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Is L-arabinose important for the endophytic lifestyle of *Pseudomonas* spp.?

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Abstract

Twenty endophytic bacteria were isolated from surface-sterilized stems and roots of cucumber plants. After removal of potential siblings and human pathogens, the remaining seven strains were identified based on their 16S rDNA as *Pseudomonas fluorescens* (2 strains) and *P. putida* (5 strains). Three strains, namely *P. fluorescens* CS1, *P. fluorescens* CR2 and *P. putida* CR3, were able to suppress tomato foot and root rot (TFRR). Special attention was paid to the characterization of the BIOLOG carbon oxidation profiles of the isolated pseudomonads in order to identify nutrients which might be important for their endophytic lifestyle. Comparative analysis of the profiles of these seven strains with those of seven rhizospheric *Pseudomonas* spp. revealed that endophytes were able to oxidize L-arabinose and 2,3-butanediol significantly more often than the rhizospheric group. An independent growth experiment performed in tubes using L-arabinose and 2,3-butanediol as sole carbon sources showed the same results as seen using BIOLOG for L-arabinose, but not for 2,3-butanediol. Since L-arabinose is one of the most abundant sugars in xylem of cucumber plants and was not detected in their rhizosphere, our data suggest that utilization of L-arabinose might be a trait contributing to the endophytic lifestyle of the isolated *Pseudomonas* endophytes.

Introduction

Plants live in association with many bacteria which can be classified as rhizobacteria, epiphytic bacteria and endophytic bacteria. Endophytic bacteria are referred to as those which are able to colonize plants internally without causing any apparent harm. Due to their endophytic lifestyle, bacterial endophytes establish a more stable and long-lasting relationship with a plant than other plant-associated bacteria do (Hardoim et al. 2008). In addition, some endophytic bacteria have beneficial effects on plants. Therefore, bacterial endophytes with plant-beneficial traits are considered to be promising bio-inoculants for agricultural application (Strobel 2006).

Once endophytes establish themselves inside a plant, some of them can stimulate plant growth and/or protect plants against phytopathogens. Endophytic bacteria are able to promote plant growth directly by the secretion of phytohormones (Spaepen et al. 2008; Sgroy et al. 2009), by nitrogen fixation (You et al. 2005; Pedraza 2008) and by phosphate solubilization (Taurian et al. 2009; Lopez et al. 2011). In addition, several beneficial bacteria contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the precursor of the plant hormone ethylene, to NH_3 and α-ketobutyrate (Glick 2005). The bacteria utilize NH_3 as a source of N and thereby decrease the ACC and ethylene levels within the plant and, as a result, can stimulate plant growth (Sun et al. 2009). The ACC-deaminase activity of endophytic bacteria and its responsibility for growth promotion of *Solanum nigrum* was reported for various strains of *Pseudomonas* (Long et al. 2008).

Endophytic bacteria can also promote plant growth indirectly via biocontrol of phytopathogens. Known mechanisms of biocontrol mediated by endophytic bacteria include (i) antibiosis through the production of antibiotics (Cho et al. 2003) or exo-enzymes (Downing and Thomson 2000) and (ii) induction of systemic resistance (Van Wees et al. 2008; Yasuda et al. 2009).

Endophytic bacteria are able to colonize intercellular spaces of the cell walls and xylem vessels which together form the plant apoplast (Compant et al. 2010). Biochemical studies of nutrients which are present in the apoplast indicate that this niche contains sugars, alcohols, amino acids, organic acids, growth factors, and mineral elements (Bacon and Hinton 2006). The question remains open of which carbon sources are utilized by endophytic bacteria in the plant apoplast.

The main aims of the present study were: (i) to isolate and identify the major culturable heterotrophic bacterial endophytes from stems and roots of cucumber plants grown in a greenhouse, (ii) to select novel beneficial strains based on their

abilities a) to promote growth of radish and/or b) to control TFRR caused by the fungus *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*Forl*), and (iii) to characterize and compare oxidation profiles of carbon sources of endophytic and rhizospheric *Pseudomonas* spp. in an attempt to identify compounds which might be involved in the endophytic lifestyle.

Materials and Methods

Isolation of endophytic bacteria

Endophytic bacteria were isolated from stems and roots of cucumber (*Cucumis sativus* L.) plants collected from greenhouses in the Tashkent area, Uzbekistan. Plant samples were treated with 70% ethanol for 3 min, followed by 4% sodium hypochlorite for 5 min, and several rinses with sterile water. To verify adequate surface sterilization, aliquots of water from the last rinsing were plated on 1/20 strength TSA control plates. Subsequently, the surface-sterilized plant samples were crushed under sterile conditions and the resulting juices were plated on 1/20 strength tryptic soy agar (TSA, Difco Laboratories, MI, USA) plates. After incubation at 28°C for 3 days colonies originating from plant juice with empty control plates were used for further analysis.

