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Genomic assisted characterization of *Pseudomonas* sp. strain Pf4, a potential biocontrol agent in hydroponics

Serena Moruzzi, Giuseppe Firrao, Cesare Polano, Stefano Borselli, Alberto Loschi, Paolo Ermacora, Nazia Loi, Marta Martini*

Department of Agricultural, Food, Environmental and Animal Sciences (DI4A), University of Udine, Via delle Scienze 206, 33100 Udine, Italy

Serena Moruzzi, tel 0039 0432 558530, email serenamoruzzi@yahoo.it
Giuseppe Firrao, tel 0039 0432 558531, email giuseppe.firrao@uniud.it, ORCID iD: https://orcid.org/0000-0002-7890-0899
Cesare Polano, tel 0039 0432 558543, email polano.cesare@spes.uniud.it
Stefano Borselli, tel 0039 0432 558525, email stefano.borselli@uniud.it
Alberto Loschi, tel 0039 0432 558522, email alberto.loschi@uniud.it
Paolo Ermacora, tel 0039 0432 558540, email paolo.ermacora@uniud.it, ORCID iD: https://orcid.org/0000-0003-0757-7956
Nazia Loi, tel 0039 0432 558536, email nazia.loi@uniud.it, ORCID iD: https://orcid.org/0000-0002-9738-9248
Marta Martini*, tel 0039 0432 558541, email marta.martini@uniud.it, ORCID iD: https://orcid.org/0000-0002-7271-5297

***CONTACT**

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ABSTRACT

In an attempt to select potential biocontrol agents against *Pythium* spp. and *Rhizoctonia* spp. root pathogens for use in soilless systems, 12 promising bacteria were selected for further investigations. Sequence analysis of the 16S rRNA gene revealed that three strains belonged to the genus *Enterobacter*, whereas nine strains belonged to the genus *Pseudomonas*. In *in vitro* assays, one strain of *Pseudomonas* sp., Pf4, closely related to *Pseudomonas protegens* (formerly *P. fluorescens*), showed noteworthy antagonistic activity against two strains of *Pythium aphanidermatum* and two strains of *Rhizoctonia solani* AG 1-IB, with average inhibition of mycelial growth >80%.

Strain Pf4 was used for *in vivo* treatments on lamb's lettuce against *R. solani* root rot in small-scale hydroponics. Pf4-treated and untreated plants were daily monitored for symptoms development and after two weeks from infection, a significant protective effect of Pf4 against root rot was recorded. The survival and population density of Pf4 on roots were also checked, demonstrating a density above the threshold value of 10^5 CFU g^{-1} of root required for disease suppression.

PCRs having as target genes involved in the synthesis of antifungal metabolites and draft genome sequencing of Pf4 demonstrated that *Pseudomonas* sp. Pf4 has the potential to produce an arsenal of secondary metabolites (*plt*, *phl*, *ofa* and *fit-rzx* gene clusters) very similar to that of the well-known biocontrol *P. protegens* strain Pf-5.

KEYWORDS

Biological control; *Rhizoctonia solani*; *Pythium* spp.; population dynamic; secondary metabolites; draft-genome sequencing.

1. Introduction

Soilless, hydroponic systems are well suited for the cultivation of many crops, including leafy vegetables. Their main feature is the possibility to control all environmental factors, i.e. nutrient

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solution supply, temperature, pH, dissolved oxygen concentration, electrical conductivity, light radiation, that translates into higher production, energy conservation, better control of growth, independence from soil quality (van Os, 1999).

Although soilless cultures have been reported as a successful alternative to the use of methyl bromide and other fumigants to avoid root-diseases caused by soil-borne pathogen microorganisms (van Os, 1999), root-diseases still occur in these systems. Sometimes disease outbreaks are even greater than in soil (McPherson, Harriman, & Pattison, 1995), promoted by suitable environmental conditions, and rapid dispersal of root-colonising agents through the cultural system (Vallance et al., 2010). The most harmful pathogenic microorganisms in hydroponic cultures are those producing zoospores, i.e. *Pythium* spp. and *Phytophthora* spp., particularly adapted to wet environment, but also *.Fusarium* spp. and *Rhizoctonia solani* are of major concern (Schnitzler, 2004; Paulitz & Bélanger, 2001). In particular, *R. solani* was recently detected in Italy on many leafy vegetables (Colla, Gilardi, & Gullino, 2012), including lamb's lettuce [*Valerianella locusta* (L.) Laterr.] (Garibaldi, Gilardi, & Gullino, 2006).

Prevention of pathogen infections, particularly in closed hydroponic systems, has become a major challenge in recent years, particularly in the light of the increasing public concern regarding the use of chemical pesticides and subsequent legislative issues (e.g., Directive 2009/128/EC). Biological control is regarded as a potentially solid alternative to the use of chemical pesticides, and can be effective also in soilless systems (Vallance et al., 2010; Postma, 2010). Since studies on suppressiveness demonstrated the potential of indigenous microflora to inhibit root diseases in hydroponic cultures (McPherson, 1998), one of the main strategies is the addition of antagonistic microorganisms to increase the level of suppressiveness (Vallance et al., 2010).

Rhizobacteria are the most efficient microorganisms against soil-borne pathogens, which occur in the environment at the interface of root and soil (Handelsman & Stabb, 1996). In particular, fluorescent pseudomonads can persistently colonize the rhizosphere (Couillerot, Prigent-Combaret, Caballero-Mellado, & Moënne-Loccoz, 2009), compete with root pathogens for micronutrients

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(especially for iron and carbon) and root surface colonization (Haas & Défago, 2005; Raaijmakers, Paulitz, Steinberg, Alabouvette, & Moënne-Loccoz, 2009), trigger Induced Systemic Resistance (ISR) response in plants (Bakker, Pieterse, & Van Loon, 2007). A major component of biocontrol potential appears to be connected with secretion: fluorescent pseudomonads that are active biocontrol agents produce secondary metabolites that act as antimicrobial compounds, i.e. 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines, pyrrolnitrin, pyoluteorin, hydrogen cyanide (HCN) (Raaijmakers, Vlami, & De Souza, 2002; Handelsman & Stabb, 1996), but also siderophores as pyoverdin, biosurfactants, extracellular lytic enzymes (Compant, Duffy, Nowak, Clément, & Barka, 2005).

Only a limited number of studies on biological control by rhizobacteria have been carried out in soilless systems and consequently a limited number of biocontrol agents have been isolated and characterized from soilless systems. Yet it is important to understand to what extent the growing system is a relevant component in determining the potential of biological control agent. Are rhizobacteria with biological control potential isolated from hydroponics different from those isolated from soil? Are they relying on different mechanisms for the control of pathogens?

In this work we selected a biocontrol agent from endogenous source, the hydroponics, characterized it for both its biocontrol performances and its genomic features, with particular reference to secondary metabolites, and compared it with other known biological agents isolated from soil. Surprisingly, the strain was not dramatically different from other previously known pseudomonads biocontrol agents, indicating that the hydroponic conditions do not significantly change the mechanisms involved in biocontrol.

2. Materials and methods

2.1. Plant pathogen strains

Fungal and oomycete pathogens were obtained from culture collection and by isolation from diseased plants. Specifically, *Pythium aphanidermatum* strain CBS 118745 and strain CBS 116664,

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were obtained from the Centraal Bureau voor de Statistiek (CBS) culture collection, and were grown on oatmeal agar (OA, oatmeal flakes boiled and filtered 30g l^{-1}, 15 g l^{-1} bacteriological agar). Whereas, fungal isolations were made in 2009 from diseased plants showing symptoms of root rot and wilting in an hydroponic farm in Friuli Venezia Giulia (FVG) region, north-eastern Italy. Sixty portions of lamb's lettuce or chicory roots and seedlings were washed in sterile distilled water, placed on water agar (WA, 20 g l^{-1} bacteriological agar) plates and incubated at 24°C for 48 h. The isolates were transferred on Petri-dishes containing OA. Fungal isolates with the morphological characters of *Rhizoctonia solani* were consistently recovered and their identity confirmed by internal transcribed spacer (ITS) analysis. DNA extraction and PCR-amplification of ITS region using the universal primers ITS1/ITS4 (White, Bruns, Lee, & Taylor, 1990) and GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) from 12 isolates of *R. solani* was carried out as previously described by Martini et al. (2009). PCR products were then digested with endonuclease *Tru*1I and visualized on a 2% agarose gel, stained with GelRed™ (Biotium Inc., Hayward, CA, USA). The subsequent restriction profiles were compared, and resulted identical to each other. Two strains of *R. solani*, TR15 and TP20, were selected for sequencing and analysis of ITS region as described by Martini et al. (2009), and successively used in this work. ITS sequences (652 bp) of *R. solani* strains TR15 and TP20 were submitted to GenBank under accessions KM589032 and KM589033 respectively. BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) analysis allowed confirmation of their morphological identification as *R. solani* and their assignment to anastomosis group AG 1-IB (Sharon, Kuninaga, Hyakumachi, & Sneh, 2006) with 100% similarity with the GenBank sequence AJ868450 of *R. solani* (*Thanatephorus cucumeris*) strain AG1 (CBS 522.96).

