

# Successful strategy for the selection of new strawberry-associated rhizobacteria antagonistic to *Verticillium* wilt

Gabriele Berg, Stefan Kurze, Arno Buchner, Elizabeth M. Wellington, and Kornelia Smalla

**Abstract:** In order to isolate and characterize new strawberry-associated bacteria antagonistic to the soil-borne pathogenic fungus *Verticillium dahliae* Kleb., rhizobacterial populations from two different strawberry species, Greenish Strawberry (*Fragaria viridis*) and Garden Strawberry (*F. × ananassa*) obtained after plating onto King's B and glycerol-arginine agar, were screened for in vitro antagonism toward *V. dahliae*. The proportion of isolates with antifungal activity determined in in vitro assay against *V. dahliae* was higher for the Garden Strawberry than for the Greenish Strawberry. From 300 isolates, 20 isolates with strong antifungal activity were selected characterized by physiological profiling and molecular fingerprinting methods. Diversity among the isolates was characterized with molecular fingerprints using amplified ribosomal DNA restriction analysis (ARDRA) and the more discriminating BOX-PCR fingerprint method. The physiological profiles were well correlated with molecular fingerprinting pattern analysis. Significant reduction of *Verticillium* wilt by bacterial dipping bath treatment was shown in the greenhouse and in fields naturally infested by *V. dahliae*. The relative increase of yield ranged from 117% (*Streptomyces albidoflavus* S1) to 344% (*Pseudomonas fluorescens* P10) in greenhouse trials, and 113% (*Streptomyces albidoflavus* S1) to 247% (*Pseudomonas fluorescens* P6) in field trials. Evaluation resulted in the selection of three effective biocontrol agents (*Pseudomonas fluorescens* P6, P10, and *Streptomyces diastochromogenes* S9) antagonistic to the *Verticillium* wilt pathogen.

**Key words:** biocontrol, molecular fingerprint, antifungal properties, *Pseudomonas*, *Streptomyces*.

**Résumé :** Le but de ce travail était d'isoler et de caractériser de nouvelles bactéries associées aux fraisiers étant antagonistes au champignon pathogène du sol *Verticillium dahliae* Kleb. Pour ce faire, des populations de rhizobactéries provenant de deux espèces différentes de fraisiers, le fraisier vert (*Fragaria viridis*) et le fraisier cultivé (*F. × ananassa*) ont été obtenues suite à leur ensemencement sur des géloses King B et glycérol-arginine, et leur antagonisme vis-à-vis de *V. dahliae* a été analysé in vitro. La proportion des isolats ayant une activité antifongique tel que déterminée par l'essai in vitro contre *V. dahliae* était plus grande chez le fraisier cultivé que chez le fraisier vert. Sur 300 isolats, 20 isolats ayant une forte activité antifongique ont été sélectionnés puis caractérisés par des méthodes de profils physiologiques et d'empreintes moléculaires. La diversité parmi les isolats a été caractérisée à l'aide d'empreintes moléculaires en utilisant la technique de l'analyse de restriction de l'ADN ribosomal amplifié (ARDRA) ainsi qu'à l'aide de la méthode plus discriminante d'obtention d'empreintes génétiques par BOX-PCR. Les profils physiologiques présentaient une bonne corrélation avec l'analyse des motifs d'empreintes moléculaires. Une réduction significative de la flétrissure causée par *Verticillium* à la suite de traitements par trempage dans des bains de cultures bactériennes fut démontrée en serre et dans des champs naturellement infectés par *V. dahliae*. L'augmentation relative des récoltes allait de 117 % (*Streptomyces albidoflavus* S1) à 344 % (*Pseudomonas fluorescens* P6) en serre, et de 113 % (*Streptomyces albidoflavus* S1) à 247 % (*Pseudomonas fluorescens* P6) dans les champs expérimentaux. Cette étude a permis la sélection de trois agents de lutte biologique (*Pseudomonas fluorescens* P6, P10, et *Streptomyces diastochromogenes* S9) qui sont antagonistes au pathogène *Verticillium*, responsable de la flétrissure.

**Mots clés :** lutte biologique, empreinte moléculaire, propriétés antifongiques, *Pseudomonas*, *Streptomyces*.

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## Introduction

The rhizosphere is the soil surrounding the root that is subject to the influence of root exudates (Lynch 1990). Intense microbial activity and greater microbial populations occur in this micro-environment, compared to the bulk soil environment, because of the release of large amounts of organic matter from roots (Lynch 1990; Sørensen 1997). The rhizosphere is also the site for interactions between plants, pathogenic microorganisms, and antagonistic rhizobacteria and fungi (Trevors and Van Elsas 1997). Bacterial antagonism toward plant pathogenic fungi may be due to the production of antibiotics, competition, or parasitism (Fravel 1988; Chet et al. 1990). The relative importance of these mechanisms may differ considerably among strains of rhizobacteria (Neiendam-Nielson et al. 1998). Antagonistic rhizobacteria have often been the focus of research in sustainable systems of agriculture (Powell 1993; Whipps 1997) because of their ability to suppress diseases caused by soil-borne plant pathogens (Weller 1988; Emmert and Handelsman 1999).

Strawberry (*Fragaria × ananassa* Duch.) is an important berry crop (FAO, Statistical Databases, Anonymous 2000). However, it is subject to attack by *Verticillium dahliae* Kleb. (Maas 1998). Microsclerotia of the pathogen develop in the senescing tissues of diseased plants and may persist in soil for several years, making chemical control problematic (Maas 1998; Fravel 1992). In the coming years, the loss of methyl bromide as a control measure for *V. dahliae* will have a great impact on the accumulation of microsclerotia in soil. One alternative to chemical control is the use of rhizobacteria-based biological control (Weller 1988; Whipps 1997). However, only a few bacterial species inhibiting the growth of *V. dahliae* have been found so far (Leben et al. 1987; Beauchamp et al. 1991; Berg 1996; Sharma and Nowak 1998). A few biocontrol agents are currently on the market, but none protect strawberries against *V. dahliae* (see [www.barc.usda.gov/psi/bpdl/bdplprod/bioprod.htm](http://www.barc.usda.gov/psi/bpdl/bdplprod/bioprod.htm)).

