Biocontrol of tomato foot and root rot by Pseudomonas bacteria in stonewool

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Chapter 2

Enrichment for enhanced competitive plant root tip colonizers

selects for a new class of biocontrol bacteria

Abstract

Our group studies tomato foot and root rot, a plant disease caused by the fungus *Forl* (*Fusarium oxysporum* f. sp. *radicis-lycopersici*). Several bacteria have been described to be able to control the disease, using different mechanisms. Here we describe a method that enables us to select, after application of a crude rhizobacterial mixture on a sterile seedling, those strains that reach the root tip faster than our best tomato root colonizer tested so far, the *Pseudomonas fluorescens* biocontrol strain WCS365. Of the five tested new isolates, four appeared to be able to reduce the number of diseased plants. Analysis of one of these strains, *P. fluorescens* PCL1751, suggests that it controls the disease through the mechanism 'competition for nutrients and niches', a mechanism novel for biocontrol bacteria. Moreover, this is the first report describing a method to enrich for biocontrol strains from a crude mixture of rhizobacteria. Another advantage of the method is that four out of five strains do not produce antifungal metabolites, which is preferential for registration as a commercial product.

Introduction

Many plant diseases are caused by phytopathogenic fungi. In order to decrease the input of agrochemicals in agriculture, biocontrol microbes are used as possible alternatives (Schippers et al., 1987; Lugtenberg and Bloemberg, 2004). We study tomato foot and root rot (TFRR), which is a disease caused by the fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*). This disease cannot efficiently be prevented by chemicals. The isolation of biocontrol bacteria involves a labor intensive screening process that, in the case of antibiosis, can be enhanced by introducing a screening step for strains that produce antifungal metabolites (AFMs) *in vitro*.

To our knowledge, no procedures have been described, which facilitate the selection of biocontrol microbes that act through other mechanisms. Kuiper and colleagues (2001) described a method to select enhanced grass root tip colonizing bacteria. In this method a mixture of rhizosphere bacteria is applied on a sterile seedling. After plant growth in a gnotobiotic system (Simons et al., 1996), those bacteria that have reached the root tip are isolated. These are subsequently used to inoculate a fresh sterile seedling, which again is allowed to grow. After three of these enrichment cycles, excellent competitive root tip colonizers were obtained (Kuiper et al., 2001). In the present paper we used this method to select enhanced tomato and cucumber root tip colonizers. Based on the notion that not only biocontrol fungi but perhaps also biocontrol bacteria exist, which act through the mechanism "competition for niches and nutrients", we screened the selected enhanced root tip colonizers for their ability to control the disease TFRR. The results are described in this paper.

Materials and Methods

Microbial strains and growth conditions

The bacterial strains used are listed in Table 1. All newly isolated strains were routinely cultured in KB (King et al., 1954) at 28°C under vigorous shaking. In some cases the synthetic medium BM (Lugtenberg et al., 1999), supplemented with 1% succinic acid, was used. *Chromobacterium violaceum* was grown in LB medium (Sambrook and Russel, 2001). *Agrobacterium tumefaciens* was grown on yeast mannitol broth (YMB) medium (Smit et al., 1987). Solid growth medium contained 1.8% agar (Difco Laboratories, Detroit, MI, USA). Spontaneous gentamycin-resistant derivatives of PCL1751 were generated by plating 100 ml of overnight culture on KB containing 20 mg of gentamycin per milliliter. After 48 h of growth spontaneous resistant colonies were collected separately and their motility was determined on plates containing 20-fold diluted KB, solidified with 0. 3%agar as described by Dekker and colleagues (1998). Non-motile Gm-resistant derivative was assigned as PCL1752. To analyze competitive growth between wild-type PCL1751 and mutant PCL1752, cells were grown overnight and diluted to a final OD_{620} of 0.1 and subsequently diluted in fresh medium in a 1:1 ratio. After growth overnight, cells were diluted 1000-fold in fresh medium and colony forming units (CFUs) were determined by plating dilutions of samples on KB plates with and without Gm. All fungi used were routinely cultivated on potato-dextrose agar (PDA, Difco Laboratories) or in Czapek-Dox liquid medium (Difco Laboratories) at 28°C under vigorous aeration. Kanamycin (50 mg ml-1), gentamycin (20 mg ml-1) and cycloheximide (100 mg ml-1) were added where applicable.