2.2. Isolation of potential bacterial biocontrol agents and preliminary screening

Bacteria strains were isolated from the rhizosphere of healthy hydroponic lamb's lettuce plants grown in the same hydroponic farm as before. Thirty root samples were collected from healthy plants, cut in 1-1.5 cm pieces, washed in sterile distilled water and transferred on WA; plates were

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incubated at 24°C for 48-72 h. Each colony was re-streaked three times, and grown in pure culture on nutrient agar medium (NA, 1 g l^{-1} beef extract, 2 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} sodium chloride, 15 g l^{-1} bacteriological agar) at 24°C for 48 h.

Fifty-one bacterial strains were preliminarily tested by a dual culture method according to Gravel, Martinez, Antoun, and Tweddell (2005) with *P. aphanidermatum* strains CBS 118745 and CBS 116664, on potato dextrose agar medium (PDA, 38 g l^{-1}). Bacteria were inoculated at one side of a Petri dish and, after 48-h incubation, a mycelium plug was placed on the opposite site of the Petri dish, approximately 5 cm apart from the bacterial inoculation point. At the same time, positive controls of fungal pathogens were prepared by placing a mycelium plug in a Petri dish. After incubation for 7 days at room temperature (about 24°C), the presence/absence of an inhibition zone between the pathogen and each bacterium was recorded. Twelve bacterial strains that proved to inhibit the tested pathogens were selected for further investigations.

2.3. Bacteria identification

DNAs from the twelve selected bacterial strains were extracted according to the procedure reported on *Current protocols in Molecular Biology* (Wilson, 1997). PCR amplification of 16S rRNA gene was performed with universal primers fD1/rP1 (Weisburg, Barns, Pelletier, & Lane, 1991). Amplifications were performed with the automated One Advanced thermocycler (EuroClone, Celbio, Milan, Italy) in 25 µl reactions containing 200 µM of each of the four dNTPs, 0.4 µM of each primer, 1.5 mM $MgCl_2$, 0.625 units of GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) and 1 µl of diluted bacterial DNA (5 ng μl^{-1}). The PCR program consisted of initial denaturation for 2 min at 94°C; 36 cycles of 1 min at 94°C, 1 min at 58°C, 2 min at 72°C; and a final extension for 8 min at 72°C.

PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, USA) and sent to Genechron laboratory, (ENEA Casaccia, Rome, Italy) for sequencing. The sequences were determined with forward and reverse primers and assembled with

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BioEdit (Hall, 1999). For bacteria identification, 16S rRNA gene sequences 1303-1409 bp long were compared with those present in GenBank using BLASTN analysis. The nucleotide sequences were deposited in GenBank.

2.4. In vitro *antagonistic activity*

The antagonistic activity of the 12 preliminarily selected bacterial strains against *P. aphanidermatum* strains CBS 118745 and CBS 116664 and *R. solani* strains TR15 and TP20 was further characterized as follows. Bacterial strains were inoculated on Petri dishes containing PDA supplemented with 3 g l^{-1} peptone and 2 g l^{-1} yeast extract, in four diametrically opposite sites, approximately 3 cm from the centre. After a 48-h incubation at 24°C, plugs of mycelium (about 5 mm in diameter) were placed in the centre of the Petri dishes. At the same time, mycelium plugs were also inoculated on Petri dishes containing only growth medium, as control reference. The plates were further incubated for 9 days, and the mycelial growth was measured daily. The assays were repeated twice, and each combination bacterial antagonist-plant pathogen was replicated at least three times. The average inhibitory effect of each strain against the two pathogens was estimated based on the percent inhibition of radial growth, calculated using the following formula (Fokkema, 1976): % inhibition = $[(C-T)\ C^{-1}] \times 100$, where C is the radial growth of the pathogen without antagonist and T is the radial growth of the pathogen in presence of the antagonist.

2.5. In vivo *activity of* Pseudomonas *sp. strain Pf4 against* Rhizoctonia solani

The bacterial strain that showed the best *in vitro* antagonistic activity, i.e. *Pseudomonas* sp. strain Pf4, was chosen for *in vivo* application with the aim to evaluate its protective effect against *R. solani* root rot and its persistence and concentration on the rhizosphere of lamb's lettuce plants growing in a soilless system. Pf4 was cultured in flasks with 50 ml of nutrient broth (NB, 1 g l^{-1} beef extract, 2 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} sodium chloride) at 24°C for 36 h, pelleted with centrifugation at 6500 rpm for 10 min at 4°C and suspended in sterile distilled water to a final

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concentration of 10^9 CFU ml^{-1}. *R. solani* was cultured in flasks with 200 ml malt extract broth (MEB, malt extract 6 g l^{-1}, maltose 1.8 g l^{-1}, dextrose 6 g l^{-1}, yeast extract 1.2 g l^{-1}) at 24°C for 14-18 d; the mycelium was rinsed with sterile distilled water and thoroughly grinded to obtain an homogeneous suspension. Lamb's lettuce plants were grown in a plant growth room, with the following conditions: temperature 26°C, photoperiod of 11 h light/13 h dark, in small scale floating systems (15 l tanks) with a standard solution widely used by horticultural farms in north-eastern Italy, as reported by Iacuzzo et al. (2011). Specifically, eight tanks were prepared, in each tank about 50 lamb's lettuce plants were grown. Bacterial treatments were carried out on four of the eight tanks (4 replicates for Pf4 treatment) and successively infected with the pathogen, the other four tanks were only infected with the pathogen (4 replicates for untreated plants). Eight additional tanks, prepared as above and not inoculated with the pathogen, served as negative controls.

Pf4 bacterial suspensions were used for three treatments: the first was applied on seeds by immersion in the bacterial suspension for 10 min, the second was applied on seedlings (approximately 10^7 CFU/seedling) about 7 days after seeding; whereas the third one was applied 18 days after seeding directly into the nutrient solution at a final concentration of 10^6 CFU ml^{-1}. Successively, Pf4-treated and untreated plants were artificially infected with the fungal pathogen. For fungal infection, a bunch of lamb's lettuce plants growing in miniaturized floating system were infected through root immersion for 2 h in the suspension of *R. solani* mycelium. Three days after the third bacterial treatment, six infected plants were put in each of the eight tanks, and used as source of inoculum. Disease development was scored daily for up to three weeks. The number of plants with *R. solani* symptoms (limping, wilting, and/or complete withering) was scored.

The experiment was repeated twice (trial I and trial II). Statistical analysis was performed separately on data obtained from each experiment. The data of disease incidence in percentage were subjected to arcsine transformation and to unpaired T-test with Welch correction using the software GraphPad InStat version 3.00 (GraphPad Software Inc., San Diego, CA, USA).

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2.5.1. Survival and population density of Pseudomonas *sp. strain Pf4 on lamb's lettuce roots in hydroponics*

In order to determine the survival and population density of the inoculated bacteria, root samples (30-300 mg) were weekly collected from two plants randomly selected from each negative control tank of trial I for a period of four weeks, starting 18 days after seeding, just before the application of bacterial suspension into the nutrient solution. Roots from Pf4-treated and untreated plants were weighed, placed in sterile distilled water (1 ml 10 mg^{-1} root tissue) and kept on a rotary shaker for 2 h. Aliquots (100 µl) of the obtained suspensions and of tenfold serial dilutions were plated in duplicate, using a spreader, onto King's B medium (20 g l^{-1} proteose peptone, 10 ml l^{-1} glycerol, 1.5 g l^{-1} K_2HPO_4, 1.5 g l^{-1} $MgSO_4 \cdot 7\ H_2O$, 15 g l^{-1} agar, pH 7.2) (King, Ward, & Raney, 1954) plates. Colonies were counted (CFU counting method) after 48 h incubation at 25°C, using UV-light.

Molecular identity of 15 colonies from each of the four weekly samplings, for a total of 60 colonies from treated plants and 60 colonies from untreated plants, was assessed by a strain-specific EvaGreen® real-time PCR method, the development of which will be described in a separate paper (Martini & Moruzzi, unpublished). Bacterial suspensions were prepared with 100 µl of sterile PCR water and bacteria scraped from the agar surface with a sterile plastic loop, successively boiled for 10 min at 99°C. 1 µl of boiled bacterial suspensions was used as a template in 20 µl-PCR reactions including 0.3 µM each primer Pfluor4GyrBF3 and Pfluor4GyrBR2, 1X Sso Fast EvaGreen SuperMix (Bio-Rad Inc., Hercules, CA, USA), and sterile H_2O. Diluted total genomic DNA (2 ng μl^{-1}) of Pf4 was used as positive control in real-time PCRs. Cycling conditions in a 96-well Bio-Rad CFX96 RealTime PCR System (Bio-Rad Inc., Hercules, CA, USA) were as follows: initial denaturation at 98 °C for 2 min; 45 cycles of 5 s at 98 °C; 5 s at 64 °C. A low resolution melting curve (ramp from 65°C to 95°C with 0.5°C increments and holding times of 5 s) was programmed at the end of the cycling reaction.