Not only is physiological and molecular characterization of potential rhizobacterial antagonists important for selecting and monitoring their fate in the rhizosphere, it is also important for their registration, patenting, and quality checks (Lemanceau et al. 1995). Several classical and molecular techniques are available for analyzing the biodiversity of microorganisms (Rademaker and De Bruijn 1997), and these have been successfully applied to rhizobacteria (Lemanceau et al. 1995; Berg 2000). Functional characterization on the basis of in vitro antagonism may be a useful tool when selecting candidate isolates as biological control agents (Neiendam-Nielson et al. 1998; Barnett et al. 1999).

In the present study, we developed a screening strategy to investigate rhizobacterial populations isolated from two different sources (strawberry species) with antagonistic activity to *Verticillium* wilt. Therefore, rhizobacteria were isolated on media selective for the genera *Pseudomonas* and *Streptomyces*, which are known for their high antagonistic potential, and screened for in vitro antagonism toward *V. dahliae*. Selected isolates were characterized by physiological profiling and molecular fingerprinting. The strains were compared using cluster analysis and the best strains were evaluated for biocontrol activity in the greenhouse and in naturally infested *Verticillium* fields.

## Materials and methods

### Isolation of bacterial antagonists and determination of the colony forming units (CFU)

Bacteria were isolated from roots of strawberry: *Fragaria × ananassa* Thuill. cv. Elsanta (Garden Strawberry) and *Fragaria viridis* (Duchesne) Weston (Greenish Strawberry). Six independent samples (based on 10 plants) were taken on October 16, 1995. Plant roots with adhering soil particles were washed for 20 min in sterile 0.85% NaCl solution. The subsequent suspension was serially diluted onto King's B-agar for isolation of *Pseudomonas* (King et al. 1954) (PSE, Gibco, Paisley, Scotland) and onto glycerol-arginine-agar for isolation of *Streptomyces* (El-Nakeeb and Lechevalier 1963) (GAA; containing glycerin (12.5 g), arginine (1.0 g), NaCl (1.0 g), K<sub>2</sub>HPO<sub>4</sub> (2.0 g), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.5 g), Fe(SO<sub>4</sub>)<sub>3</sub>·6H<sub>2</sub>O (0.01 g), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.001 g), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.001 g), MnSO<sub>4</sub>·H<sub>2</sub>O (0.001 g) (all from Merck, Darmstadt, Germany), agar (15 g) (Difco, Detroit, Mich.), and distilled water (1 L). Plates were incubated for 7 d at 20°C, and bacterial colonies were counted. The fluorescent pseudomonads were isolated on King's B under UV-light (254 and 366 nm). Isolates were purified and selected isolates (12 h culture, 30°C) were stored in nutrient broth (Sifin, Berlin, Germany) with 15% glycerol at -80°C.

### Bioassay for in vitro inhibition of fungal growth

Antifungal activity was determined in an in vitro assay on Waksman agar (WA; containing: proteose-peptone (5 g) (Merck), glucose (10 g) (Merck), meat extract (3 g) (Chemex, München, Germany), NaCl (5 g) (Merck), agar (20 g) (Difco), distilled water (1 L), pH 6.8). A suspension of hyphal fragments of fungi (48 h culture, 20°C, Waksman broth) was plated on agar and after 30 min, bacteria were streaked as a broad band on the same surface. The plant pathogens *Verticillium dahliae*, *V. albo-atrum*, *Fusarium culmorum* (ascomycetes with a chitin-glucan-containing cell wall), *Rhizoctonia solani* (a basidiomycete with a chitin-glucan-containing cell wall), and *Pythium ultimum*, *Phytophthora cactorum* (oomycetes with a cellulose-glucan-containing cell wall) were used in this study. Generally, the phytopathogenic fungi grew as well as the rhizobacteria on Waksman agar. Zones of inhibition were measured after 5 days of incubation at 20°C. Inhibition was clearly discerned by limited growth or the complete absence of fungal mycelium in the inhibition zone surrounding a bacterial colony. The fungal strains *Verticillium albo-atrum* DSM 12233 and *Rhizoctonia solani* DSM 63010 were obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The other phytopathogenic fungi were from the strain collection of the University of Rostock, Department of Microbiology.

### Bacterial identification

The standardized micro-method API 20 NE (BioMérieux, Marcy-L'Etoile, France) was used for Gram-negative bacteria. Characterization of *Streptomyces* species was with the 41 morphological and physiological diagnostic characters outlined by Williams et al. (1983). Three identification statistics were used to assess the reliability of identifications of unknown strains. The Wilcoxon probability was used to assign an identity to an unknown strain, and scores of 0.8 and above indicated a positive identification. Taxonomic distance (*d*) determined the distance of the unknown from the centroid of the cluster group to which the unknown has been assigned; scores of 0.4 and below were taken to indicate a position within the cluster. The standard error of *d* gave a measure of the variation in test results of the unknown around the centroid of a cluster with which it was identified; scores of 2.0 or less were typical of strains grouped within the cluster, and a nega-

tive score indicated the unknown was closer to the centroid than average.

### Production of secondary metabolites

Antibiosis to *V. dahliae* by the bacterial strains was assayed on Waksman agar plates (15 mL) containing 5 mL of the sterile culture filtrate (64 h culture). The pH was adjusted to be between 7 and 8. A 5-mm plug from agar plates of *V. dahliae* was placed in the center of each plate. As a control, prepared WA plates (20 mL) were similarly inoculated with mycelial plugs. Colony growth was measured for 10 days and the reduction (%) in linear growth of the fungi was calculated. Siderophore production was assayed according to the method of Schwyn and Neilands (1987). The Aquaquant 14417-Testsystem (Merck) with culture broth (64 h culture) of the isolates was used to detect cyanide production.