Isolation of microbes from roots of tomato and cucumber plants and enrichment of enhanced competitive root tip colonizers

Three-month-old tomato and cucumber plants with adhering rhizosphere soil were collected from greenhouses just outside Tashkent, Republic of Uzbekistan. Roots and adhering rhizosphere soil (total 25 g) of each plant species were shaken

vigorously for 2 h in 50 ml of sterile PBS. The samples were diluted, plated on 20-fold diluted solidified Tryptic Soy agar (1/20 TSA; Difco Laboratories) supplemented with cycloheximide (100 mg ml-1), and incubated overnight at 28°C. Subsequently all colonies were scraped together from the plate and suspended in 10 ml PBS. The resulting suspension was used to inoculate germinated sterile seedlings of tomato (cultivar Carmello, Syngenta, Enkhuizen, the Netherlands)and cucumber (cultivar Grendel, Syngenta, Enkhuizen, the Netherlands) for enrichment (Fig. 1) using the gnotobiotic system described by Simons and colleagues (1996). Those rhizobacteria that succeeded to reach the 1-cm-long root tip after growth for 7 days in the gnotobiotic quartz sand moisturized with plant nutrient solution (Hoffland et al., 1989), estimated to contain 1% of the total number of root colonizing bacteria (Simons et al., 1996), were subjected to two more selection cycles to enrich for the best enhanced competitive root tip colonizers (Fig. 1).
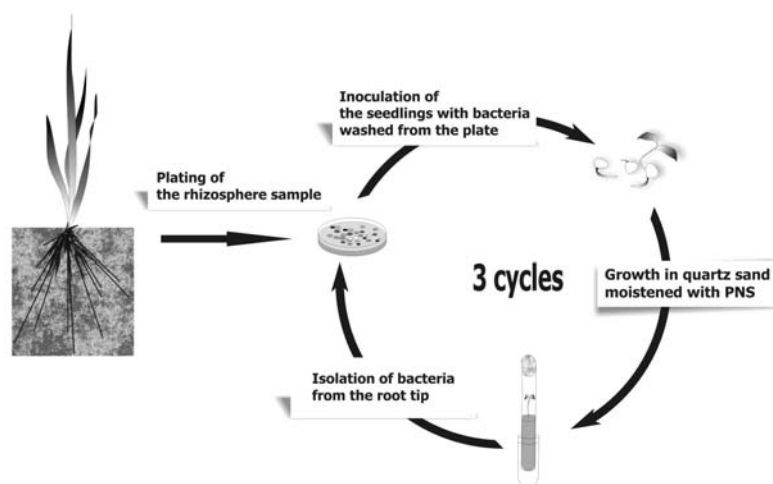

Fig. 1. Selection of enhanced root colonizing bacteria. Microbes were isolated from the rhizospheres of tomato and cucumber plants, grown in diluted TSB and used to inoculate sterile germinated seedlings of the same plant species as the rhizobacteria were derived from. These are tomato cultivar Carmello and cucumber cultivar Grendel. After growth in the gnotobiotic system, those microbes that had reached the 1 cm long root tip were shaken off the root tip, plated on KB agar, judged for colony diversity, and the cells from the combined colonies were used for another enrichment cycle. After a total of three cycles the bacteria from the root tip were plated and selected for competitive root colonization experiments.