2.6.* In vitro *screening for genes associated with antibiotic production in* Pseudomonas *sp. Pf4

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Bacterial strain Pf4 was examined by PCR for the presence of genes involved in antibiotic production using gene-specific primers. Table 1 lists the target genes and PCR primer sets used for the detection of genes encoding the selected antibiotics: 2,4-diacetylphloroglucinol (2,4-DAPG), phenazine-1-carboxylic acid, pyrrolnitrin, pyoluteorin, hydrogen cyanide. All primers sets were used in PCR mixtures with a total volume of 25 µl containing dNTPs 200 µM each, $MgCl_2$ 1.5 mM, each primer 0.4 µM, 0.625U GoTaq Flexi (Promega, Madison, WI, USA). The PCR cycling conditions were: initial denaturation for 2 min at 94°C; 34 cycles of 1 min at 94°C, 40 s at 68°C (or 62/64°C) (Table 1), 1 min at 72°C; and a final extension for 8 min at 72°C. PCR products were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and captured with a DigiDoc-It imaging system (UVP, Cambridge, United Kingdom).

2.7. Library preparation, draft genome sequencing, assembly and annotation.

Genomic DNA was prepared for sequencing by the Nextera DNA sample preparation kit (Illumina), according to the manufacturer's instructions. Sequencing was performed on an Illumina MiSeq platform using indexed paired-end 300-nucleotide v2 chemistry at the Istituto di Genomica Applicata (Udine, Italy). Paired reads were assembled into contigs using the A5-miseq pipeline (Tritt, Eisen, Facciotti, & Darling, 2012).

Automated annotation of *Pseudomonas* sp. Pf4 draft genome sequence was performed using the RAST server (Aziz et al., 2008) and the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Orthologs inference and comparison with *P. protegens* Pf-5 was achieved with the standalone OMA program (http://omabrowser.org/standalone/).

Secondary metabolite production clusters were examined using the antiSMASH program (Medema et al., 2011). Sequence (BLAST) analysis of gene clusters for the synthesis of antibiotics, exoenzyme, cyclic lipopeptide, siderophores, toxin, and of Gac/Rsm homologues in *Pseudomonas* sp. Pf4 was conducted and similarities to those in *P. protegens* and other closely related

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Pseudomonas spp. strains was recorded (Loper et al., 2012; Takeuchi et al., 2015; Flury et al., 2016; Garrido-Sanz et al., 2016).

Contig 8 sequence of *Pseudomonas* sp. Pf4 containing the *fit-rzx* cluster was scanned for regions of genomic islands, putative signatures of HGT, using the IslandViewer3 website (Dhillon et al., 2015) with the algorithms IslandPick (Langille, Hsiao, & Brinkman, 2008), SIGI-HMM (Waack et al., 2006) and IslandPath-DIMOB (Hsiao, Wan, Jones, & Brinkman, 2003).

2.8. Phylogenetic analysis based on MLSA

For the MLSA-based phylogenies a total of 28 *Pseudomonas* strains of *P. chlororaphis* (including *P. protegens*- and *P. saponiphila*-related strains) and *P. corrugata* subgroups in the *P. fluorescens* group according to Mulet, Lalucat, and García-Valdés (2010) and Mulet et al. (2012) were analysed, comprising Pf4, 10 type strains (Gomila, Peña, Mulet, Lalucat, & García-Valdés, 2015) and 17 *Pseudomonas* strains whose complete or draft genome are available in the databases. The sequences of *gyrB*, *rpoD* and *rpoB* housekeeping genes along with the 16S rDNA gene sequence were retrieved from the genomic annotation, if available, and by performing BLASTN on the genomic sequence if otherwise. Genes for the type strains were retrieved from the PseudoMLSA database (http://www.uib.es/microbiologiaBD/Welcome.php).

The sequences of four genes were cut and concatenated as described by Mulet et al. (2010), and successively aligned with CLUSTAL W from the Molecular Evolutionary Genetics Analysis program-MEGA7 (Kumar, Stecher, & Tamura, 2016). The maximum parsimony (MP) tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm, implemented in the MEGA7, with search level 3 in which the initial trees were obtained by the random addition of sequences (10 replicates). *P. syringae* ATCC19310 type strain was used as an outgroup taxon to root the tree. Bootstrapping (500 replicates) was performed to estimate the stability and support for the inferred clades.

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3. Results

3.1. Isolations and preliminary screenings

Bacterial colonies isolated from thirty lamb's lettuce root samples were used in preliminary dual culture tests with two *P. aphanidermatum* strains (CBS 118745 and 116664). Among the 51 bacterial strains tested, 12 strains showed growth limiting activity, as summarized in Table 2. After 4 days of incubation, three of the 12 bacteria showed an inhibition zone of more than 10 mm, while four showed an inhibition zone ranging from 1 to 10 mm. The remaining five bacteria showed a reduced inhibition zone, although no physical contact was observed between the bacterial and the oomycete growth.

The identification of the 12 bacterial strains was preliminary carried out by sequence analysis using BLASTN of PCR amplified ribosomal DNAs, that resulted about 1303-1409 bp in length (accession numbers listed in Table 2). According to the sequence analysis, three bacterial strains (En8, En10, En12) with 16S rDNA gene sequence similarities of 99.2-99.3% among them belonged to *Enterobacter* spp., showing sequence identities of about 99% with three different *Enterobacter* sp. strain sequences deposited in GenBank, while the other nine strains belonged to *Pseudomonas fluorescens* group. Specifically, six strains (Pf1, Pf2, Pf3, Pf4, Pf5, Pf11) were closely related to *P. protegens* showing a 99-100% sequence similarity with strain CHA0^T (=DSM 19095^T) (AJ278812), two strains (Pf6 and Pf7) to *P. fluorescens* with 99% similarity with strain ATCC 13525^T (AF094725) and one strain (Pf9) to *P. poae* with 99% similarity with strain DSM 14936^T (AJ492829).

3.2. In vitro *antagonistic activity*

The results of *in vitro* antagonism tests of each of the 12 bacterial strains towards the plant pathogens *P. aphanidermatum* and *R. solani* are shown in Figures 1A and 1B respectively. Since *P. aphanidermatum* strains CBS 118745 and CBS 116664, and the *R. solani* strains TR15 and TP20 showed a nearly identical behaviour, combined data for each species are shown. The data from all

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replicates of the two experiments were also combined (Figure 1). Examples of the recorded bacterial antagonisms are given in Figure 2. All bacterial strains demonstrated the ability to inhibit the growth of both fungal pathogens, at least in the first 2-3 days of incubation, however bacterial strain Pf4 exhibited the highest inhibitory activity against both pathogens *P. aphanidermatum* and *R. solani* with 91.78% and 83.70% inhibition, after 2 and 3 days of incubation respectively. After 9 days of incubation, its inhibitory activity was still very high showing 88.89% and 66.17% of inhibition against *P. aphanidermatum* and *R. solani*, respectively (Figure 1). Interestingly, *P. aphanidermatum* could not be recovered from plates where it was incubated together with Pf4, suggesting that Pf4 had a fungicidal activity against it.

In addition to Pf4, *P. aphanidermatum* was strongly inhibited also by bacterial strain Pf9 (*P. poae*) and En8 (*Enterobacter* sp.) that showed 56.39% and 51.81% inhibition of growth after 9 days, respectively, and moderately inhibited by Pf2 (*P. protegens*) with 43.47% inhibition. In presence of the other strains, *P. aphanidermatum* was only slightly inhibited (between 14.68% and 30.56%).

Furthermore, *R. solani* was strongly inhibited also by bacterial strains Pf6 (*P. fluorescens*) and Pf7 (*P. fluorescens*), that showed respectively 65.42% and 64.89% inhibition of growth after 9 days; these bacteria were effective as Pf4 at the end of the assay, but less effective than it after 2, 3 and 7 days of incubation. *R. solani* was moderately inhibited by En8 and Pf9 (with 43.09% and 42.35% inhibition, respectively), and slightly or not inhibited (between 0% and 14.81%) in presence of the other strains.

3.3. In vivo *activity of* Pseudomonas *sp. strain Pf4 against* Rhizoctonia solani

Pf4-treated and untreated lamb's lettuce plants were artificially infected with the fungal pathogen *R. solani* in order to test the protective effect of Pf4. In both groups of plants the first symptoms of disease appeared at 6 days after fungal infection (dpi) and developed very fast, especially on untreated plants (Figure 3). In fact, on untreated plants there was a sudden rise at 7 dpi, and then the number of symptomatic plants increased constantly; on Pf4-treated plants, there was a sudden rise

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at 8-9 dpi, and a slow progression of the disease until 14 dpi. After 14 days, no new infections were observed, neither on untreated or treated plants. In any case, plants infected by *R. solani* showed a sudden shrivelling of leaves, and withered completely in 1-2 days; roots and crown became yellowish-brown and rotted.

Figure 4 with data of disease incidence from the two trials (four replicates each), shows the effects of Pf4 inoculation on lamb's lettuce plants infected with *R. solani* at 14 dpi, when the maximum number of wilted plants was reported. Untreated plants showed a very high disease incidence in both trials with an average disease incidence equal to 91.10 ± 7,59% (mean of four replicates ± SD) in trial I and 89.23 ± 15.05% in trial II; whereas plants treated with Pf4 showed a much lower disease incidence, even though the protection effect in the two trials showed some difference. Namely, Pf4-treated plants exhibited a very high protection against *R. solani* in the first trial with an average disease incidence equal to 25.17 ± 5.78% and a lower degree of protection in the second trial with an average disease incidence of 55.60 ± 6.97%. Nevertheless, statistical analysis showed that Pf4 displayed an extremely significant (P value is 0.0006, Welch's approximate t = 9.757 with 4 degrees of freedom) and significant (p value is 0.0313, Welch's approximate t = 3.832 with 3 degrees of freedom) biocontrol activity in trial I and II respectively, against the unprotected control with pathogen alone.