Microbial strains and growth conditions

All isolated bacterial strains and eight *Pseudomonas* rhizospheric strains (*P. fluorescens* WCS365, PCL1444 and PCL1751 and *P. putida* PCL1760, PCL1759, PCL1758, PCL1603 and PCL1445), which belong to the collection of Institute of Biology Leiden, were grown and maintained on full strength TSA. The rhizospheric strains used for a comparative analysis originate from the following plants: avocado (*P. putida* 1603 and 1760), Barmultra grass (*P. fluorescens* 1444 and P. putida 1445), tomato (*P. putida* 1758 and 1759) and potato (*P. fluorescens* WCS365). *P. fluorescens* PCL1751, which is an excellent root colonizer (Kamilova et al. 2005) and is naturally resistant to kanamycin, was used as a reference strain for competitive cucumber root tip colonization experiments. Kanamycin was used at the final concentration of 50 µg ml^{-1}.

The fungi *Aspergillus niger*, *Forl, F. solani* and the oomycete *Pythium ultimum* were routinely cultivated on potato-dextrose agar (PDA, Difco Laboratories). To obtain spores for biocontrol experiments, *Forl* was routinely grown on Czapek-Dox liquid medium (Difco Laboratories) and incubated on a rotary shaker at 150 rpm at 28 °C.

Molecular characterization of endophytic strains

Amplified ribosomal restriction analysis (ARDRA) and identification of endophytic strains was performed as described previously (Malfanova et al. 2011). Briefly, 16S rRNA gene was amplified, cut with four different restriction enzymes and the resulting fragments were separated using a 2% agarose gel. Those strains which gave a unique restriction pattern and appeared morphologically distinct were sent for sequencing to Service XS, Leiden, the Netherlands. Species they belonged to were identified as sharing at least 99% homology with those of known species.

Characterization of potential plant-beneficial traits

Characterization of potential plant-beneficial traits such as the production of exo-enzymes (β-glucanase, cellulase, chitinase, lipase and protease), antifungal metabolites (AFM) and auxins was performed as described previously (Malfanova et al. 2011). The presence of ACC deaminase was judged by growth on 1-aminocyclopropane-1-carboxylate (ACC) as the sole N-source according to Belimov et al. (2005).

Plant growth promotion

Endophytic bacteria were tested for their ability to promote the growth of radish plants. This was done by soaking seeds of radish in a bacterial cell suspension (adjusted to 10^8 cfu/ml) in phosphate buffered saline solution (PBS) for 15 minutes. As a negative control, seeds were treated with sterile PBS. The treated seeds were subsequently planted in non-sterile potting soil and grown under greenhouse conditions at 80% humidity and 16 h of daylight. Each variant consisted of four replicates with five seeds each. After two weeks of growth, the fresh weight of the roots was determined. All experiments were performed at least twice.

Biocontrol of tomato foot and root rot

Biocontrol of TFRR in soil was performed according to Kamilova et al. (2005) with small modifications. Briefly, tomato seeds of cultivar Carmello (Syngenta, Enkhuizen, the Netherlands) were coated with bacteria by dipping the seeds in a suspension of 1% (w/v) methylcellulose (Sigma, St Louis, MO, USA) in PBS containing 10^8 cfu/ml. The treated seeds were then planted in non-sterile potting soil supplemented with 10^7 *Forl* spores per kg. 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 light. After 3 weeks of growth, plants were removed from soil and examined for

symptoms of foot and root rot, such as brown spots, lesions, wilting or even death. Only roots without any of these symptoms were referred to as healthy and all others were scored as diseased. All experiments were performed at least twice.

Biocontrol of TFRR in stonewool substrate was conducted as described by Validov et al. (2007). Briefly, tomato seeds were placed in stonewool plugs which had been soaked in advance in Plant Nutrient Solution (PNS) (Wageningen UR Greenhouse Horticulture, Bleiswijk, the Netherlands) supplemented with *Forl* spores (10^7 spores/L) and bacterial cells (10^6 cfu/ml). In the negative control PNS was supplemented with spores only. Plants were grown for 14 days under greenhouse conditions at 80% humidity and 16 h of daylight. The plants were then removed from the stonewool and examined for symptoms of foot and root rot. All experiments were performed twice. Homogeneity of variance and analysis of variance (ANOVA) at p = 0.05 were conducted with SPSS software (Chicago, IL, USA).