### Production of cell-wall-degrading enzymes

Chitin degradative ability was screened by plating onto chitin-agar-plates (CA) containing: nutrient broth (Sifin) 1.62 g, NaCl (0.5 g), M9 salts (6 g), Chitin (2 g), CaCl<sub>2</sub> (14.7 g), MgSO<sub>4</sub> (246.6 g), Thiamin-HCl (1 g) (all from Sigma, Deisenhofen, Germany), Bacto-Agar (Difco) (15 g), and distilled water (1 L). Clearance halos indicating enzymatic degradation were measured after 5 days of incubation at 30°C.  $\beta$ -1,3-Glucanase activity was determined by measuring the production of reducing sugars from laminarin (Fluka, Buchs, Switzerland). The standard assay (1 mL) contained the enzyme extract, 2.5 mg laminarin, and 100 mM acetate buffer pH 5.2. The laminarin substrate was dissolved in acetate buffer by heating at 60°C before use. The reaction mixture was incubated for 1 h at 50°C. Total reducing sugars were assayed colorimetrically at 530 nm and expressed as glucose equivalents.

### DNA extraction and PCR assay using BOX-PCR

Genomic DNA from each strain was extracted by the method of Wilson (1987). BOX element oligonucleotide primers with the sequence of 5'-CTACGGCAAGGCGACGCTGACTGACG-3' were synthesized by MWG Biotech (Ebersberg, Germany). The BOX-PCR was performed as described by Rademaker and De Bruijn (1997).

### ARDRA

Amplified ribosomal DNA restriction analysis (ARDRA) was performed by means of PCR. Amplified 16S rDNA fragments were digested according to Massol-Deya et al. (1995) using the enzymes *AluI* and *HaeIII* (New England Biolabs, Schwalbach, Germany).

### Biological control in the greenhouse

Unsterilized soils (sandy loam, pH 6.9) were infested with *V. dahliae* according to Berg et al. (1994). Polypropylene boxes (0.7 L) were filled with infested soil and strawberry frigo plants (harvested as runner plants in autumn, stored at a temperature of -2°C during winter) cv. Elsanta (Janssen, Kalkar, Germany) were planted into the soil. Plant roots were dipped in a suspension of the rhizobacterial strain ( $2 \times 10^9$  CFU·mL<sup>-1</sup>) for 15 min and planted into infested soil. Spontaneous mutants resistant to rifampicin ( $100 \mu\text{g}\cdot\text{mL}^{-1}$ , Fluka, Buchs, Switzerland) of candidate rhizobacteria were used for all biocontrol experiments. Control plants without treatment were dipped in tap water and planted in infested soil (untreated control). In each experiment, six pots containing plants grown in soil without pathogen inoculum, and treated or not with inoculant bacteria, served as negative controls. Thirty replicates of each treatment were performed in a randomized complete block design of three blocks. The experiments were conducted under greenhouse conditions (artificial light, long day) at average temperatures of 24°C for ten weeks. Every week after inoculation, disease incidence was recorded and numbers of buds and blossoms

were counted. After 10 weeks, five independent rhizosphere samples (soil adhering to roots) based on 5 plants were collected. CFUs of spontaneous rifampicin resistant mutants on nutrient agar containing 100 ppm rifampicin were determined after incubation of 5 days at a temperature of 20°C. Additionally, the fresh and dry weights of fruits were measured. Experiments were repeated two times using the identical test design.

### Biological control of Verticillium wilt in field trials

The field trials I (1997, location Stuthof) and II (1998, location Goorstorf) were carried out in Germany (Mecklenburg-Western Pomerania) in areas naturally infested by *V. dahliae*. The density of microsclerotia of *V. dahliae* in soil was determined according to the soil dilution method of Termorshuizen et al. (1998). Soil population density of *V. dahliae* in Stuthof and Goorstorf was 40 and 21 microsclerotia · g<sup>-1</sup> soil (Table 2). Further soil parameters at both locations were analyzed by the Institute for Agricultural Analysis and Research (LUFA, Rostock, Germany). In Stuthof, the soil type was loamy sand, pH = 5.5, 1.7% organic matter and the following contents of nutrients (mg · 100 g<sup>-1</sup> soil): P<sub>2</sub>O<sub>5</sub>, 12; K<sub>2</sub>O, 15; Mg, 6. In Goorstorf, the soil quality was sand, pH = 5.7, 1.3% organic matter and the following contents of nutrients (mg · 100 g<sup>-1</sup> soil): P<sub>2</sub>O<sub>5</sub>, 16; K<sub>2</sub>O, 8; Mg, 11. In trials I and II, strawberry plants cv. Elsanta were grown with approximately 2000 plants in randomized complete block design with six replicates. The strawberries were planted as frigo plants in May. Candidate rhizobacteria ( $2 \times 10^9$  CFU · mL<sup>-1</sup>) were diluted with tap water (v/v) 1:1 and were applied via a 15-minute dipping bath treatment of the roots in the rhizobacteria suspension immediately prior to planting. As controls, tap water was used instead of bacterial suspension. In July of the same year, the strawberry fruits were harvested and the fruit weight was measured. Additionally, wilted plants were counted. Five independent rhizosphere samples based on 10 plants were collected, and CFUs of spontaneous rifampicin resistant mutants on nutrient agar containing 100 ppm rifampicin were determined.