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Table 1. List of microorganisms used in this study

Strains	Characteristics	Reference or source
Bacteria		
WCS365	*Pseudomonas fluorescens* ; excellent competitive root colonizer; biocontrol strain of tomato foot and root rot	Geels and Schippers, 1983; Simons et al.1996;Dekkers et al., 2000;
PCL1285	Tn5luxAB derivative of WCS365, comparable with wild type in root colonization ability; Km ^{r}	De Weert et.al., 2004
PCL 1391	*P. chlororaphis*; efficient competitive root colonizer; biocontrol strain of tomato foot and root rot which produces phenazine-1-carboximide	Chin–A–Woeng et al. 1998
PCL1751	Wild-type *P. fluorescens*, isolated from Uzbekistan tomato rhizosphere	This study
PCL1752	Spontaneous nonmotile mutant of *P. fluorescens* PCL1751; Gm^r	This study
PCL1753	Wild-type *P. fluorescens*, isolated from Uzbekistan tomato rhizosphere	This study
PCL1754	Wild-type *P. putida*, isolated from Uzbekistan cucumber rhizosphere	This study
PCA0067	Wild-type *Pantoae agglomerans*, isolated from Uzbekistan cucumber rhizosphere	This study
PCA0081	Wild-type *Aeromonas hydrophila*, , isolated from Uzbekistan tomato rhizosphere	This study
CV026	*Chromobacterium violaceum* N-AHL reporter strain	Milton et al, 1997
NT1	*Agrobacterium tumefaciens* NT1 N-AHL reporter strain harboring pJM749 containing a lacZ reporter fused to a tra gene of which expression is dependent on TraR	Piper et al., 1993
Fungi		
LBOP17	*Pythium ultimum*; causes damping-off and fruit rot of tomato	IPO-DLO, Wageningen, The Netherlands
R3-11A	*Gaeumannomyces graminis pv.tritici* (Ggt), causes take-all disease of wheat and other cereals.	Raaijmakers and Weller, 1998
ZUM2076	*Botrytis cinera*, causes gray mold of tomato	Novartis Seeds BV, Enkhuizen, The Netherlands
ZUM2372	*Alternaria dauci* isolated from carrot seeds. Pathogen of carrot.	Novartis Seeds BV, Enkhuizen, The Netherlands
ZUM2407	*Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*); causal agent of tomato foot and root rot	IPO-DLO, Wageningen, The Netherlands

Competitive tomato root tip colonization assay

Seeds of tomato were sterilized, allowed to germinate, and the seedlings were inoculated with a 1:1 mixture of two bacterial strains and planted in the gnotobiotic quartz sand system as described by Simons and colleagues (1996). Plants were grown in climate-controlled chambers with 16 h of day light at 24°C during 7 days. To estimate competitive root tip colonization, the root tip (1 cm) with adhering rhizosphere soil and was cut off and shaken vigorously for 15 min in 1.0 ml PBS to remove the bacteria. Dilutions of the bacterial suspensions were plated onto KB and on KB supplemented with Km to determinate the numbers of Km-resistant and Km-sensitive bacteria in the suspension. All colonization experiments were performed in 10-fold. The average number of bacteria and the standard deviation were calculated. The non-parametric Wilcoxon–Mann–Whitney test (Sokal and Rohlf, 1981) for mixed inocula was used to perform statistics.

Preliminary characterization of plant growth promotion traits

To test AFM production *in vitro*, 0.5 ×0.5 cm agar plugs of each fungus were stabbed in the centre of PDA and KB agar plates that were subsequently inoculated with individual bacterial test strains at a distance of 3.0 cm from the fungus. Bacterial strains that caused an inhibition zone of at least2 mm were judged as positive. Hydrogen cyanide was detected using cyanide indicator paper (Castric, 1975), protease on 10% milk agar plates (Brown and Foster, 1970), chitinase on plates containing colloidal chitin (Shimahara and Takiguchi, 1988) and β-glucanase on plates containing lichenan (Sigma, St. Louis, MO, USA) (Walsh et al., 1995). Production of biosurfactant was determined using the drop collapsing assay (Jain *et* al., 1991). Phase variation was judged as described by Van den Broek and colleagues (2003). Motility was tested as described by Dekker and colleagues (1998) on 0. 3% agar.