Carbon oxidation/utilization assay

Seven *Pseudomonas* strains isolated from roots and stems of cucumber plants and seven rhizospheric strains of *Pseudomonas* spp. of different plant origin were tested for their ability to oxidize various carbon sources using BIOLOG GN2 Microplates (Biolog Inc., Hayward, CA, USA). Bacteria were grown overnight at 28 °C under aeration (150 rpm), harvested by centrifugation and subsequently resuspended in 0.85% (w/v) NaCl solution to a final OD_{590} of 0.15. Aliquots of 150 µl were inoculated in each well of a 96-wells microplate using a multichannel pipette. Plates were covered with a lid and incubated statically at 28˚C for 48 hours. The appearance and intensity of a purple color, caused by the reduction of the tetrazolium salt, was read using a microplate reader at OD_{590} and by direct observation. Only results with values of $OD_{590} > 0.3$ and the visible presence of the purple color were scored as positive. In the case of border values between 0.29-0.3 and/or absence of purple color in the test wells, the results were scored as "+/-". To check whether there is a significant difference in carbon oxidation abilities between the endophytic and rhizospheric group, the Chi-square test (p=0.05) was performed using SPSS software.

To verify results obtained using the Biolog assay, we performed independent growth experiments. Endophytic and rhizospheric bacteria were grown overnight in LB broth and washed twice with 0.85% NaCl to remove traces of extracellular carbon. Subsequently bacteria were inoculated in M9 minimal medium containing (i) 0.2% (w/v) L-arabinose or 2,3-butanediol (experiment), (ii) 0.2% (w/v) glucose (positive

control), and (iii) no added carbon source (negative control) to a final OD_{590} of 0.15. Falcon tubes containing 5 ml of each suspension were incubated for 24 h at 28˚C and 150 rpm. Subsequently the bacterial growth was scored spectrophotometrically at OD_{590}. All experiments were performed at least twice.

Competitive cucumber root tip colonization

In order to verify the ability of rhizospheric pseudomonads to efficiently colonize the rhizosphere of cucumber plants, competitive root tip colonization experiments were performed as described by Kamilova et al. (2005). Briefly, surface-sterilized cucumber seeds were inoculated with a 1:1 mixture of two bacterial strains (experiment vs. reference strain). The treated seeds were then planted in a gnotobiotic sand system (Simons et al. 1996) and grown for 7 days under greenhouse conditions. Subsequently, one cm of the root tip was cut off and vigorously shaken for 15 min to remove the adhered bacteria. Suspensions with bacterial cells were then diluted and plated on TSB with and without Km. The number of Km-sensitive (experiment) and Km-resistant (reference) colonies was determined and the average of each group was calculated. All colonization experiments were performed in five replicates.

Results

Isolation and characterization of endophytic bacteria

Endophytic bacteria were isolated from stems and roots of greenhouse-grown cucumber plants. The controls showed that all living microorganisms on the plant surface were killed or became nonculturable.

A total of 20 strains were randomly chosen from the colonies obtained after plating plant juices on 1/20 strength TSA. To eliminate potential siblings, these strains were compared for their colony morphology, motility, their ARDRA patterns and production of the exo-enzymes β-glucanase, cellulase, chitinase, lipase and protease. Eleven strains which were indistinguishable with respect to the mentioned traits were considered as likely siblings and not further studied.

The nine remaining strains were screened for their antagonistic activity towards four pathogens, their ability to produce auxin, and their growth on ACC as the sole N-source (Table 1). Strain CR3 is antagonistic towards *P. ultimum*, but not *A. niger*, *F. solanum* or *Forl* and does not secrete any exo-enzymes. None of the other strains showed any antagonism against these phytopathogens or production of exo-enzymes

Table 1. Overview of plant-beneficial traits of selected endophytic bacteria

Strain	Antifungal activity[a]	Exo-enzymes[b]	Auxin[c]	ACC[d]
CS1	-	P	-	+
CS4	-	-	+	-
CS5	-	-	++	-
CS6	-	-	-	-
CS8	-	-	+	-
CR2	-	-	-	-
CR3	Pu	-	+	-
CR6	-	-	+	-
CR9	-	-	-	-

[a] Pu, *Pythium ultimum.*

[b] P, protease.

[c] Auxin level after growth in medium supplemented with tryptophan: ++ >80 µg/ml, + >10-20 µg/ml, - < 10 µg/ml. Auxin level after growth in medium without tryptophan was zero for all strains.

[d] ACC **(**1-aminocyclopropane-1-carboxylate), growth on ACC as the sole N-source.

except for CS1 which showed protease activity.

Five strains, namely CR3, CR6, CS4, CS5 and CS8 produce detectable amounts of auxins, but only in the presence of tryptophan in the growth medium. The level of auxin secreted by strain CS5 is more than 80 µg/ml. In the case of CS4 and CR3, the auxin level in the media with tryptophan is more than 15 µg/ml. Two other strains, namely CR6 and CS8 produce appr. 10 µg/ml of auxins.

The only strain able to utilize ACC as the sole nitrogen source is CS1.

Molecular identification of endophytic strains

The strains were identified based on comparison of their rRNA sequences with database sequences from correctly identified strains (Table 2). The following species were isolated from stems and roots of cucumber plants: one *Bacillus cereus* strain, two *P. fluorescens* strains, five *P. putida* strains and one *Stenotrophomonas maltophilia*. To see whether these strains are safe to be applied in the field as biocontrol and/or plant-growth promoting strains, we evaluated to which risk group (Anonymous 1998) they belong. Two strains, namely *B. cereus* and *S. maltophilia* belong to risk group 2, representing potential human pathogens. Therefore, they were excluded from further experiments. This left us with seven pseudomonads.