### Statistical analysis

Data of the physiological profiling were converted to a binary code, and interisolate relationships were measured by the Euclidian metric unweighted pair-group average method using the STATISTICA program (StatSoft, Hamburg, Germany). Molecular fingerprint patterns generated for each strain were compared and grouped by using the Gelcompare program (Kortrijk, Belgium). Differences between the treatments in the biocontrol experiments were determined by a two-sided test of binomial proportion ( $P < 0.05$ ) by Statistical Product and Service Solutions for Windows, Rel. 8.0.0. (SSPS Inc., Chicago, Ill.).

## Results

### Isolation of strawberry-associated rhizobacteria

The abundance of culturable bacteria in the strawberry rhizospheres was determined on King's B medium for isolation of *Pseudomonas* strains and GAA, glycerol-arginine-agar for the preferential isolation of *Streptomyces* species. The plate counts on GAA were lower than on King's B for both strawberry species. Higher population densities of rhizobacteria were found from the Greenish Strawberry:  $2.2 \times 10^9$  SD  $\pm$  1.1 (pseudomonads) and  $1.3 \times 10^8$  SD  $\pm$  1.0 (streptomycetes) CFU per g wet root. The Garden Strawberry plants had lower population densities of rhizobacteria:  $9.5 \times 10^8$  SD  $\pm$  4.6 (pseudomonads) and  $1.1 \times 10^7$  SD  $\pm$  0.2 (streptomycetes) CFU per g wet root.

**Table 1.** Strains of *Pseudomonas* and *Streptomyces* isolated from *Fragaria* × *ananassa* Thuill. cv. Elsanta (Garden Strawberry) and *Fragaria viridis* (Duchesne) Weston (Greenish Strawberry) grown in Mecklenburg-Western Pomerania (Germany). Pseudomonads were isolated on King's B agar; streptomycetes were isolated on glycerol-arginine-agar.

No.	Plant species	Identification species	ID*
P1	<i>Fragaria</i> × <i>ananassa</i>	<i>Pseudomonas fluorescens</i>	0,89
P2	<i>Fragaria</i> × <i>ananassa</i>	<i>Pseudomonas fluorescens</i>	0,89
P3	<i>Fragaria</i> × <i>ananassa</i>	<i>Pseudomonas fluorescens</i>	0,89
P4	<i>Fragaria</i> × <i>ananassa</i>	<i>Pseudomonas chlororaphis</i>	0,82
P5	<i>Fragaria</i> × <i>ananassa</i>	<i>Pseudomonas chlororaphis</i>	0,82
P6	<i>Fragaria</i> × <i>ananassa</i>	<i>Pseudomonas fluorescens</i>	0,89
P7	<i>Fragaria</i> × <i>ananassa</i>	<i>Pseudomonas fluorescens</i>	0,89
P8	<i>Fragaria</i> × <i>ananassa</i>	<i>Pseudomonas fluorescens</i>	0,89
P9	<i>Fragaria viridis</i>	<i>Pseudomonas fluorescens</i>	0,63
P10	<i>Fragaria viridis</i>	<i>Pseudomonas fluorescens</i>	0,63
S1	<i>Fragaria</i> × <i>ananassa</i>	<i>Streptomyces albidoflavus</i>	0,98
S2	<i>Fragaria</i> × <i>ananassa</i>	<i>Streptomyces rimosus</i>	0,95
S3	<i>Fragaria</i> × <i>ananassa</i>	<i>Streptomyces diastatochromogenes</i>	0,92
S4	<i>Fragaria viridis</i>	<i>Streptomyces exfoliatus</i>	0,84
S5	<i>Fragaria</i> × <i>ananassa</i>	<i>Streptomyces albidoflavus</i>	0,85
S6	<i>Fragaria viridis</i>	<i>Streptomyces albidoflavus</i>	0,69
S7	<i>Fragaria viridis</i>	<i>Streptomyces rimosus</i>	0,61
S8	<i>Fragaria viridis</i>	<i>Streptomyces albidoflavus</i>	0,95
S9	<i>Fragaria viridis</i>	<i>Streptomyces diastatochromogenes</i>	0,54
S10	<i>Fragaria viridis</i>	<i>Streptomyces albidoflavus</i>	0,95

\*Index of identification according to API (BioMérieux) for *Pseudomonas* and according to Williams et al. (1983) for *Streptomyces*.

### Screening for isolates antagonistic to *Verticillium dahliae*

A total of 600 isolates from both rhizospheres were randomly selected and screened for their ability to suppress the pathogen *V. dahliae* in an in vitro bioassay. A large proportion of the bacterial isolates from both rhizospheres antagonized *V. dahliae*. The percentage of isolates with antifungal activity from the Garden Strawberry plant was higher (47%) than for the Greenish Strawberry (29%). The efficiency of antifungal activity in vitro was also plant species-dependent: the percentage of strong antifungal isolates (inhibition zone in dual culture assay of more than 10 mm) was higher from the rhizospheres of cultivated Garden Strawberry (21%) than for the Greenish Strawberry (6%).

### Physiological profiling of selected strawberry-associated rhizobacteria

Twenty isolates with strong antifungal activity and different morphological characteristics were selected for further investigation (Table 1). Half of the selected potential antagonists isolated from King's B agar produced fluorescent pigments. We were able to identify this group by means of the API system (API20NE) and additional physiological tests (xylose and sorbitol assimilation). The enzymatic properties were identical for all *Pseudomonas* strains whereas differences in some nutritional properties were found. The isolates P4 and P5 could not use xylose and sorbitol, and therefore were determined as *Pseudomonas chlororaphis*. All other fluorescent strains that were able to assimilate xylose were identified as *Pseudomonas fluorescens*. Ten isolates from GAA with colony morphology typical of *Streptomyces* species were also identified by their physiological and morpho-

logical characteristics. They belonged to 4 different species: *S. albidoflavus*, *S. diastatochromogenes*, *S. exfoliatus*, and *S. rimosus*. Altogether six bacterial species antagonistic to *Verticillium* wilt were identified. Most of the *Pseudomonas* isolates were obtained from the Garden Strawberry and most of the *Streptomyces* isolates were isolated from the Greenish Strawberry.