3.3.1. Survival and population density of Pseudomonas *sp. strain Pf4 on lamb's lettuce roots in hydroponics*

The survival and population density of Pf4 on the rhizosphere of lamb's lettuce plants growing in small scale floating systems, as determined by CFU counting method, is reported in Figure 5. Lines A and C show the overall CFU counts on King's B agar of fluorescent pseudomonads on the roots of Pf4-treated and untreated plants, respectively.

On treated plants, CFU counts ranged from 2 x 10^5 to 1.5 x 10^7, and on untreated plants from 0 to 1 x 10^5. Data obtained from colony counting were then adjusted on the basis of the results of

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molecular analysis (Figure 5; lines B and D) carried out on randomly sampled fluorescent colonies. In each sample taken from treated roots, 80% to 100% of the colonies gave a positive reaction (Figure 5, line B) with specific primers Pfluor4gyrB F3/R2, displaying a Ct range between 9 and 17 and a unique melting peak at 86.0°C; whilst in samples collected from untreated roots none of the fluorescent colonies gave a positive reaction (Figure 5, line D). CFU counts of Pf4, over a time span longer than the average growing cycle of lamb's lettuce in hydroponics, ranged between 1.60 x 10^5 and 1.29 x 10^7 CFU g^{-1} of root tissue. In particular, Pf4 went across a quick increase in the first week after its inoculation in the tanks, rising the initial concentration of 5.00 x 10^5 to a maximum of 1.29 x 10^7 CFU g^{-1} of root tissue; then Pf4 slowly decreased in the following weeks reaching the minimum concentration of 1.60 x 10^5 CFU g^{-1} of root tissue after four weeks.

3.4. In vitro *screening for genes associated with antibiotic production in* Pseudomonas *strain Pf4*

PCR primers sets for conserved sequences of genes involved in the biosynthesis of five antibiotics were targeted against Pf4 strain. Of the five genes investigated, those involved in the synthesis of 2,4 DAPG (*phlD*), pyrrolnitrin (in both loci *prnD* and *prnC*), pyoluteorin (in both loci *pltC* and *pltB*) and in cyanide production (in both loci *hcnBC* and *hcnAB*) were detected in *Pseudomonas* sp. Pf4, although in locus *hcnAB* a faint PCR signal was obtained even with less stringent PCR conditions. Whereas, gene sequence for phenazine-1-carboxylic acid wasn't detected in Pf4. In all cases where a positive signal was obtained, the PCR products were of the expected size.

3.5. Genome-wide sequence data

We conducted draft-genome sequencing to obtain information on strain Pf4. The Illumina sequencing provided 1,149,353,940 nts of 300 nts reads that passed the quality check. Sequencing of the Pf4 library provided 3,828,938 reads which were assembled into 36 contigs (N50 = 688,889;

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largest contig: 1,018,138) for a total of 6,832,152 nts (a coverage of 100.9X). The G+C content was 62.5%, which is similar to that of other sequenced *Pseudomonas* sp. genomes.

Automated annotation of the *Pseudomonas* sp. Pf4 draft genome sequence using the NCBI pipeline assigned a total of 5,907 candidate protein coding-genes, with 1,324 (22.41%) annotated as hypothetical proteins. The assembly predicted a total of 62 tRNA and 11 (6 5S, 3 16S, 2 23S) rRNA sequences. The draft genome sequence of *Pseudomonas* sp. Pf4 has been deposited in the DDBJ/EMBL/GenBank database under the accession no. LUUD00000000 The BioProject designation for this project is PRJNA315258 and the BioSample accession no. is SAMN04554942.

Four gene clusters (*hcn*, *plt*, *prn*, and *phl*) encoding the enzymes for the synthesis of the typical antibiotics of *P. protegens* were found in the genomic sequence of strain Pf4 (Tables 3 and S1), which supported the results obtained by PCR analyses for all four antibiotic biosynthetic genes described above. The *hcn* and *phl* gene clusters showed high homology (91-99% and 92-99% respectively) with those of *P. protegens* strains (CHA0^{T}, Pf-5 and Cab57) (Gross & Loper, 2009; Takeuchi, Noda, & Someya, 2014) and closely related *Pseudomonas* sp. Os17 and St29 (Takeuchi et al., 2015). The *plt* gene cluster showed very high homology (98-100%) only with that of *P. protegens* strains; and the *prn* gene cluster showed high homology (92-98%) with those of *P. protegens* strains and *P. chlororaphis* strains (Table S1).

Other typical gene clusters encoding factors associated to biocontrol found in the Pf4 genome and highly similar to their homologs in *P. protegens* and/or *Pseudomonas* sp. Os17 and St29 (Tables 3 and S1) include the *aprA* gene cluster (for the major extracellular protease AprA); the genes associated with the Gac/Rsm signal transduction pathway; the gene clusters for pyoverdine, found in the Pf4 genome at four different loci (Gene ID 17855-17860, 29340-29435, 04660-04610, and 04555-04545) as reported in Pf-5 (Gross & Loper, 2009) and Cab57 (Takeuchi et al., 2014); and the genes associated with the synthesis of other siderophores (i.e. enantio-pyochelin, hemophore biosynthesis and ferric-enterobactin receptor) (Tables 3 and S1).

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Among more uncommon genes encoded in the Pf4 genome we found the gene cluster for orfamides (82-85% similar to that of *P. protegens*), and the complete *rzx* gene cluster (approximately 79 kb, with the highest homology 98-99% to that of Pf-5) encoding analogs of the antimitotic macrolide rhizoxin in *P. protegens* Pf-5 (Loper, Henkels, Shaffer, Valeriote, & Gross, 2008), just upstream the *fit* cluster (with the highest homology 89-97% to that of *P. protegens* strains) (Figure 6, Table S1) encoding a functional insect toxin reported in *P. protegens* Pf-5 (Péchy-Tarr et al., 2008).

The homology search of the gene cluster over the entire genome suggested that the known pathways for the synthesis of phenazine may not be present in the Pf4 strain, confirming PCR results described above.

3.6. Phylogenetic analysis based on MLSA

A phylogenetic tree (Figure 7) was generated based on the concatenated sequences with a total length of 3712 nucleotides in the following order: 16S rRNA (1288 nt), gyrB (798 nt), rpoD (711 nt), and rpoB (915 nt).

In the phylogenetic tree, three well-supported clades can be distinguished, two of them including *P. protegens*-/*P. saponiphila*-related strains (*P. protegens* clade) and *P. chlororaphis*-related strains (*P. chlororaphis* clade) respectively, both belonging to *P. chlororaphis* subgroup according to Mulet et al. (2010; 2012), and the third clade (*P. corrugata* clade) corresponding to *P. corrugata* subgroup (Mulet et al., 2010; 2012).

Phl^+ Plt^+ *Pseudomonas* strain Pf4 represents a separate branch in the well-supported *P. protegens* clade, which includes Phl^+ Plt^+ *Pseudomonas* strains closely related to *P. protegens* species (Ramette et al., 2011) (Figure 7, Table 3) and Phl^+ Plt^- *Pseudomonas* strains closely related to *P. saponiphila* (Takeuchi et al., 2015; Wu et al., 2016).

In the MLSA of these four genes, sequence similarity of Pf4 was 97.28% with *P. protegens* $CHA0^T$ and 96.8% with *P. saponiphila* DSM 9751^T, demonstrating that Pf4 is a member of *P. chlororaphis* subgroup, most closely related to *P. protegens* strains.

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4. Discussion

A pool of bacterial microorganisms was isolated from roots of healthy lamb's lettuce plants growing in floating system in a farm in which a *R. solani* root rot outbreak occurred in 2009, with the aim to select microorganisms well adapted to soilless environment and synchronized with the pathogen in time and space (Postma, 2010). Molecular identification based on 16S rRNA gene sequences revealed that nine of the 12 selected bacteria belonged to genus *Pseudomonas* (six strains most closely related to *P. protegens*, two to *P. fluorescens* and one to *P. poae*), and three to *Enterobacter*. Bacteria from these genera are common inhabitants of rhizosphere, both in soil and in soilless system, and are well known as biocontrol agents against diseases caused by soil-borne fungal pathogens (Couillerot et al., 2009; Haas & Défago, 2005; Pliego, Ramos, de Vicente, & Cazorla, 2011).

Pf4, the isolate showing the strongest antagonistic *in vitro* activity was further characterized. It was able to clearly inhibit the growth of both pathogens *Pythium aphanidermatum* and *Rhizoctonia solani in vitro;* it was then shown in *in vivo* tests with pre-treatment of lamb's lettuce plants growing in hydroponics to reduce significantly *R. solani* disease incidence, despite some inconsistency in the degree of the suppressive activity in the two trials. Whether the variability in the efficacy could be ascribed to the growing system (soilless) or due to factors not associated to the growing system, such as poor host colonization by the biocontrol agent or variable expression of genes involved in disease suppression, as reported for experiments carried out in soil (Raaijmakers et al., 2002) could not be ascertained and deserves further investigations.