The production of auxin was determined by a colorimetric method. Briefly, test strains were inoculated in BM/succinate without or with tryptophan (100 mg ml-1) and incubated at 28°C at 150 rpm min^{-1}. After 1, 4 and 8 days of cultivation,

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aliquots of bacterial cultures were centrifuged at 13 000 rpm for 10 min. Two milliliters of supernatant fluid was added to a tube with 100 ml 10 mM orthophosphoric acid and 4 ml of Salkowski reagent (Gordon and Weber, 1951). The mixture was incubated at room temperature for 30 min and absorbance of the developed pink color was read at 530 nm. The indole-3-acetic acid (IAA) concentration in the culture was determined by using a calibration curve of pure IAA as a standard. Autoinducers were extracted from supernatant fluids using dichloromethane and the activity of the extracts was analyzed using *Chromobacterium* (Milton et al., 1997) and *Agrobacterium* (Piper et al., 1993) reporter strains as described by Chin-A-Woeng and colleagues (2001).

Isolation of tomato root exudates

Tomato root exudate was isolated as described by Simons and colleagues (1997). Briefly, batches containing 100 sterile seedlings were placed in 100 ml PNS and cultivated in a climate-controlled growth chamber at 24°C, 70% relative humidity and 16 h of daylight. After 14 days, sterility was tested, and root exudate of sterile samples was collected, filtered through 0. 22 mm filters and kept at 4°C until use.

Strain identification

Strains were identified after colony PCR (Williams et al., 1990) for amplification of 16S rDNA. The PCR products were sequenced by ServiceXS (Leiden, the Netherlands) and analyzed for homology using BLAST (Altschul et al., 1997). Amplified ribosomal DNA restriction analysis was performed according to Vaneechoutte and colleagues (1990).

Growth of bacteria in tomato root exudate

Bacteria were pre-grown overnight in 20-fold diluted TSB medium. Cells were spun down, washed three times in PBS, and used for inoculation of tomato root exudate to a final concentration of approximately 10^4 CFU ml-1. The suspension

was incubated at 21°C under aeration at 150 rpm. Growth was measured by dilution plating on KB.

Biocontrol of TFRR using seedling inoculation

Tomato seeds cultivar Carmello were coated with bacteria by dipping the seeds in a mixture of 1% (w/v) methylcellulose (Sigma, St Louis, MO, USA) and 10^9 CFU ml^{-1}bacteria in PBS. *Forl* spores were prepared as described by Chin-A-Woeng and colleagues (1998). Tomato seeds were placed in non-sterile potting soil (Jonkind grond B.V., Aalsmeer, the Netherlands) infested with *Forl* spores (2×10^6 spores kg-1). For each treatment, 96 plants were tested in eight trays of 12 plants each. Plants were grown in a greenhouse at 21–24°C, 70% relative humidity and 16 h daylight. After 15–21 days of growth, plants were removed from the soil, and the plants roots were examined for foot and root rot symptoms as indicated by browning and lesions. Only roots without any disease symptoms were classified as healthy. Differences in disease level among treatments were determined by analysis of variance (ANOVA) and mean comparisons were performed by Fisher's least-significant difference test (a = 0. 05), using SPSS software (SPSS, Chicago, IL, USA). All experiments were performed at least twice. In all biocontrol experiments positive controls consisted of application of *P. fluorescens* WCS365, causing induced systemic resistance (Gerrits and Weisbeek, 1996), and *P. chlororaphis* PCL1391, a phenazine producing TFRR biocontrol strain (Chin-A-Woeng et al., 1998), which requires delivery of this AFM along the root for biocontrol activity (Chin-A-Woeng et al., 2000).

Induction of resistance against TFRR

The root system and hypocoteledons of 3-week-old tomato plants (cv. Moneymaker, purchased from Rijnsburg Zaadhandel, Rijnsburg, the Netherlands) were split and each half of the root was replanted in separate pots, whereas the stem remained intact. After 1 week one part of the root system was inoculated with bacteria (10^9 CFU per plant in 5 ml PBS) or with PBS in control plants. After another week the other part of the root system was challenged by adding to each plant 5 ml PBS containing 10^6 *Forl* spores. Three weeks after challenging, roots were analyzed

for the presence of lesions. Seventeen or 23 plants were grown per treatment. The difference in health conditions (healthy or sick plants) between two different treatments was statistically analyzed using chi-squared goodness-of-fit test (Heath, 1995).