All of the isolates inhibited the growth of *V. dahliae* (Table 2). The isolates P4 and P5, belonging to *Pseudomonas chlororaphis* were only active against *V. dahliae*. Four isolates of *Streptomyces* species (S1, S2, S3, S9) were active against all of the tested fungi. The majority of the isolates were active against *Rhizoctonia solani*, *Phytophthora cactorum*, and *Pythium ultimum* while only six isolates (all from *Streptomyces*) were active against *Fusarium culmorum*. The antifungal activity was strain-specific.

All *Pseudomonas* and *Streptomyces* isolates tested produced antibiotics, and had siderophore activity (Table 2). While all the *Pseudomonas* strains produced cyanide, none of the streptomycete isolates did. The reverse was true for chitinase activity: all the *Streptomyces* isolates had chitinase activity, whereas none of the pseudomonads did. The *Pseudomonas* isolates P1, P2, P3, P5, and P10 produced the fungal cell-wall degrading enzyme  $\beta$ -1,3-glucanase. The in vitro bioactivity of isolates was species-dependent.

### Molecular fingerprinting of selected strawberry-associated rhizobacteria

Molecular fingerprints of the different strains of *Pseudomonas* and *Streptomyces* isolated from the rhizosphere were generated by ARDRA (amplified ribosomal DNA restriction analysis) and BOX-PCR. The PCR amplification of 16S rDNA

**Table 2.** In vitro antagonism against several pathogenic fungi and production of antifungal agents by *Pseudomonas* and *Streptomyces* strains.

Isolate	In vitro antagonism <sup>a</sup>						Production of antifungal agents				
	<i>V. d.</i>	<i>V. a.</i>	<i>F. c.</i>	<i>R. s.</i>	<i>P. c.</i>	<i>P. u.</i>	Antibiosis <sup>b</sup>	Siderophore <sup>c</sup>	HCN <sup>d</sup>	Glucanase <sup>e</sup>	Chitinase <sup>f</sup>
P1	++	++	—	++	++	++	+	+	+	+	—
P2	++	—	—	++	++	++	++	+	+	+	++
P3	++	—	—	++	++	++	++	++	+	+	++
P4	+	—	—	—	—	—	+	+	+	—	—
P5	++	++	—	++	++	++	+	+	+	—	—
P6	++	++	—	++	++	++	+	+	+	—	—
P7	++	—	—	++	++	++	++	+	+	—	—
P8	++	++	—	++	++	++	+	+	+	—	—
P9	++	—	—	++	—	—	++	+	+	—	—
P10	++	—	—	++	++	++	++	+	+	+	—
S1	+	+	+	+	+	+	++	+	—	—	+
S2	++	++	++	++	++	++	++	+	—	—	+
S3	+	++	++	++	++	++	++	+	—	—	+
S4	+	—	—	—	—	—	+	+	—	—	+
S5	+	+	—	++	++	+	++	+	—	—	+
S6	+	+	++	++	++	—	+	+	—	—	+
S7	+	—	+	—	+	—	++	+	—	—	+
S8	+	—	—	—	++	—	++	+	—	—	+
S9	+	+	+	+	++	+	++	+	—	—	+
S19	+	+	—	++	+	+	++	+	—	—	+

<sup>a</sup>Activity against phytopathogenic fungi: *V. d.* = *Verticillium dahliae*, *V. a.* = *Verticillium albo-atrum*, *F. c.* = *Fusarium culmorum*, *R. s.* = *Rhizoctonia solani*, *P. c.* = *Phytophthora cactorum*, *P. u.* = *Pythium ultimum*; dual culture assay: — represents 0–5 mm wide zone of inhibition, + represents 5–10 mm wide zone of inhibition, ++ represents >10 mm wide zone of inhibition.

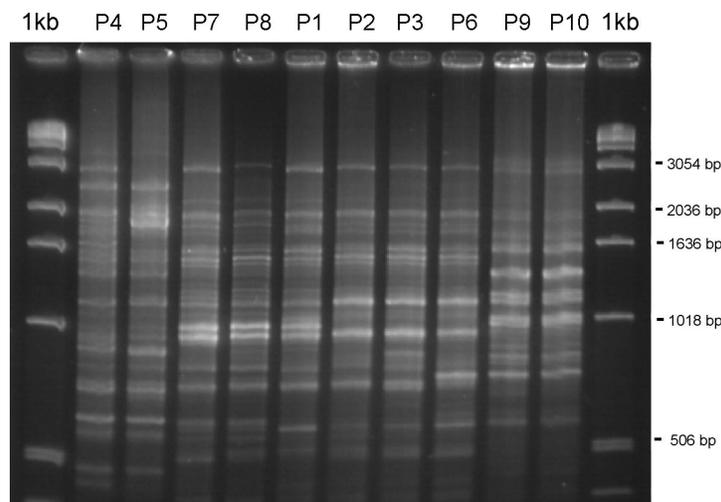
<sup>b</sup>Bioassay antibiosis: Sterile filtrate test against *V. dahliae*, inhibition zones: ++ 100–60%, + 60–30%, – 30–1%.

<sup>c</sup>Siderophore activity (++ represents 20 mm wide orange zone, + represents 5–20 mm wide orange zone, – represents 5–3 mm orange zone).

<sup>d</sup>Production of HCN (Cyanide; Merck). Schnelltest 14417. — represents <0.001 mg·L<sup>-1</sup>.

<sup>e</sup>β-1,3-Glucanase activity (++ >10 U·mL<sup>-1</sup>, + 10–1 U·mL<sup>-1</sup>, — 0.1–1 U·mL<sup>-1</sup>).