During *in vivo* test (trial I), the persistence and concentration of Pf4 on the rhizosphere were monitored by a conventional culturing method and molecular analysis, that demonstrated that the totality or majority of the fluorescent pseudomonads from treated roots corresponded to Pf4, while in the case of untreated ones none of the fluorescent pseudomonads resembled Pf4. Hence, Pf4 was capable of surviving at high level of population in the rhizosphere for a period of 4 weeks starting

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18 days after seeding, therefore exceeding the entire lamb's lettuce growing cycle in floating system. The population dynamics were consistent with those reported in literature for soil (Haas & Défago, 2005), i. e. artificially inoculated biocontrol agent initially colonize roots at 10^7-10^8 CFU g^{-1}, then decline within few weeks. The lowest colonization level shown by Pf4 was 1.60 x 10^5 CFU g^{-1} of lamb's lettuce root, corresponding to the threshold population density (10^5 - 10^6 CFU g^{-1} of root) that must be reached by *Pseudomonas* spp. strains for effective disease suppression in soil (Haas & Défago, 2005).

Since the fluorescent pseudomonads population level of untreated plants was quite similar at the end of the monitoring period, we could confirm previous works (Vallance et al., 2010) indicating that also in soilless cultures a bacterial population could naturally and quickly develop without artificial inoculation, even though starting with a "microbiological vacuum" (Postma, 2010).

In order to shed light on the mechanisms underlying the biocontrol properties of *Pseudomonas* sp. Pf4, PCRs having as target genes encoding antibiotic synthesis and draft genome sequencing were undertaken. Indeed, both methods showed the presence in Pf4 of genes involved in the biosynthesis of typical *P. protegens* secondary metabolites, such as genes clusters *hcn*, *plt*, *prn*, and *phl*, involved in the production of hydrogen cyanide, pyoluteorin, pyrrolnitrin and 2,4-DAPG, respectively. The biosynthesis of pyoluteorin was claimed (Garrido-Sanz et al., 2016) to be specific of *P. protegens* within the *P. fluorescens* group; however the results of this study and of that of Flury et al. (2016) demonstrated that also other *Pseudomonas* spp. strains (i.e. Pf4, PH1b, CMR5c and CMAA1215, Table 3 and Fig. 7) in the *P. chlororaphis* subgroup harbour *plt* gene cluster.

In addition to the above, also other gene clusters coding for extracellular enzymes as *apr* gene cluster and siderophores as *pch*, *has* and *pfe* gene clusters, besides Gac/Rsm homologues and small regulatory RNAs, showed high homology with *P. protegens* strains, as well as with *Pseudomonas* sp. Os17 and St29, supporting the notion of a close relatedness of Pf4 to both groups of fluorescent pseudomonads. Interestingly, Pf4 also has the biosynthetic potential for metabolites that are less universally spread among the fluorescent pseudomonads; in particular, with our genomic drafting

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we discovered in Pf4 the gene clusters for the cyclic lipopeptide orfamide A, for the insect toxin FitD and for rhizoxin analogs, recently identified natural products discovered through genomics-guided approaches. Orfamide A, a biosurfactant influencing swarming motility of Pf-5, was shown to function as an antifungal agent, to lyse oomycete zoospores, and to act as an insecticidal agent (Gross & Loper, 2009; Ma et al., 2016). The gene cluster for orfamides, which has been identified in strain Pf-5 mining *Pseudomonas* genomes (Gross et al., 2007) was also found in the genomes of other *P. protegens* strains, CHA0^T and Cab57 (Takeuchi et al., 2014), and of *P. protegens*-related strains (i.e. *Pseudomonas* spp. CMR5c, CMR12a, CMAA1215, PH1b) (Ma et al., 2016). The Fit insect toxin cluster was first identified in *P. protegens* Pf-5, in which the production of this toxin has been associated with the lethality of this strain for the tobacco hornworm *Manduca sexta* (Péchy-Tarr et al., 2008). The complete gene cluster has also been identified in *P. protegens* CHA0^T and several other *P. protegens* strains, in closely related *Pseudomonas* spp. Os17, St29 and CMR5c, in *P. chlororaphis* strains O6, 30–84 and many others, suggesting that Fit toxin is consistently and exclusively shared by strains belonging to the *P. chlororaphis* subgroup [corresponding to sub-clade 1 after Loper et al. (2012)] (Loper et al., 2012; Péchy-Tarr et al., 2013; Takeuchi et al., 2015; Garrido-Sanz et al., 2016; Flury et al., 2016).

Rhizoxins are 16-membered polyketide macrolides that exhibit significant phytotoxic, antifungal and antitumoral properties by binding to b-tubulin, thereby interfering with microtubule dynamics during mitosis. The complete *rxz* cluster has been initially reported in *P. protegens* Pf-5 (Loper et al., 2008). This cluster has been found to be absent from two other fully sequenced *P. protegens* strains, CHA0^T and Cab57 (Takeuchi et al., 2014), but present in *P. protegens* PF and closely related *Pseudomonas* sp. Os17 (Takeuchi et al., 2015; Loper et al., 2016) in the *P. fluorescens* group.

In Pf4 the rhizoxin biosynthesis gene cluster is adjacent to the gene cluster encoding for the production of the FitD insect toxin. To date only few other closely related *Pseudomonas* spp. strains, *P. protegens* strains Pf-5 and PF and the related strain *Pseudomonas* sp. Os17, are known to

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have the Fit and rhizoxin gene clusters linked (i.e. the *fit-rzx* cluster) in their genomes. As in *P. protegens* Pf-5 and *Pseudomonas* sp. Os17, the genomic region with the *fit-rzx* gene clusters of Pf4 did not showed the characteristics of a genomic island, although Loper et al. (2016) suggested that the *fit-rzx* clusters of Pf-5 and closely related strains have a complex evolutionary history that includes HGT. Loper et al. (2016) demonstrated that the *fit-rxz* cluster confers oral and injectable toxicity to a broader set of insects than either the *fit* or *rzx* clusters alone, therefore Pf4 represents a potential bacteria that may exhibit oral toxicity towards agriculturally relevant insect pests as Pf-5. Testing *in vivo* insecticidal activity would be an interesting address for future research on Pf4.

Draft genome of Pf4 allowed also to obtain the sequence of the housekeeping *rpoD*, *gyrB* and *rpoB* genes, which represent the three genes besides the 16S rRNA gene used in the multilocus sequence analysis (MLSA) developed by Mulet et al. (2010) and proved to be a useful tool for *Pseudomonas* spp. identification at the species level (Gomila et al., 2015). MLSA is a major contribution to accurate identification, needed since a large number of strains with disease suppression potential are reported as *P. fluorescens*, but only some of them are presently retained within this species (Bossis, Lemanceau, Latour, & Gardan, 2000; Mulet et al., 2010). Mulet et al. (2010) established a similarity of 97.0% in the MLSA of these four genes as the threshold value for strains in the same species in the genus *Pseudomonas*. The sequence similarity obtained between Pf4 and *P. protegens* CHA0^T or *P. saponiphila* DSM 9751^T (97.28 and 96.80% respectively) and the phylogenetic analysis indicated that Pf4 potentially belong to a novel *Pseudomonas* species, as it forms a clearly distinct lineage within the *P. protegens* clade (Figure 7) in the *P. chlororaphis* subgroup defined according to Mulet et al. (2010; 2012).

5. Conclusions

Pf4 displayed the ability to inhibit the growth of *R. solani* and *P. aphanidermatum in vitro*, and the capacity to suppress root rot caused by *R. solani in vivo*, on lamb's lettuce plants grown in hydroponics. Despite the fact that it was isolated from the roots of plants in hydroponic culture, Pf4

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was not only at the taxonomic level, but also at the genomic level, rather similar to other strains of *Pseudomonas* spp. that have been isolated from soil and shown to be active biocontrol agent in soil. In particular, it could be inferred from the drafted genome sequence that Pf4 has the potential to produce an arsenal of secondary metabolites very similar to that of the well-known biocontrol *P. protegens* strain Pf-5. Actually, Pf4 is the only not-*P. protegens* strain among those analysed of closely related *Pseudomonas* spp., which is more like Pf-5 in the type of secondary metabolites produced. Moreover, Pf4 can colonize lamb's lettuce roots for the entire growth cycle of this crop in floating system at a density of 10^5-10^7 CFU g^{-1} of root, therefore above the threshold required for suppression of root diseases in soil. This work support the notion that key factors conferring the ability to suppress root diseases in soil are also of paramount relevance in hydroponics. After the recent discovery that certain pseudomonads cannot only suppress fungal plant diseases but also have the potential to control insect pests, the results of this work further widen the application targets of the so called *P. chlororaphis* subgroup, adding value to their use as biocontrol agents and opening up new industrial opportunities toward the development of unique biopesticides for biological control of plant diseases and pests using the same product in different growth environments.