Plant growth promotion

For the evaluation of the effect of bacterial isolates on the growth of tomato and cucumber plants, seeds were coated with bacterial mixtures as described in the biocontrol assay and grown under greenhouse conditions in non-sterile potting soil. Each variant consisted of three replicas with eight seeds per replica. After 3 weeks of growth fresh and dry weight of shoots was determined and analyzed using analysis of variance followed by Fisher's least-significant-difference test (a = 0. 05), using SPSS software (SPSS, Chicago, IL, USA). All experiments were performed at least twice.

Results and discussion

Isolation of enhanced root colonizing bacteria

The mixtures of rhizosphere bacteria from cucumber and tomato plants, cultivated for 12 weeks under greenhouse conditions in soil that had never been treated with fungicides were, respectively, used to inoculate seedlings of the same plants and enhanced competitive root tip colonizers were enriched as described in the *Experimental procedures* section and illustrated in Fig. 1. After each cycle we observed that diversity of colonies in terms of color, size, transparency, etc. somewhat decreased. This observation was true for both types of plants. After the third cycle of enrichment, 16 colonies were randomly chosen to test in competitive tomato root tip colonization assays against PCL1285 (Table 1), a kanamycin-resistant derivative of the best known competitive tomato root tip colonizer *P. fluorescens* WCS365 (Lugtenberg *et al* ., 2001). Seven of the newly isolated strains appeared to be better competitive colonizers than *P. fluorescens* WCS365 or its colonization-proficient Km-resistant derivative PCL1285. The latter strains showed

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log 10 [(cfu + 1)/ cm of root tip] root tip colonization values between 4.6 and 5.0 (Table 2). The nine strains derived from the enrichment procedure, which were worse competitive colonizers than PCL1285, showed log 10 [(cfu + 1)/cm of root tip] values between 3.7 and 4.5 (results not shown). Ten rhizosphere isolates, randomly chosen before the enrichment procedure was started, appeared to be 100- to 1000-fold poorer competitive colonizers than the best new isolates: the number of bacteria isolated from the root tip varied from not detectable (the detection limit has a log 10 [(cfu + 1)/cm of root tip] value of 2.7) to a log 10 [(cfu + 1)/cm of root tip] value of 3.7. The seven isolates that showed enhanced competitive root colonizing ability were subjected to the amplified ribosomal DNA restriction analysis (ARDRA) procedure to identify putative siblings. Three out of the seven best colonizers appeared to be siblings (data not shown). The resulting five unique isolates (Table 2) were subjected to further characterization. Note that two of these isolates are derived from cucumber but are able to colonize tomato roots efficiently. The lesson of the ARDRA result is that it is advisable to check for siblings prior to starting the labour-intensive colonization and biocontrol work. Furthermore, we conclude that the used enrichment procedure not only works for the monocot grass but also for the dicot tomato. The enrichment method makes it easy to isolate better competitive tomato root tip colonizers than *P. fluorescens* WCS365, a strain that we considered as the best tomato root colonizer for almost a decade.

Taxonomic characterization of enhanced competitive colonizers

Gram staining showed that all five isolates with enhanced competitive root colonizing ability are Gram-negative. Nucleotide sequencing of amplified 16S rDNA fragments, obtained after colony polymerase chain reaction (PCR), and comparative analysis with the DNA databases, revealed that the isolated enhanced competitive root tip colonizing strains belong to genera *Pseudomonas* , *Aeromonas* and *Pantoae* (Table 1)*.*

Table 2. Competitive tomato root tip colonization ability of the newly isolated strains in competition with WCS365 or PCL1285, a Tn5luxAB derivative of WCS365 [x]