Table 2. Molecular identification of endophytic strains[a] and risk group classification[b]

Strain	Bacterial species[c]	Identity (%)	Phylum	Risk group
CS1	*Pseudomonas fluorescens*	99	β-Proteobacteria	1
CS4	*Pseudomonas putida*	99	β-Proteobacteria	1
CS5	*Pseudomonas putida*	99	β-Proteobacteria	1
CS6	*Stenotrophomonas maltophilia*	99	β-Proteobacteria	2
CS8	*Bacillus cereus*	99	Firmicutes	2
CR2	*Pseudomonas fluorescens*	100	β-Proteobacteria	1
CR3	*Pseudomonas putida*	99	β-Proteobacteria	1
CR6	*Pseudomonas putida*	99	β-Proteobacteria	1
CR9	*Pseudomonas putida*	99	β-Proteobacteria	1

[a] After elimination of siblings.

[b] Risk group 1 includes bacteria which are safe to be applied in the field; risk group 2 includes potential human and/or plant pathogens (Anonymous, 1998).

[c] Sequences have been submitted to GenBank under accession numbers JX010776-JX010784.

Plant growth promotion

Four auxin-producing strains, namely CR3, CR6, CS4 and CS5, and one ACC-utilizing strain, CS1, were tested for their ability to promote radish growth under greenhouse conditions. Radish was chosen as the model plant because its roots secrete a high level of tryptophan on filter paper (Kamilova et al. 2006) which can be used by many beneficial bacteria as the precursor of auxin. However none of the tested strains showed plant growth promotion (results not shown).

Biocontrol of tomato foot and root rot

All seven *Pseudomonas* spp. were tested for their ability to suppress TFRR in soil and stonewool. Two strains, namely *P. fluorescens* CR2 and *P. putida* CR3, significantly decreased disease symptoms of tomato plants in soil from 38% in the non-inoculated control to 22% and 24%, respectively (Fig 1a). Significant biocontrol activity of these strains was also found in the second soil experiment. In the stonewool biocontrol experiments strains CR2 and CR3 were not significantly active but another strain, *P. fluorescens* CS1, was able to suppress TFRR symptoms from 71% to 41% in the first and from 58% to 36% in the second experiment (Fig 1b).

Carbon oxidation assay

Seven endophytic and rhizospheric *Pseudomonas* spp. were characterized and compared in respect to their carbon oxidation profiles in order to identify carbon sources which might be important for their endophytic lifestyle (Table 3). Out of 95

Table 3. Carbon sources oxidized by endophytic and rhizospheric pseudomonads[a]

Carbon source (GN2)	R[b]	E[b]	Carbon source (GN2)	R[b]	E[b]
Dextrin	2	2	D-glucuronic acid	6	7
Glycogen	2	2	Alpha-hydroxybutyric acid	6	6
NAC-D-glucosamine	3	1	Gamma-hydroxibutyric acid	1	1
L-arabinose*	**2**	**7**	**P-hydroxiphenylacetic acid***	**5**	**1**
D-arbitol	3	2	Itaconic acid	0	1
D-fructose	7	6	Alpha-keto- butyric acid	4	3
D-galactose	2	2	Malonic acid	4	5
M-inositol	1	2	Sebacic acid	1	0
D-mannitol	3	2	Succinamic acid	5	3
D-mannose	7	6	Glucuronamide	5	5
D-psicose	2	2	L-alanil-glycine	7	6
D-sorbitol	1	2	Glyciyl-L-Glutamic acid	1	0
Sucrose	1	3	D-serine	5	5
D-trehalose	1	2	L-threonine	6	7
Succinic acid mono-methyl ester	5	3	Urocanic acid	3	5
Acetic acid	6	7	Inosine	6	6
Formic acid	6	7	Uridine	2	2
D-galactonic acid lactone	2	2	Phenylethylamine	6	5
D-galacturonic acid	6	6	**2,3-butanediol***	**1**	**6**
D-glucosaminic acid	2	2	D,L-alpha-glycerol phosphate	1	2

[a] Does not include carbon sources which oxidized by all or none of the tested strains (see Results).

[b] Number of strains out of the seven tested pseudomonads which oxidized the carbon source.