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Disclosure statement

No potential conflict of interest was reported by the authors

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Table S1. Sequence analysis of gene clusters for the synthesis of antibiotics, exoenzyme, cyclic lipopeptide, siderophores, and toxin, and of Gac/Rsm homologues in *Pseudomonas* sp. Pf4 and similarities to those in *P. protegens* strains (CHA0^T, Pf-5, Cab57) and in other most closely related *Pseudomonas* sp. strains (Os17, St29). Similarity to *P. chlororaphis* strains was also verified in the case of *prn* and *fit* gene clusters.

Tables

Table 1. Target genes encoding enzymes involved in the biosynthesis of several antibiotics and primer sets used for their amplification in *Pseudomonas* sp. Pf4 strain from this study.

Target gene (antibiotic)	Primer	Sequence (5'-3')	Annealing T°	Expected size of PCR product	Reference
phlD (2,4-DAPG)	Phl2a Phl2b	GAGGACGTCGAAGACCACCA ACCGCAGCATCGTGTATGAG	62°C	745	Raaijmakers, Weller, & Thomashow, 1997
phzCD (phenazine-1-carboxylic acid)	PCA2a PCA3b	TTGCCAAGCCTCGCTCCAAC CCGCGTTGTTCCTCGTTCAT	68°C	1150	Raaijmakers *et al.*, 1997
prnD (pyrrolnitrin)	PRND1 PRND2	GGGGCGGGCCGTGGTGATGGA YCCCGCSGCCTGYCTGGTCTG	68°C	786	de Souza & Raaijmakers, 2003
prnC (pyrrolnitrin)	PrnCf PrnCr	CCACAAGCCCGGCCAGGAGC GAGAAGAGCGGGTCGATGAAGCC	64°C	720	Mavrodi *et al.*, 2001
pltC (pyoluteorin)	PLTC1 PLTC2	AACAGATCGCCCCGGTACAGAACG AGGCCCGGACACTCAAGAAACTCG	68°C	438	de Souza & Raaijmakers, 2003
pltB (pyoluteorin)	PltBf PltBr	CGGAGCATGGACCCCCAGC GTGCCCGATATTGGTCTTGACC	68°C	791	Mavrodi *et al.*, 2001
hcnBC (hydrogen cyanide)	Aca Acb	ACTGCCAGGGGCGGATGTGC ACGATGTGCTCGGCGTAC	62°C	587	Ramette, Frapolli, Défago, & Moënne-Loccoz, 2003
hcnAB (hydrogen cyanide)	PM2 PM7-26R	TGCGGCATGGGCGTGTGCCATTGCTG CCTGG CCGCTCTTGATCTGCAATTGCAGGCC	68°C	570	Svercel, Duffy, Défago, 2007

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Table 2. Preliminary data of antagonistic activity against *P. aphanidermatum* after 4 days of incubation and molecular identification based on BLASTn analysis of 16S rRNA gene sequences with corresponding GenBank accession numbers of 12 selected bacterial strains. Abbreviation: Pf, bacteria belonging to *P. fluorescens* group; En, bacteria belonging to *Enterobacter* spp.

Bacterial strain ID	Antagonistic activity*	Accession No.	GenBank closest relative (accession no.)	% similarity
Pf1	++	KM589020	*Pseudomonas protegens* CHA0^T (AJ278812)	99%
Pf2	+++	KM589021	*Pseudomonas protegens* CHA0^T (AJ278812)	100%
Pf3	+	KM589022	*Pseudomonas protegens* CHA0^T (AJ278812)	99%
Pf4	+++	KM589023	*Pseudomonas protegens* CHA0^T (AJ278812)	100%
Pf5	+	KM589024	*Pseudomonas protegens* CHA0^T (AJ278812)	99%
Pf6	++	KM589027	*Pseudomonas fluorescens* ATCC[2] 13525^T (AF094725)	99%
Pf7	+	KM589028	*Pseudomonas fluorescens* ATCC[2] 13525^T (AF094725)	99%
En8	+++	KM589029	*Enterobacter* sp. TM 1.3 (DQ279307)	99%
Pf9	++	KM589026	*Pseudomonas poae* DSM[1] 14936^T (AJ492829)	99%
En10	+	KM589030	*Enterobacter* sp. 638 (CP000653)	99%
Pf11	++	KM589025	*Pseudomonas protegens* CHA0^T (AJ278812)	99%
En12	+	KM589031	*Enterobacter aerogenes* KNUC5012 (JQ682638)	99%

* +: <1 mm inhibition zone; ++: 1 to 10 mm inhibition zone; +++: >10 mm inhibition zone.
[1] DSM: Deutsche Sammlung von Mikroorganismen.
[2] ATCC: American Type culture Collection.

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Table 3. Overview on presence (+)/absence (-) of secondary metabolites biosynthetic gene clusters in *P. protegens* and closely related *Pseudomonas* spp. strains. Except Pf4 isolated in the present work from roots in hydroponics, all the other strains were isolated moslty from roots of plants grown in soil.

Species	Strain	Gene cluster											
		hcn[a]	*plt*[a]	*prn*[a]	*phl*[a]	*aprA*[a]	*pvd*[a]	*pch*[a]	*has*[a]	*pfe*[a]	*ofa*[a]	*fit*[a]	*rzx*[a]
P. protegens	CHA0[T]	+	+	+	+	+	+	+	+	+	+	+	-
	Cab57	+	+	+	+	+	+	+	+	+	+	+	-
	Wayne1	+	+	+	+	+	+	+	+	+	+	+	-
	Pf-5	+	+	+	+	+	+	+	+	+	+	+	+
	PF	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas spp.	Pf4	+	+	+	+	+	+	+	+	+	+	+	+
	Os17	+	-	-	+	+	+	+	+	+	-	+	+
	St29	+	-	-	+	+	+	+	+	+	-	+	-
	NZI7	+	-	-	+	+	+	+	+	+	-	-	-
	PH1b	+	+	-	-	+	+	+	+	+	+	+	-
	CMR5c	+	+	+	+	+	+	+	+	+	+	+	-
	CMAA1215	-	+	-	+	+	+	+	+	+	+	+	-

[a]*hcn*, for hydrogen cyanide; *plt*, for pyoluteorin; *prn*, for pyrrolnitrin; *phl*, for 2,4-diacetylphloroglucinol; *aprA*, for major extracellular protease AprA; *pvd*, for pyoverdine; *pch*, for enantio-pyochelin; *has*, for hemophore biosynthesis; *pfe*, for ferric-enterobactin receptor; *ofa*, for orfamide; *fit*, for FitD toxin; *rzx*, for rhizoxin.

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Figure legends

Figure 1. Antagonistic activity (% inhibition of fungal growth, y axis) of 12 potential antagonistic bacterial strains (x axis) against *P. aphanidermatum* CBS 118745 and CBS 116664 (A), and *R. solani* TR15 and TP20 (B), under *in vitro* conditions after 2 or 3 days of incubation respectively, and at the end of the experiments (9 days of incubation). Error bars indicate standard deviations.

Figure 2. (A-L) Growth of *P. aphanidermatum* cultures at 1, 2 and 9 days of incubation with different bacterial antagonists: A-C, Pf4 (strain with maximum antagonistic activity); D-F, Pf5 (strain with minimum antagonistic activity); G-I, En8 (strain with strong antagonistic activity); J-L, pure culture of *P. aphanidermatum*. Control colony reached the maximum diameter in 2 days (K); at that time even the less efficient strains showed a quite high inhibition activity, ranging between 32.41% and 68.13% (E). No physical contact was observed for the entire duration of the assay between all the bacteria tested, including those showing low inhibition activity (F), and the mycelium of *P. aphanidermatum*.

(M-X) Growth of *R. solani* cultures at 2, 3 and 9 days of incubation with different bacterial antagonists: M-O, Pf4; P-R, Pf5; S-U, En8; V-X, pure culture of *R. solani*. Control colony reached the maximum diameter in 3 days (W), and even the less efficient strains showed at that time a significant inhibition, ranging between 31.94% and 61.67% (Q). In some cases, a change in *R. solani* mycelium colour becoming darker brown (R), or a change in the shape of the colony edges becoming uneven and jagged (O), were observed.

Figure 3. Incidence (% of symptomatic plants per total number of plants observed) dynamics of root rot caused by *R. solani* on lamb's lettuce plants, Pf4-treated (Pf4+) or untreated (Pf4-), from 5 to 16 dpi.

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Figure 4. Data of disease incidence (% of symptomatic plants per total number of plants observed) of root rot caused by *R. solani* in the two trials at 14 dpi on Pf4-treated or untreated lamb's lettuce plants. Error bars indicate standard deviations.

Figure 5. Population density of Pf4 (log10 CFU g^{-1} of root tissue) on lamb's lettuce roots in hydroponics determined by CFU counting method. Lines A: CFU of fluorescent pseudomonads g^{-1} of treated roots; B: CFU of Pf4 g^{-1} of treated roots; C: CFU of fluorescent pseudomonads g^{-1} of untreated roots; D: CFU of Pf4 g^{-1} of untreated roots.

Figure 6. Genetic organization of the *fit* (for FitD toxin, in red) and *rzx* (for rhizoxin analogs, in blu) gene clusters in the genome of Pf4, obtained using SnapGene software (from GSL Biotech; available at snapgene.com).