Competing strains	Competitive root tip colonization [lg(cfu+1/cm) root tip][y]	
	Test strain	Reference strain
PCL1751 vs WCS365	5.30 ± 0.37 (a)	4.67 ± 0.58 (b)
PCA0081 vs PCL1285	5.40 ± 0.25 (a)	4.60 ± 0.75 (b)
PCL1753 vs PCL1285	5.70 ± 0.62 (a)	4.70 ± 0.57 (b)
PCA0067 vs PCL1285	5.95 ± 0.35 (a)	4.85 ± 0.72 (b)
PCL1754 vs PCL1285	5.60 ± 0.30 (a)	5.00 ± 0.60 (b)

The 16S rDNA sequences of the new isolates show the following percentages of homology with that of the following strains: strain PCL1751 99% with *P. fluorescens* 62e, strain PCL1753 99% with *P. fluorescens* Q2-87, strain PCL1754 99% with *P. putida* ATCC11250, strain PCA0081 99% with *Aeromonas hydrophila* 45/90 and strain PCA0067 99% with *Pantoea agglomerans* strain JCM1236. The sequences of the five new isolates have been deposited in GenBank under the following accession numbers. *Aeromonas hydrophila* PCA0081, AY900170; *P. agglomerans* PCA0067, AY900169; *P. putida* PCL1754, AY9001168; *P. fluorescens* PCL1751, AY9001171 and *P. fluorescens* PCL1753, AY9001172.

Potential biocontrol traits

The five enhanced competitive colonizers (Table 2) were tested for a number of potential biocontrol traits (Table 3). Only one strain, *P. fluorescens* PCL 1753, is antagonistic towards (four out of five of the tested) phytopathogenic fungi. It also produces autoinducer and hydrogen cyanide (HCN). None of the strains has biosurfactant activity and only two strains secrete at least one of the five tested exoenzymes. Strain PCL1753 is able to induce AHL reporter genes, in both *Chromobacterium violaceum* and *Agrobacterium tumefaciens* (Table 3). In the last

case the signal was weaker. None of these isolates showed colony phase variation (results not shown).

Table 3. Overview of possible plant growth promoting and biocontrol properties of the five newly isolated enhanced tomato root tip colonizers

Strain	Antifungal activity [a]					Exo-enzyme activity					Secondary metabolites			
	Forl	*P.ultimum*	*A.dauchi*	*B.cinera*	*Ggt*	protease	lipase	β-gluconase	cellulase	chitinase	AI [b]	HCN	BS	IAA [c]
PCL1751	-	-	-	-	-	+	-	-	-	-	-	-	-	-
PCA0081	-	-	-	-	-	+	+	-	-	+	-	-	-	10.2 ± 0.6
PCA0067	-	-	-	-	-	-	-	-	-	-	-	-	-	19.8 ± 2.3
PCL1753	-	+	+	+	+	-	-	-	-	-	+	+	-	7.5 ± 1.8
PCL1754	-	-	-	-	-	-	-	-	-	-	-	-	-	n.d.

[a] Antifungal activities were tested as inhibition of growth *in vitro*. Results obtained on PDA and KB were similar.
[b] Abbreviations: AI, autoinducer; BS, biosurfactant; IAA, indole-3-acetic acid; n.d., not determined.
[c] Values represent amounts of IAA in µg/ml in the spent culture medium after 8 days of cultivation of the bacteria in the BM medium supplemented with 1% succinic acid and tryptophan (100 µg/ml).

Auxin production and effect on plant growth

Auxin production was tested in the absence and presence of the auxin precursor tryptophan. All strains reached the stationary phase within 24 h, but no auxin was detectable at that time. It appeared that in 4- and 8-day-old cultures *P. agglomerans* PCA 0067, *A. hydrophila* PCA 0081 and *P. fluorescens* PCL1753 (Table 3), as well as the well known efficient competitive root tip colonizer and biocontrol strain *P. fluorescens* WCS365 (result not shown), produce auxin. Another biocontrol strain, *P. chlororaphis* PCL1391 (not shown) and new isolate *P. fluorescens*

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PCL1751 (Table 3), did not produce a detectable amount of auxin. No auxin production was detected in cells grown in the absence of tryptophan.