[*] Carbon sources which were differently oxidized between the rhizospheric (R) and the endophytic (E) group based on Chi-square analysis ($p < 0.05$).

different carbon sources, as many as 34 were oxidized by all tested strains. These include one sugar (alpha-D-glucose), 12 organic acids (cis-acetonic, citric, D-gluconic, beta-hydroxybutyric, alpha-ketoglutaric, alpha-ketovaleric, D,L-lactic, propionic, quinic, D-saccharic, succinic and bromosuccinic acid), 13 amino acids (D-alanine, L-alanine, L-asparagine, L-aspartic, L-glutamic, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-pyroglutamic acid, L-serine and gamma-amino butyric acid) and eight other compounds. A total number of 21 carbon sources were not oxidized by any of the strains. These comprise 11 sugars (D-cellobiose, L-fucose, gentiobiose, alpha-D-lactose, lactulose, maltose, D-melibiose, beta-methyl-D-glucoside, D-raffinose, L-rhamnose and turanose), three sugar alcohols (adonitol, i-erythritol and xylitol), one amino acid (L-phenylalanine), one amino acid derivative (glycil-L-aspartic acid) and five other compounds.

Three out of the 40 remaining carbon sources gave significantly different oxidation

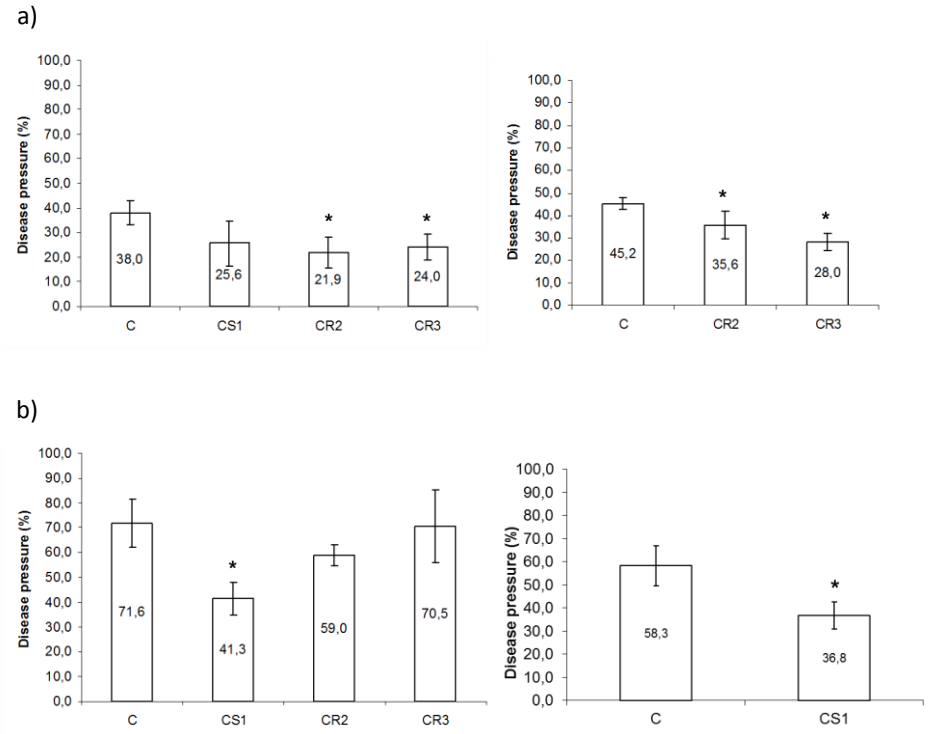

Fig. 1. Biocontrol of TFRR by endophytic *Pseudomonas* spp. a) in soil; b) in stonewool substrate. Numbers inside the columns present the percentage of sick plants. Bars indicate confidence interval ($p < 0.05$). Statistically different values are indicated with asterisks. C, uninoculated control.

profiles between the endophytic and rhizospheric group, namely L-arabinose, 2,3-butanediol and p-hydroxyphenylacetic acid (Table 3). L-arabinose was oxidized by all seven endophytes and only by two rhizospheric strains, namely *P. fluorescens* strains WCS365 and PCL1444 ($p < 0.05$). Six out of seven endophytic pseudomonads (*P. fluorescens* CS1 and CR2 and *P. putida* CS5,CR3, CR6 and CR9) and one out of seven rhizospheric ones (*P. fluorescens* WCS365) were able to oxidize 2,3-butanediol ($p < 0.05$). Para-hydroxyphenylacetic acid was oxidized by five rhizospheric and one endophytic strain ($p < 0.05$).

The results of an independent growth experiment, as judged by an increase in the optical density (590 nm) compared to M9 medium without added carbon source, using L-arabinose as the sole carbon source showed the same results as the BIOLOG experiments obtained for L-arabinose. However for 2, 3-butanediol as the sole carbon source, growth was measured for all endophytic strains and for six out of the seven rhizosperic strains.

Competitive cucumber root tip colonization experiment

To check whether the tested rhizospheric strains which originate from different plant hosts are cucumber rhizosphere competent and therefore can serve as suitable controls for the cucumber endophytes, we evaluated these strains in a competitive root tip colonization experiment against *P. fluorescens* PCL1751 (Kamilova et al. 2005), an excellent root colonizer. It appeared that all strains colonized the cucumber root tip in competition with *P. fluorescens* PCL1751 and therefore are cucumber rhizosphere competent (Table 4).