Figure 7. MP phylogenetic tree of strains belonging to *P. chlororaphis* and *P. corrugata* subgroups based on four-gene (16S rRNA, *gyrB*, *rpoD* and *rpoB*) MLSA scheme of Mulet et al. (2010; 2012). Bootstrap values over 50% are indicated in the tree.

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Table S1.

Sequence analysis of gene clusters for the synthesis of antibiotics, exoenzyme, cyclic lipopeptide, siderophores, and toxin, and of Gac/Rsm homologues in *Pseudomonas* sp. Pf4 and similarities to those in *P. protegens* strains (CHA0^{T}, Pf-5, Cab57) and other most closely related *Pseudomonas* sp. strains (Os17, St29). Similarity to *P. chlororaphis* strains was also verified in the case of *prn* and *fit* gene clusters.

Gene ID (NCBI)	Gene name (ID for PFL)	Position (NCBI)	Size of product (amino acids)	% amino acid homology	*Pseudomonas* sp.
***hcn* gene cluster (for hydrogen cyanide)**					
A1348_23065	*hcnA* (2577)	6: 391003–391320 (+)	105	98 97	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_23070	*hcnB*	6: 391317–392726 (+)	469	95 91	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_23075	*hcnC* (2579)	6: 392719–393972 (+)	417	99 96	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
***plt* gene cluster (for pyoluteorin)**					
A1348_17270	*pltM* (2784)	4: 360091–361599 (–)	502	99	*P. protegens* strains
A1348_17275	*pltR*	4: 361596–362627 (–)	343	98	*P. protegens* strains
A1348_17280	*pltL*	4: 363114–363380 (+)	88	100	*P. protegens* strains
A1348_17285	*pltA*	4: 363394–364743 (+)	449	100	*P. protegens* strains
A1348_17290	*pltB*	4: 364776–372152 (+)	2458	98	*P. protegens* strains
A1348_17295	*pltC*	4: 372201–377525 (+)	1774	99	*P. protegens* strains
A1348_17300	*pltD*	4: 377576–379210 (+)	544	98-99	*P. protegens* strains
A1348_17305	*pltE*	4: 379212–380354 (+)	380	99	*P. protegens* strains
A1348_17310	*pltF*	4: 380351–381844 (+)	497	99	*P. protegens* strains
A1348_17315	*pltG*	4: 381848–382630 (+)	260	99	*P. protegens* strains
A1348_17320	*pltZ*	4: 382636–383307 (–)	223	99	*P. protegens* strains
A1348_17325	*pltI*	4: 383383–384396 (+)	337	99	*P. protegens* strains
A1348_17330	*pltJ*	4: 384393–386162 (+)	589	99	*P. protegens* strains
A1348_17335	*pltK*	4: 386172–387314 (+)	380	99	*P. protegens* strains
A1348_17340	*pltN*	4: 387331–388437 (+)	368	99	*P. protegens* strains
A1348_17345	*pltO*	4: 388449–389945 (+)	498	98-99	*P. protegens* strains
A1348_17350	*pltP* (2800)	4: 390011–390616 (+)	201	99	*P. protegens* strains
***prn* gene cluster (for pyrrolnitrin)**					
A1348_27080	*prnA* (3604)	8: 330759–332375 (–)	538	96 94-96	*P. protegens* strains *P. chlororaphis* strains
A1348_27075	*prnB*	8: 329674–330759 (–)	361	92-95 92	*P. chlororaphis* strains *P. protegens* strains
A1348_27070	*prnC*	8: 327929–329632 (–)	567	97-98 95-97	*P. protegens* strains *P. chlororaphis* strains
A1348_27065	*prnD* (3607)	8: 326813–327904 (–)	363	94-96 94	*P. chlororaphis* strains *P. protegens* strains
***phl* gene cluster (for 2,4-diacetylphloroglucinol)**					

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A1348_10485	*phlH* (5951)	2: 363678–364352 (–)	224	93-94 90	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_10490	*phlG*	2: 364495–365379 (+)	294	96 93	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_10495	*phlF*	2: 365432–366034 (–)	200	97	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_10500	*phlA*	2: 366497–367585 (+)	362	96 94-95	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_10505	*phlC*	2: 367615–368811 (+)	398	99	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_10510	*phlB*	2: 368824–369264 (+)	146	99 96-99	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_10515	*phlD*	2: 369473–370522 (+)	349	99 98	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_10520	*phlE* (5958)	2: 370633–371910 (+)	425	92	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
***apr* gene cluster**					
A1348_26990	*aprA* (3210)	8: 308831-310279 (–)	482	96 93	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_26985	*Inh* (PFL_3209)	8:308354..308737 (-)	128	84 96	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_26980	*aprD*	8: 306344–308137 (–)	597	95 94	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_26975	*aprE*	8: 305013–306347 (–)	444	97-98 96-97	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_26970	*aprF* (3206)	8: 303649–305010 (–)	453	98 94	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
Gac/Rsm homologues in *Pf4*					
A1348_03275	*gacS* (4451)	0: 690217–692970 (–)	917	97	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_25980	*gacA* (3563)	7: 486282–486866 (+)	194	100	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_03020	*rsmA* (4504)	0: 641626–641814 (+)	62	100	*Pseudomonas* spp.
A1348_09780	*rsmE* (2095)	2: 219078–219797 (+)	239	96 92	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_15270	*retS* (0664)	3: 607391–610177 (–)	928	97	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_28385	*ladS* (5426)	9: 172345–174711 (+)	788	93 91	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
small regulatory RNAs					
—	*rsmZ* (6285)	1: 506535–506661 (+)	127 nt	99 98	*P. protegens* strains *Pseudomonas* sp. Os17, St29
—	*rsmY* (6291)	2: 73788–73906 (+)	118 nt	100 99	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
—	*rsmX* (6289)	10:86797–86915 (+)	119 nt	98 97-98	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
***pvd* gene cluster (for pyoverdine)**					
A1348_17855	*pvdQ* (2902)	4: 506592–508925 (+)	777	91 85	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_17860	*fpvR* (2903)	4: 508978–509979 (–)	333	91 90	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_29340	*pvdA* (4079)	10: 26184–27521 (–)	445	88	*P. protegens* strains *Pseudomonas* sp. Os17, St29
A1348_29345	*fpvI*	10: 27719–28201 (–)	160	85 84	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_29350	RND efflux	10: 28524–29696 (+)	390	96	*Pseudomonas* sp. Os17, St29

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	Transporter (4081)				*P. protegens* strains
A1348_29355	ABC efflux Transporter (4082)	10: 29697–31670 (+)	657	97 91	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_29360	RND efflux Transporter (4083)	10: 31678–33069 (+)	463	95 76-77	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_29365	PFL_4084	10: 33186–33485 (+)	99	94 90 47-49	*Pseudomonas* sp. St29 *Pseudomonas* sp. Os17 *P. protegens* strains
A1348_29370	PFL_4085	10: 33514–33951 (+)	145	62-63	*P. protegens* strains
A1348_29375	*pvdP* (4086)	10: 34004–35632 (–)	542	95 59	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_29380	*pvdM*	10: 35806–37155 (+)	449	99 95 71-74	*Pseudomonas* sp. Os17 *Pseudomonas* sp. St29 *P. protegens* strains
A1348_29385	*pvdN*	10: 37188–38474 (+)	428	99 91 68-69	*Pseudomonas* sp. Os17 *Pseudomonas* sp. St29 *P. protegens* strains
A1348_29390	*pvdO*	10: 38522–39412 (+)	296	100 76 66	*Pseudomonas* sp. Os17 *Pseudomonas* sp. St29 *P. protegens* strains
A1348_29395	*pvdF*	10: 39445–40464 (+)	339	100	*Pseudomonas* sp. Os17
A1348_29400	*pvdE*	10: 40789–42444 (+)	551	100 79 74-75	*Pseudomonas* sp. Os17 *Pseudomonas* sp. St29 *P. protegens* strains
A1348_29405	*fpvA*	10: 42552–45035 (+)	827	100 42 39-41	*Pseudomonas* sp. Os17 *Pseudomonas* sp. St29 *P. protegens* strains
A1348_29410	*pvdD*	10: 45701–56242 (–)	3513	99 53-54 45	*Pseudomonas* sp. Os17 *P. protegens* strains *Pseudomonas* sp. St29
A1348_29415	*pvdJ* (4094)	10: 56263–59334 (–)	1023	99 37 35-36	*Pseudomonas* sp. Os17 *Pseudomonas* sp. St29 *P. protegens* strains
A1348_29425	*pvdI* (4095)	10: 60472–69768 (–)	3098	97 63 48	*Pseudomonas* sp. Os17 *Pseudomonas* sp. St29 *P. protegens* strains
A1348_29430	Siderophore-interacting protein (4096)	10: 69943–70911 (+)	322	91 85	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_29435	PFL_4097	10: 71090–71830 (–)	246	98 97 91	*Pseudomonas* sp. St29 *Pseudomonas* sp. Os17 *P. protegens* strains
A1348_04660	PFL_4169	0: 999307–1000530 (–)	407	99 93-94 90	*Pseudomonas* sp. Os17 *P. protegens* strains *Pseudomonas* sp. St29
A1348_04655	PFL_4170	0: 998771–999310 (–)	179	99 94-96 88	*Pseudomonas* sp. Os17 *P. protegens* strains *Pseudomonas* sp. St29
A1348_04650	PFL_4171	0: 998433–998771 (–)	112	97 93-95 94	*Pseudomonas* sp. Os17 *P. protegens* strains *Pseudomonas* sp. St29
A1348_04645	PFL_4172	0: 997864–998436 (–)	190	100 98 84-85	*Pseudomonas* sp. St29 *Pseudomonas* sp. Os17 *P. protegens* strains
A1348_04640	PFL_4173	0: 996899–997828 (–)	309	98 98	*P. protegens* strains *Pseudomonas* sp. St29