To test whether the ability to produce auxin has a significant influence on plant growth, tomato seeds were inoculated with the newly selected enhanced colonizing bacteria and grown in potting soil for 21 days. Measurements of fresh and dry weight of tomato shoots (see *Experimental procedures* section for details) showed that inoculation with these bacteria did not cause a significant effect on plant growth (results not shown). We conclude that none of the new isolates promotes tomato in the absence of a pathogen.

Biocontrol of tomato foot and root rot

Of the five enhanced colonizers isolated after three enrichment cycles, the strains *P. fluorescens* PCL1751 and *P. agglomerans* PCA0067 significantly control TFRR and do so to a similar extent as our standard biocontrol strain WCS365 (Table 4). Strains *A. hydrophila* PCA 0081 and *P. fluorescens* PCL1753 showed significant biocontrol of TFRR in one experiment. A reduction of diseased plants was also found in the two other experiments, but the effect was not significant in those cases. *Pseudomonas putida* strain PCL1754 failed to control TFRR in any of the two performed biocontrol experiments. We conclude that four out of the five enhanced colonizers derived from the enrichment procedure have a moderate- to-good biocontrol activity. Interestingly, the two best biocontrol strains *P. fluorescens* PCL1751 and *P. agglomerans* PCA0067 are not antagonistic (Table 3). The results in Tables 2 and 4 with *P. putida* strain PCL1754 show that excellent colonization is not sufficient for biocontrol. Consultation of a classification list of bacteria in safety risk groups (Anonymous, 1998) showed that four of the five isolates fall in risk group 2 and that one of the two consistent new biocontrol strains, *P. fluorescens* PCL1751, is the safest one for application as it falls in risk group 1. Therefore, we continued with this strain.

Table 4. Effect of enhanced competitive tomato root tip colonizers on control of tomato foot root rot

ExP.No	Percentage of diseased plants [x] Test strains					
	None	PCL1751	PCA0081	PCA0067	PCL1753	PCL1754
1	75±15 (a)	51±15(b)		57±8(b)		
2	49±12(a)	27±12(b)	43±15(a)	33±10(b)	40±6(a)	40±11(a)
3	84±8(a)		68±16(b)		64±16(b)	80±10(a)
4	68±9(a)		60±9 (a)		65±15(a)	

[x] The percentage of diseased plants was determined 2-3 weeks after inoculation. Per strain, 96 plants in 8 trays of 12 plants were tested. For statistics, a variance analysis followed by Fisher's least- significant-difference test (α=0.05), using *SPP*S software (*SPP*S Inc., Chicago, Il., USA), was used

Competition for niches and nutrients

PCL1752 was isolated as a spontaneous mutant of *P. fluorescens* PCL1751 impaired in motility when tested on semisolid King's medium B (KB). The mutant was as competitive as the wild type when grown 1:1 in competition with its parent in KB and in tomato root exudate. We conclude that the non-motile mutant has intact housekeeping genes.

In competitive tomato root tip colonization assays, in which sterile seedlings were inoculated with mutant and wild-type cells in equal numbers, followed by plant growth in the gnotobiotic sand system (Simons et al., 1996), the mutant was completely outcompeted by its parental strain both in the middle part of the root and on the root tip. Whereas the wild type reached 5×10^5 cfu per centimetre of root tip, the mutant was not recovered at all from the root tip. In biocontrol experiments in potting soil under greenhouse conditions, the mutant showed no significant biocontrol activity against TFRR, in contrast to the wild type (results not shown). We therefore conclude that competitive colonization, or competition for niches, is required for biocontrol activity of *P. fluorescens* PCL1751.