Discussion

General remarks about the isolated endophytes

After developing the protocol for the isolation of endophytic bacteria from cucumber plants, we used a similar strategy as described by Validov et al. (2007) for the elimination of siblings and potential pathogens. The fact that 11 out of the 20 strains are siblings indicates that the diversity among the isolated endophytes is low. Out of the nine remaining strains, two strains belong to risk group 2 (Table 2), indicating the presence of potential human pathogens among these endophytes. This phenomenon has been reported previously for both rhizospheric (Berg et al. 2005; Egamberdiyeva et al. 2008) and endophytic bacteria (Malfanova et al. 2011).

Table 4. Competitive cucumber root tip colonization experiment.

Competing strains[a]	cfu/cm of root tip[b]	
	Test strain	**Reference strain**
365 vs 1751	$(3{,}52\pm0{,}43)*10^4$	$(4{,}27\pm0{,}39)*10^4$
1444 vs 1751	$(2{,}60\pm1{,}41)*10^5$	$(4{,}03\pm0{,}48)*10^5$
1445 vs 1751	$(9{,}81\pm4{,}1)*10^3$	$(1{,}35\pm1{,}64)*10^5$
1603 vs 1751	$(5{,}43\pm4{,}2)*10^3$	$(2{,}21\pm0{,}37)*10^5$
1758 vs 1751	$(2{,}00\pm0{,}35)*10^4$	$(7{,}65\pm2{,}78)*10^4$
1759 vs 1751	$(2{,}40\pm0{,}54)*10^4$	$(4{,}90\pm0{,}72)*10^4$
1760 vs 1751	$(5{,}14\pm0{,}66)*10^4$	$(3{,}38\pm0{,}39)*10^5$

[a] The tested strains were inoculated on cucumber seeds in a 1:1 ratio with the reference strain *P. fluorescens* PCL1751.

[b] The average number of Km-sensitive (test) and Km-resistant (reference) colonies after plating the suspension with bacterial cells washed from the cucumber root tip.

Seven remaining bacteria were identified as *Pseudomonas* spp. of which members have been found as endophytes of different plants (Mercado-Blanco and Bakker 2007; Ramesh et al. 2008). Several representatives of this group, namely *P. fluorescens* and *P. putida*, are widely known for their various plant-beneficial traits which include production of antifungal metabolites and exo-enzymes, ACC-deaminase activity and secretion of phytohormones (Mercado-Blanco and Bakker 2007). In our study only a few identified pseudomonads displayed these characteristics (Table 1). The most common beneficial trait was the production of auxin in the presence of its precursor L-tryptophan.

Plant growth promotion

Auxin-producing strains were further tested under greenhouse conditions for their ability to promote the growth of radish roots. Auxins are known to be essential for plant physiology because they affect the root and shoot architecture (Spaepen et al. 2009). To our surprise, none of the tested pseudomonads had a significant effect on the root biomass of radish plants. The same results were obtained on tomato and cucumber plants (data not shown). The amount of L-tryptophan secreted by cucumber and tomato plants is low (1.8 and 7.4 ng per seedling, respectively), while the amount secreted by radish plants exceeds 0.29 µg per seedling (Kamilova et al. 2006) and is sufficient to stimulated microbial IAA production in nutrient rich medium (Kravchenko et al. 2004). However in nutrient poor soil, addition of high amounts of either L-tryptophan or IAA (up to 3 mg per kg soil) does not lead to a significant increase in radish root weight (Frankenberger et al. 1990). This may explain the absence of plant growth promotion in our experiments and highlights the importance of the substrate used during such investigations.

Biocontrol of TFRR

Biocontrol results (Fig 1) indicated that *P. fluorescens* strains CS1 and CR2 and *P. putida* strain CR3 have a strong ability to control TFRR. However, the biocontrol effect of these strains was substrate-dependent. Apparently, the biocontrol mechanisms used by the different strains do not necessarily function well in both substrates. A similar effect was reported previously by Validov et al. (2009) who found that flagellar motility, which is a key trait for the biocontrol ability of PCL1751 in potting soil, is not important during colonization of stonewool by *P. putida* PCL1760.

Despite the fact that the selected biocontrol strains do not produce exo-enzymes and antifungal metabolites against *Forl in vitro*, they were able to suppress the disease caused by the fungus *in vivo*. This observation is in agreement with other reports (Berg and Hallmann 2006; Malfanova et al. 2011), indicating that *in vitro* and *in vivo* beneficial activity of some bacteria is not necessarily correlated. Possible mechanisms of biocontrol include induction of systemic resistance (ISR) and competition for niches and nutrient (CNN) (Lugtenberg and Kamilova 2009). It is tempting to speculate that CNN is likely to be involved in biocontrol mediated by the selected endophytic pseudomonads. This mechanism has been proven for *P. fluorescens* PCL1751 and *P. putida* PCL1760 which also do not inhibit *Forl* in the plate assay but show significant biocontrol of TFRR in stonewool (Kamilova et al. 2005; Validov et al. 2007; Validov et al. 2009). Since the three strains which showed biocontrol did only so on one of the two substrates, we did not study the mechanism(s) of action.