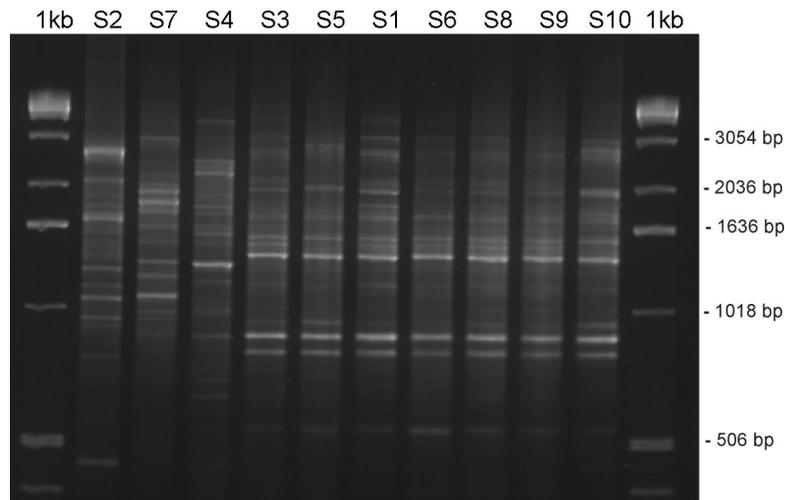
<sup>f</sup>Chitinase activity: plate assay (+ represents hydrolysis, — represents no hydrolysis).

**Fig. 1.** BOX fingerprints of *Pseudomonas* isolates (lanes 2–11). Lanes 1 and 12 contain the molecular size marker ladder (1 kb). The sizes of the marker bands are indicated on the right of the figure.

for each isolate resulted in an amplification fragment (data not shown). The *Pseudomonas* restriction analysis of the amplified DNA with the enzyme *AluI* produced three recognizable ARDRA patterns, while only two patterns were found with the enzyme *HaeIII* (data not shown). Overall, five

different patterns were found. *Pseudomonas* strains were analyzed by PCR using BOX primers (Fig. 1). This method was more discriminating than ARDRA, and some of the profiles were unique. The majority of the *Streptomyces* isolates (80%) showed identical ARDRA-patterns. The patterns ob-

**Fig. 2.** BOX fingerprints of *Streptomyces* isolates (lanes 2–11). Lanes 1 and 12 contain the molecular size marker ladder (1 kb). The sizes of the marker bands are indicated on the right of the figure.



**Table 3.** Grouping of *Pseudomonas* isolates according to cluster analysis based on physiological and molecular fingerprints.

No.	Species	Origin <sup>a</sup>	Cluster group <sup>b</sup>			
			Physiology <sup>c</sup>	Antifungal activity <sup>d</sup>	Mechanisms <sup>e</sup>	Molecular fingerprint <sup>f</sup>
P1	<i>P. fluorescens</i>	1	I	I	I	I
P2	<i>P. fluorescens</i>	1	I	II	I	II
P3	<i>P. fluorescens</i>	1	I	II	I	II
P4	<i>P. chlororaphis</i>	1	III	III	II	III
P5	<i>P. chlororaphis</i>	1	III	III	I	III
P6	<i>P. fluorescens</i>	1	I	I	II	IV
P7	<i>P. fluorescens</i>	1	I	II	II	V
P8	<i>P. fluorescens</i>	1	I	I	II	V
P9	<i>P. fluorescens</i>	2	II	II	II	VI
P10	<i>P. fluorescens</i>	2	II	II	I	VI

<sup>a</sup>Root sample: 1 = *F. ananassa*, 2 = *F. viridis*.

<sup>b</sup>Data of physiological and molecular characteristics were clustered using UPGMA. Strains showing a level of similarity of 90% were clustered into the same group.

<sup>c</sup>Based on physiological properties obtained with API system.

<sup>d</sup>Based on the in vitro antagonism against several pathogenic fungi.

<sup>e</sup>Based on produced antifungal agents.

<sup>f</sup>Based on ARDRA and BOX-PCR.

tained by using BOX-PCR were more diverse than with ARDRA but showed a similar grouping of the isolates (Fig. 2). *Streptomyces rimosus* and *S. exfoliatus* gave a BOX-PCR profile completely different from the other strains.

### Comparison of physiological and molecular properties

The origin, taxonomic classification, and grouping of phenotypic and molecular patterns according to clusters of *Pseudomonas* isolates are shown in Table 3. The two isolates of *P. chlororaphis* formed a separate cluster in the physiological characteristics, antifungal activity, and in their molecular fingerprint. Analysis of the molecular fingerprints by cluster analysis revealed that groups I, II, IV, V comprised isolates of *P. fluorescens* isolated from Garden Strawberry, while the two *P. fluorescens* isolates from the Greenish Strawberry belonged to group VI. Additionally, the isolates from the Greenish Strawberry, P9 and P10, were similar and grouped into the same cluster by their physiological charac-

teristics, antifungal activity, and in their molecular fingerprint, but not by their antifungal mechanisms. The origin, classification, and grouping of phenotypic and molecular patterns according to clusters of *Streptomyces* isolates are shown in Table 4. The data were more heterogeneous than those for *Pseudomonas*. Seven major molecular groups were defined at 90% similarity level. Six groups of them contained only one isolate. The isolates of the species *S. diastatochromogenes* and *S. albidoflavus* fell into the same cluster (I). On the other side, isolates of *S. albidoflavus* belonged to four different molecular groups (I, II, III, VII). The isolates of *S. rimosus* were in different molecular cluster groups (V, VI) but the strains showed a similarity of 70.5%.

### Evaluation of biocontrol activity in the greenhouse

Four rhizobacterial strains (P6, P10, S1, S9) were selected for evaluation of biocontrol activity, two from the Garden Strawberry (P6, S1), and two from the Greenish Strawberry (P10, S9). These strains showed strong in vitro antagonism

**Table 4.** Grouping of *Streptomyces* isolates according to cluster analysis based on physiological and molecular fingerprints.