To test whether the enhanced colonizers can efficiently grow on exudate, the major nutrient source in the rhizosphere, the growth of the five enhanced colonizers was compared with that of five random strains isolated from the starting

material before enrichment. All five enhanced colonizers, as well as *P. fluorescens* WCS365, reached densities of 2×10^7 cfu ml^{-1} within 24 h and remained at that level for the next 48 h. In contrast, the best growing control strain reached a density of 7×10^6; the other control strains reached a maximum value of 3×10^5. The observation that the enhanced colonizers, as well as *P. fluorescens* WCS365 (not shown), grow much better on exudate than random rhizobacteria shows that the enrichment method (Fig. 1) selects for strains that utilize exudates components efficiently for growth. It suggests that competition for nutrients plays a major role in the biocontrol activity of the enhanced colonizers. Therefore this result, combined with the lack of biocontrol by the non-motile mutant, suggests that strain PCL1751 acts through competition for niches and nutrients.

Induction of systemic resistance against TFRR

Strains *P. fluorescens* WCS365 and *P. fluorescens* PCL1751 have in common that they are not antagonistic *in vitro*, do not secrete exo-enzymes, are excellent competitive tomato root tip colonizers, grow to the same high cell density on tomato root exudates and can control TFRR. However, their mechanisms of biocontrol seem to be different: WCS365 causes ISR, at least in *Arabidopsis thaliana* (Gerrits and Weisbeek, 1996) whereas our present results indicate that PCL1751 acts through competition for niches and nutrients. To test whether the two strains really use different mechanisms for their biocontrol action we decided to test, using the split root system described by Kroon (1992), whether the strains can induce resistance towards TFRR in tomato. In this system one of the root parts is treated with a putative biocontrol agent to allow induction of resistance and the other root part is challenged 1 week later with the pathogen.

The results are shown in Table 5. When no bacteria were added to one of the parts of the root system, 70–88% of the plants showed disease symptoms in the part of the root system that had been treated with *Forl*. Strain *P. fluorescens* PCL1751 appeared not to be able to prevent disease in the split root system. In contrast, strain *P. fluorescens* WCS365 showed significant biocontrol. It reduced the number of diseased plants in the tomato split root system to 39–47%. No

introduced *Pseudomonas* bacteria could be recovered from the non-inoculated root part. This result indicates that the induced resistance is systemic. The results of Table 5 therefore show that strain *P. fluorescens* WCS365, in contrast to *P. fluorescens* strain PCL1751, can systemically induce resistance against TFRR. This is the first time that it is shown that *P. fluorescens* WCS365 induces resistance in tomato plants without direct contact with the pathogenic fungus.

Plant growth promotion

For the evaluation of the effect of bacterial isolates on the growth of tomato and cucumber plants, seeds were coated with bacterial mixtures as described in the biocontrol assay and grown under greenhouse conditions in non-sterile potting soil. Each variant consisted of three replicas with eight seeds per replica. After 3 weeks of growth fresh and dry weight of shoots was determined and analysed using analysis of variance followed by Fisher's least-significant-difference test (a = 0.05), using SPSS software (SPSS, Chicago, IL, USA). All experiments were performed at least twice.

Table 5. Induction of systemic resistance against tomato foot and root rot by *P. fluorescens* PCL1751 and *P. fluorescens* WCS365 using a tomato split root system [x]

Microorganism(s) present	Experiment 1		Experiment 2	
	Healthy	Sick	Healthy	Sick
(i) *Forl*	7	16(a)	2	15(a)
(ii) PCL1751 and *Forl*	5	18(a)	5	12(a)
(iii) WCS365 and *Forl*	14	9(b)	9	8(b)

[x] Twenty three (exp.1) or seventeen (exp.2) plants were grown (i) in the presence of *Forl* and the absence of bacteria, (ii) in the presence of *Forl* and *P. fluorescens* PCL1751, and (iii) in the presence of *Forl* and *P. fluorescens* WCS365. Three weeks after addition of *Forl* spores, the diseased plants were scored. Values in one column followed by a different letter are significantly different from each other (P≤0.05), according to the chi-squared goodness-of-fit test (Heath, 1995). For details of inoculation and growth conditions, see Materials and Methods section.

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