Utilization of carbon sources by endophytic and rhizospheric pseudomonads

Out of the 95 different carbon sources tested, three were significantly differentially oxidized between the rhizospheric and the endophytic group ($p<0.05$). An independent growth experiment using M9 medium confirmed the BIOLOG result in the sense that L-arabinose is utilized by all endophytic pseudomonads while only two out of the seven tested rhizospheric strains could use it as their sole carbon source. Interestingly, L-arabinose is one of the major sugars present in the apoplast of different plants, including cucumbers (Iwai et al. 2003). Therefore our results suggest that utilization of L-arabinose might be a trait contributing to the endophytic lifestyle of the *Pseudomonas* endophytes isolated from cucumber plants.

Our suggestion about the role of L-arabinose may be extended to other endophytes and plants because Prakamhang et al. (2009) found that all 51different endophytes isolated from rice were able to use L-arabinose as well as glucose as their sole carbon sources. L-arabinose was not detected in the root exudate of cucumber plants (Kamilova et al. 2006). This fact together with the results from the competitive cucumber colonization experiment, which showed that all tested rhizospheric strains are able to reach the tip of the root at high density (see Table 4), suggests that the root tip colonization ability of the rhizospheric strains is not dependent on utilization of L-arabinose. The results also suggest that those rhizosperic strains which are able to utilize L-arabinose have an enhanced possibility of becoming endophytes.

Further analysis of the BIOLOG results showed that 2, 3-butanediol was differentially oxidized. However this could not be fully verified in an independent growth experiment although the endophytic strains were able to reach a higher cell density level than the rhizospheric strains (data not shown). It is interesting to note that 2, 3-butanediol is a volatile signaling molecule involved in plant growth regulation and triggering of ISR (Ryu et al. 2003). As can be expected from signal molecules, they can be degraded and several pseudomonads apparently are able do so.

In conclusion, the results of the present study show that among the seven isolated *Pseudomonas* cucumber endophytes three strains have biocontrol activity. Moreover, we also found that, in contrast to most rhizospheric *Pseudomonas* spp., endophytic pseudomonads were able to utilize L-arabinose, one of the most abundant sugars in the xylem fluid of various plants.

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References

Anonymous. 1998. Sichere Biotechnologie. Eingruppierung biologischer Agenzien: Bacterien, BG Chemie, Merkblatt B 006 8/98 ZH 1/346. Heidelberg, Germany: Jedermann-Verlag Dr Otto Pfeffer oHG.

Bacon CW, Hinton DM. 2006. Bacterial endophytes: the endophytic niche, its occupants, and its utility. In: Gnanamanickam SS (ed) Plant-associated bacteria. Springer, the Netherlands, pp. 155–194.

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR. 2005. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol. Biochem. 37: 241–250.

Berg G, Hallmann J. 2006. Control of plant pathogenic fungi with bacterial endophytes. In: Schulz B, Boyle C, Sieber T (eds) Microbial root endophytes. Springer-Verlag, Berlin, pp. 53-69.

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

Cho S, Lim W, Hong S. et al. 2003. Endophytic colonization of balloon flower by antifungal strain *Bacillus* sp. CY22. Biosci. Biotech. Biochem. 67: 2132 – 2138.

Compant S, Clément C, Sessitsch A. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42: 669-678.

Downing K, Thomson J. 2000. Introduction of the *Serratia marcescens* chiA gene into an endophytic *Pseudomonas fluorescens* for the biocontrol of phytopathogenic fungi. Can J. Microbiol. 46: 363–369.

Egamberdiyeva D, Kamilova F, Validov S, Gafurova L, Kucharova Z, Lugtenberg B. 2008. High incidence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown in salinated soil in Uzbekistan. Environ. Microbiol. 10: 1–9.

Frankenberger WT, Chang AC, Arshad M. 1990. Response of *Raphanus sativus* to the auxin precursor, L-tryptophan applied to soil. Plant Soil 129: 235-241.

Glick BR. 2005. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol. Lett. 251: 1–7.

Hardoim PR, van Overbeek LS, Elsas JD. 2008. Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol. 16: 463-471.

Iwai H, Usui M, Hoshino H, Kamada H, Matsunaga T, Kakegawa K, Ishii T, Satoh S. 2003. Analysis of sugars in squash xylem sap. Plant Cell Physiol. 44: 582-587.

Kamilova F, Kravchenko LV, Shaposhnikov AI, Azarova T, Makarova N, Lugtenberg BJJ. 2006. Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. Mol Plant-Microbe Interact. 19: 250–56.

