau *et al.*, 1988) and reinforces the need to perform tests under nonsterile conditions.

W34 is a consistently competitive colonizer of the root of *Cucumis sativus* under *in vitro* conditions as well as in non-sterilized soil. Its population is stable in the rhizosphere of this plant for a period of time sufficient to allow application as bioagent against pathogens such as *Pythium* that attack the seedlings at an early stage. Inoculation of the seeds was demonstrated to be a feasible and efficient approach in this context. Even when inoculated together with the well-established biocontrol strain 2-79, a considerable number of bacteria of strain W34 was active in the rhizosphere. We found evidence that 2-79 can coexist with W34 on the roots of *Cucumis sativus*. The possibility of a combined application of both strains should therefore be considered in the future to achieve a broader range of biocontrol effects.

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