No.	Species	Origin <sup>a</sup>	Cluster group <sup>b</sup>			
			Physiology <sup>c</sup>	Antifungal activity <sup>d</sup>	Mechanisms <sup>e</sup>	Molecular fingerprint <sup>f</sup>
S1	<i>S. albidoflavus</i>	1	I	I	I	VII
S2	<i>S. rimosus</i>	1	V	I	I	VI
S3	<i>S. diastatochromogenes</i>	1	II	I	I	I
S4	<i>S. exfoliates</i>	2	III	IV	I	IV
S5	<i>S. albidoflavus</i>	1	I	II	I	I
S6	<i>S. albidoflavus</i>	2	IV	III	I	I
S7	<i>S. rimosus</i>	2	VI	VI	II	V
S8	<i>S. albidoflavus</i>	2	VIII	V	I	II
S9	<i>S. diastatochromogenes</i>	2	II	I	I	I
S10	<i>S. albidoflavus</i>	2	I	II	I	III

<sup>a</sup>Root sample: 1 = *F. ananassa*, 2 = *F. viridis*.

<sup>b</sup>Data of physiological and molecular characteristics were clustered using UPGMA. Strains showing a level of similarity of 90% were clustered into the same group.

<sup>c</sup>Based on physiological properties obtained with identification matrix according Williams (1983). Strains showing a level of similarity of 60% were clustered into the same group.

<sup>d</sup>Based on the in vitro antagonism against several pathogenic fungi.

<sup>e</sup>Based on produced antifungal agents.

<sup>f</sup>Based on ARDRA and BOX-PCR.

toward the fungal pathogens tested and were from different physiological and molecular cluster groups. When strawberry plants were treated with *P. fluorescens* P10, *S. albidoflavus* S1, and *S. diastatochromogenes* S9 significantly reduced the incidence of disease in the greenhouse (Table 5). The other strain showed also an effect but P6 did not significantly control the pathogen. The *Pseudomonas* treatment enhanced the yield of strawberry fruits up to 344% (Table 5). All the strains were able to colonize the rhizosphere of strawberry. Ten weeks after inoculation, rhizobacteria were reisolated at population densities of  $1 \times 10^5 - 3 \times 10^7$  CFU per g wet root.

#### Evaluation of biocontrol activity in the field

The strains P6 and P10 were tested in a field trial in Stuthof while strains S1 and S9 were tested in another field trial in Goorstorf. In field trial I, statistically significant ( $P = 0.004$ ) reductions of symptoms were observed in plots treated with strain *Pseudomonas* compared with untreated control plots (Table 5). In field trial II, infection by soil borne pathogens did not occur. Thus, significant differences in health between the control and the plots treated with *Streptomyces* could not be detected. All four bacterial strains enhanced yield under field conditions. However, the yield enhancement was lower for the plants treated with *Streptomyces* strains that were grown in a field site where no *Verticillium* infection occurred. *Pseudomonas fluorescens* P6 increased the yield significantly by about 247%. All strains were able to colonize the strawberry rhizosphere under field conditions. Three months after inoculation, the population densities of applied rhizobacteria were  $7 \times 10^3 - 2 \times 10^6$  CFU per g wet root.

#### Discussion

In order to isolate new biocontrol agents to protect strawberries against *Verticillium* wilt, a successful strategy for an effective selection and evaluation of potent biocontrol agents was developed. In the present study, the in vitro screening of

300 isolates resulted in a selection of 20 isolates, which could effectively suppress *V. dahliae* in vitro. Rhizobacteria from the genera *Pseudomonas* and *Streptomyces*, which are known for their high antagonistic potential, were preferentially selected. The group of fluorescent pseudomonads included the species *P. chlororaphis* and *P. fluorescens*, and these species have been reported to be the most frequently beneficial and antifungal rhizobacteria (Leben et al. 1987; De Freitas and Germida 1990). Although *Streptomyces* species have been studied extensively for their antifungal properties (Huddleston et al. 1997; Yuan and Crawford 1995), the *Streptomyces* species found in the present study were not previously reported as *Verticillium* antagonists. According to the result of cluster analysis of physiological profiling and molecular fingerprinting of the 20 strains, four strains were selected to screen for biocontrol efficacy against *V. dahliae* in situ. In biocontrol experiments in the greenhouse and in fields naturally infected by *Verticillium*, bacterial treatment resulted in an efficient suppression of the pathogen. An additional indirect effect was the increased yield in strawberries. On the basis of these results it was possible to patent the three best biocontrol agents *Pseudomonas fluorescens* P6, P10, and *Streptomyces diastatochromogenes* S9 (Berg et al. 1999).

As first point of the strategy two different strawberry species from different origins were included in the investigations. The plants of the Greenish Strawberry were obtained from a natural ecosystem (dry calcareous grassland) in a conserved area where they are native. In contrast, the Garden Strawberry is a hybrid of *F. chiloensis* (L.) Duchesne and *F. virginiana* Duchesne (both from American origin). In the 18th century, they were bred for cultivation in Europe. We found that percentage of antifungal bacteria was higher in the rhizosphere of the Garden Strawberry than in the rhizosphere of the Greenish Strawberry. Compared to percentages of antagonistic bacteria in the rhizospheres of other plants, for example maize 11–37% (15), weeds up to 18% (Kremer et al. 1990), oilseed rape 16% (Berg 1996), and potato 1–

**Table 5.** Biological control of Verticillium wilt and yield increase in strawberries treated with *Pseudomonas fluorescens* P6 and P10, *Streptomyces albidoflavus* S1, and *Streptomyces diastatochromogenes* S9 under greenhouse and field conditions.