Kamilova F, Validov S, Azarova T, Mulders I, Lugtenberg B. 2005. Enrichment for enhanced competitive root tip colonizers selects for a new class of biocontrol bacteria. Environ. Microbiol. 7: 1809–1817.

Kravchenko LV, Azarova TS, Makarova NM, Tikhonovich IA. 2004. The Effect of tryptophan present in plant root exudates on the phytostimulating activity of rhizobacteria. Microbiology 73: 156-158.

Long HH, Schmidt DD, Baldwin IT. 2008. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PloS one 3: e2702.

Lopez BR, Bashan Y, and Bacilio M. 2011. Endophytic bacteria of *Mammillaria fraileana*, an endemic rock-colonizing cactus of the southern Sonoran Desert. Arch. Microbiol. 193: 527–41.

Lugtenberg B, Kamilova F 2009. Plant-growth-promoting rhizobacteria. Ann. Rev. Microbiol. 63: 541-56.

Malfanova N, Kamilova F, Validov S, Shcherbakov A, Chebotar V, Tikhonovich I, Lugtenberg B. 2011. Characterization of *Bacillus subtilis* HC8, a novel plant-beneficial endophytic strain from giant hogweed. Microb. Biotech. 4: 523-532.

Mercado-Blanco J, Bakker PAHM. 2007. Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. Antonie van Leeuwenhoek 92: 367-89.

Pedraza RO. 2008. Recent advances in nitrogen-fixing acetic acid bacteria. Int. J. Food Microbiol. 125: 25–35.

Prakamhang J, Minamisawa K, Teamtaisong K, Boonkerd N, Teaumroong N. 2009. The communities of endophytic diazotrophic bacteria in cultivated rice (*Oryza sativa* L.). Appl. Soil Ecol. 42: 141-149.

Ramesh R, Joshi AA, Ghanekar MP. 2008. Pseudomonads: major antagonistic endophytic bacteria to suppress bacterial wilt pathogen, *Ralstonia solanacearum* in the eggplant (*Solanum melongena* L.). World J. Microbiol. Biotech. 25: 47-55.

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW. 2003. Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Science (USA) 100: 4927-4932.

Sgroy V, Cassán F, Masciarelli O, Del Papa MF, Lagares A, Luna V. 2009. Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte *Prosopis strombulifera*. Appl. Microbiol. Biotech. 85: 371–81.

Simons M, van der Bij AJ, Brand I, de Weger LA, Wijffelman CA, Lugtenberg BJJ. 1996. Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. Mol. Plant-Microbe Interact. 9: 600–607.

Spaepen S, Dobbelaere S, Croonenborghs A, Vanderleyden J. 2008. Effects of *Azospirillum brasilense* indole-3-acetic acid production on inoculated wheat plants. Plant Soil 312: 15–23.

Spaepen S, Vanderleyden J, Okon Y. 2009. Plant Growth-promoting actions of rhizobacteria. In: van Loon LC (ed) Plant innate immunity. Elsevier, pp. 283-320.

Strobel G. 2006. *Muscodor albus* and its biological promise. J. Indust. Microbiol. Biotech. 33: 514-522.

Sun Y, Cheng Z, Glick BR. 2009. The presence of a 1-aminocyclopropane-1-carboxylate (ACC) deaminase deletion mutation alters the physiology of the endophytic plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN. FEMS Microbiol. Lett. 296: 131-136.

Taurian T, Anzuay MS, Angelini JG, Tonelli ML, Ludueña L, Pena D, Ibáñez F, Fabra A. 2009. Phosphate-solubilizing peanut associated bacteria: screening for plant growth-promoting activities. Plant Soil 329: 421–431.

Validov S, Kamilova F, Qi S, Stephan D, Wang JJ, Makarova N, Lugtenberg B. 2007. Selection of bacteria able to control *Fusarium oxysporum* f. sp. *radicis lycopersici* in stonewool substrate. J. Appl. Microbiol. 102: 461–471.

Validov SZ, Kamilova F, Lugtenberg BJJ. 2009. *Pseudomonas putida* strain PCL1760 controls tomato foot and root rot in stonewool under industrial conditions in a certified greenhouse. Biol. Control 48: 6-11.

Van Wees SCM, Van der Ent S, Pieterse CMJ. 2008. Plant immune responses triggered by beneficial microbes. Curr. Opin. Plant Biol. 11: 443–448.

Yasuda M, Isawa T, Shinozaki S, Minamisawa K, Nakashita H. 2009. Effects of colonization of a bacterial endophyte, *Azospirillum* sp. B510, on disease resistance in rice. Biosci. Biotech. Biochem. 73: 2595-2599.

You M, Nishiguchi T, Saito A, Isawa T, Mitsui H, Minamisawa K. 2005. Expression of the *nifH* gene of a *Herbaspirillum* endophyte in wild rice species: daily rhythm during the light–dark cycle. Appl. Environ. Microbiol. 71: 8183– 8190.