Experiment	Treatment *	No. of wilted plants	% Wilted plants $\pm$ SD	Yield (g)	% Increase yield $\pm$ SD
Greenhouse					
	<i>P. fluorescens</i> P6	5.46	81.5 $\pm$ 59.8	55.4	323.5 $\pm$ 53.6
	<i>P. fluorescens</i> P10	4.46	66.6 $\pm$ 8.3	59.0	344.2 $\pm$ 28.1
	<i>S. albidoflavus</i> S1	1.50	31.6 $\pm$ 5.7	20.5	117.0 $\pm$ 39.0
	<i>S. diastatochromogenes</i> S9	2.80	59.1 $\pm$ 14.3	54.2	309.4 $\pm$ 52.7
	Untreated control	10	100	17.5	
Field					
	<i>P. fluorescens</i> P6	236.5	124.7 $\pm$ 7.9	20280	247.3 $\pm$ 9.55
	<i>P. fluorescens</i> P10	193.5	102.1 $\pm$ 8.7	11580	140.1 $\pm$ 4.55
	<i>S. albidoflavus</i> S1	0	0	9260	112.9 $\pm$ 7.04
	<i>S. diastatochromogenes</i> S9	0	0	9520	116.1 $\pm$ 7.73
	Untreated control	189.6	100 $\pm$ 5.3	8200	

\*Dipping bath treatment of roots.

11% (Lottmann et al. 1999), the percentage of antagonistic isolates, especially from the Garden Strawberry, was relatively high. The cultivated Garden Strawberry was grown under agricultural field conditions with a high population density of *Verticillium microsclerotia* in the soil. The fact that antagonistic rhizobacteria were accumulated in pathogen-infested soils has been described before (Berg 1996). The plant species or cultivar and especially the composition of root exudates plays a key role in the diversity of rhizobacterial populations colonizing the roots (Kremer et al. 1988; Siciliano et al. 1998) and can influence the frequency of antagonistic bacteria. However, the type of soil has also an important influence on the diversity of rhizobacteria (Sørensen 1997). A diversity of different antagonistic bacteria was found for the two strawberry species. The bacterial species *Pseudomonas fluorescens*, *Streptomyces albidoflavus*, *S. rimosus*, and *S. diastatochromogenes* were found in rhizospheres of both *F. viridis* and *F.  $\times$  ananassa*, while *Pseudomonas chlororaphis* was only found in the rhizosphere of *F.  $\times$  ananassa*, and *S. exfoliatus* in the rhizosphere of *F. viridis*. The origins of the most successful biocontrol bacteria evaluated in this study were the Greenish Strawberry (P10 and S9) and the Garden Strawberry (P6).

The next step involved characterizing selected bacteria by physiological profiling and molecular fingerprinting methods. According to these criteria, bacteria were selected for in planta investigations. These characterizations resulted in the discovery of interesting properties for the rhizobacteria tested. All of the rhizobacteria were able to produce antibiotics, and these metabolites play a major role in disease suppression in some bacteria, acting in concert with other mechanisms such as competition and parasitism (Fravel 1988). Additionally, the production of siderophores was found for all the strains tested. Hamdan et al. (1991) acknowledged the role of siderophores in disease suppression. In this study, some of the *Pseudomonas* isolates produced glucanase, while all of the *Streptomyces* isolates had chitinolytic activity. In some cases, microorganisms capable of lysing other organisms can serve as a powerful tool for biological control (Chet et al. 1990). The bacterial isolates were also genotypically characterized by molecular methods based on analysis of polymorphisms of DNA. The BOX-PCR-method was

highly discriminating and allowed the grouping of the isolates. ARDRA allowed differentiation of the *Pseudomonas* isolates while it was not possible to distinguish *Streptomyces* isolates with this method. In general, a positive correlation was found in this study between groupings of isolates established by the phenotypic and genotypic characterization, especially for the *Pseudomonas* isolates.

The third point of the strategy was the evaluation of selected biocontrol candidates in greenhouse and field trials. The success of biological approaches to control plant diseases must be judged by their performance under field conditions. Unfortunately, discrepancies exist between the antagonistic effect under in vitro conditions and the corresponding in situ efficacy (Weller and Cook 1983). We found a good correlation between the effect in vitro, in the greenhouse and in the field. The biocontrol efficacy in the greenhouse under defined abiotic and biotic conditions was higher than in the field. Under field conditions, the incidence of Verticillium wilt is influenced by the initial inoculum density of microsclerotia, the cropping history of the field site, the abiotic conditions such as weather conditions and stress, soil parameters, and other pathogens such as nematode infestation (Harris and Yang 1996). In our study, different amounts of microsclerotia in the different soils of the field trials were established. In field trial I, the potential of infectious microsclerotia was much higher, with 40 microsclerotia  $\cdot$  g<sup>-1</sup> soil, than in field trial II with 21 microsclerotia  $\cdot$  g<sup>-1</sup> soil, although at mean values from 1–2 microsclerotia  $\cdot$  g<sup>-1</sup> soil, the fungi are able to infect the susceptible strawberry cultivar Elsanta (Harris and Yang 1996). There was a correlation between the population density of microsclerotia in soil and the disease incidence of strawberry plants. The cropping history of a site is also a guide to wilt risk. Plants which are potent amplifiers of soil inoculum of *V. dahliae* such as potato, cotton, brassica crops, sugar beet, and miscellaneous vegetables are dangerous, and enhance the potential risk of an infection. Field trial I was grown after oilseed rape, and this crop is responsible for the high inoculum of *V. dahliae* in northern Germany. In contrast, field trial II was planted on a former barley field, a non-host plant of Verticillium.

Ideally, the candidate bacteria should be screened on the plants rather than in vitro, however, in many situations it is

impractical to conduct large-scale screening trials on the whole plant. In the present study, we developed a rational strategy to conduct preliminary screening in vitro combined with physiological profiling and molecular fingerprinting, and to move to secondary screens in planta which are more relevant to the natural situation as soon as the numbers of potential antagonists have been reduced to appropriately.

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