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				96	*Pseudomonas* sp. Os17
				98	*P. protegens* strains
A1348_04635	PFL_4174	0: 996159–996902 (–)	247	98	*Pseudomonas* sp. St29
				97	*Pseudomonas* sp. Os17
A1348_04630	PFL_4175	0: 995246–996145 (–)	299	99	*P. protegens* strains
				99	*Pseudomonas* sp. Os17, St29
A1348_04625	PFL_4176	0: 994262–995245 (–)	327	97	*Pseudomonas* sp. Os17, St29
				93	*P. protegens* strains
A1348_04620	PFL_4177	0: 993202–994029 (–)	275	94-95	*Pseudomonas* sp. Os17, St29
				88-90	*P. protegens* strains
A1348_04615	PFL_4178	0: 992415–992639 (+)	74	99	*P. protegens* strains
				99	*Pseudomonas* sp. Os17, St29
A1348_04610	*pvdH* (4179)	0: 990920–992332 (+)	470	97	*Pseudomonas* sp. Os17, St29
				95-96	*P. protegens* strains
A1348_04555	*pvdL* (4189)	0: 963956–976972 (+)	4338	97	*Pseudomonas* sp. Os17, St29
				95-96	*P. protegens* strains
A1348_04550	*pvdS*	0: 963033–963581 (–)	182	100	*P. protegens* strains
				99	*Pseudomonas* sp. Os17, St29
A1348_04545	*pvdY* (4191)	0: 962639–962992 (+)	117	70-71	*P. protegens* strains
				67	*Pseudomonas* sp. Os17, St29
***pch* cluster (for enantio-pyochelin)**					
A1348_15840	*pchR* (3497)	4: 49492–50394 (–)	300	97	*Pseudomonas* sp. Os17, St29
				95	*P. protegens* strains
A1348_15845	*pchD*	4: 50770–52437 (+)	555	90	*P. protegens* strains
				88	*Pseudomonas* sp. Os17, St29
A1348_15850	*pchH*	4: 52421–54175 (+)	584	90	*Pseudomonas* sp. Os17, St29
				89	*P. protegens* strains
A1348_15855	*pchI*	4: 54172–55935 (+)	587	87	*Pseudomonas* sp. Os17, St29
				86-87	*P. protegens* strains
				88	*Pseudomonas* sp. Os17
A1348_15860	*pchE*	4: 55928–59398 (+)	1156	88	*P. protegens* strains
				87	*Pseudomonas* sp. St29
A1348_15865	*pchF*	4: 59395–64815 (+)	1806	94	*P. protegens* strains
				93-94	*Pseudomonas* sp. Os17, St29
A1348_15870	*pchK*	4: 64827–65927 (+)	366	85-86	*P. protegens* strains
				84	*Pseudomonas* sp. Os17, St29
A1348_15875	*pchC*	4: 65924–66703 (+)	259	93-94	*Pseudomonas* sp. Os17, St29
				90	*P. protegens* strains
A1348_15880	*pchB*	4: 66727–67050 (+)	107	85	*Pseudomonas* sp. Os17, St29
				84	*P. protegens* strains
A1348_15885	*pchA* (3488)	4: 67043–68476 (+)	477	89	*P. protegens* strains
				86	*Pseudomonas* sp. Os17, St29
***has* gene cluster (for hemophore biosynthesis)**					
A1348_28615	*hasI* (5380)	9: 223960–224481 (+)	173	96-97	*P. protegens* strains
				95	*Pseudomonas* sp. Os17, St29
A1348_28620	*hasS*	9: 224545–225558 (+)	337	93	*P. protegens* strains
				87	*Pseudomonas* sp. Os17, St29
A1348_28625	*hasR*	9: 225690–228395 (+)	901	95-96	*P. protegens* strains
				95	*Pseudomonas* sp. Os17, St29
A1348_28630	*hasA*	9: 228479–229096 (+)	205	97	*P. protegens* strains
				92	*Pseudomonas* sp. Os17, St29
A1348_28635	*hasD*	9: 229315–231099 (+)	594	97-98	*P. protegens* strains
A1348_28640	*hasE*	9: 231096–232445 (+)	449	96	*P. protegens* strains
A1348_28645	*hasF* (5374)	9: 232442–233779 (+)	445	94-95	*P. protegens* strains
***pfe* gene cluster (for ferric-enterobactin receptor)**					
A1348_23430	*pfeR* (2665)	6: 473816–474508 (–)	230	93-94	*Pseudomonas* sp. Os17, St29
				92-93	*P. protegens* strains

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A1348_23425	*pfeS*	6: 472479–473816 (–)	445	96-97 94-95	*Pseudomonas* sp. Os17, St29 *P. protegens* strains
A1348_23420	*pfeA* (2663)	6: 470135–472375 (–)	746	95-97 96	*P. protegens* strains *Pseudomonas* sp. Os17, St29
***ofa* gene cluster (for orfamide A)**					
A1348_18430	*ofaA* (2145)	5: 35808–42188 (–)	2126	82	*P. protegens* strains
A1348_18425	*ofaB*	5: 22429–35544 (–)	4371	85	*P. protegens* strains
A1348_18420	*ofaC* (2147)	5: 7709–22432 (–)	4907	84	*P. protegens* strains
***fit* gene cluster (for FitD toxin)**					
A1348_26560	*fitA* (2980)	8: 199520–201661 (–)	713	96 93 88-91	*P. protegens* strains *Pseudomonas* sp. Os17, St29 *P. chlororaphis* strains
A1348_26555	*fitB*	8: 198135–199523 (–)	462	96-97 93 88-92	*P. protegens* strains *Pseudomonas* sp. Os17, St29 *P. chlororaphis* strains
A1348_26550	*fitC*	8: 195973–198132 (–)	719	97 88-92 90	*P. protegens* strains *P. chlororaphis* strains *Pseudomonas* sp. Os17, St29
A1348_26545	*fitD*	8: 186846–195857 (–)	3003	93-94 77-83 80	*P. protegens* strains *P. chlororaphis* strains *Pseudomonas* sp. Os17, St29
A1348_26540	*fitE*	8: 185262–186767 (–)	501	93-96 85-87 86	*P. protegens* strains *P. chlororaphis* strains *Pseudomonas* sp. Os17, St29
A1348_26535	*fitF*	8: 181945–185181 (–)	1078	89 77 67-75	*P. protegens* strains *Pseudomonas* sp. Os17, St29 *P. chlororaphis* strains
A1348_26530	*fitG*	8: 181031–181948 (+)	305	95-96 88 82-88	*P. protegens* strains *Pseudomonas* sp. Os17, St29 *P. chlororaphis* strains
A1348_26525	*fitH* (2987)	8: 180030–181010 (+)	326	90-91 75-81 80	*P. protegens* strains *P. chlororaphis* strains *Pseudomonas* sp. Os17, St29
***rzx* gene cluster (for rhizoxin)**					
A1348_26520	hypothetical protein PFL_2988	8: 179502–179906 (+)	134	98 84	*P. protegens* Pf-5 *Pseudomonas* sp. Os17
A1348_26515	*rzxB* (2989)	8: 158807–178849 (–)	6680	98 79	*P. protegens* Pf-5 *Pseudomonas* sp. Os17
A1348_26510	*rzxC*	8: 143811–158636 (–)	4941	98 81	*P. protegens* Pf-5 *Pseudomonas* sp. Os17
A1348_26505	*rzxD*	8: 131692–143814 (–)	4040	98 80	*P. protegens* Pf-5 *Pseudomonas* sp. Os17
A1348_26500	*rzxH*	8: 130286–131695 (–)	469	99 90	*P. protegens* Pf-5 *Pseudomonas* sp. Os17
A1348_26495	*rzxE*	8: 117720–130220 (–)	4166	98 80	*P. protegens* Pf-5 *Pseudomonas* sp. Os17
A1348_26490	*rzxF*	8: 110029–117654 (–)	2541	98 78	*P. protegens* Pf-5 *Pseudomonas* sp. Os17
A1348_26485	*rzxI*	8: 109125–109991 (+)	288	99 88	*P. protegens* Pf-5 *Pseudomonas* sp. Os17
A1348_26480	*rzxG*	8: 106937–108964 (–)	675	98 84	*P. protegens* Pf-5 *Pseudomonas* sp. Os17
A1348_26475	*rzxA* (2997)	8: 99945–107012 (–)	2355	98 74	*P. protegens* Pf-5 *Pseudomonas* sp. Os17

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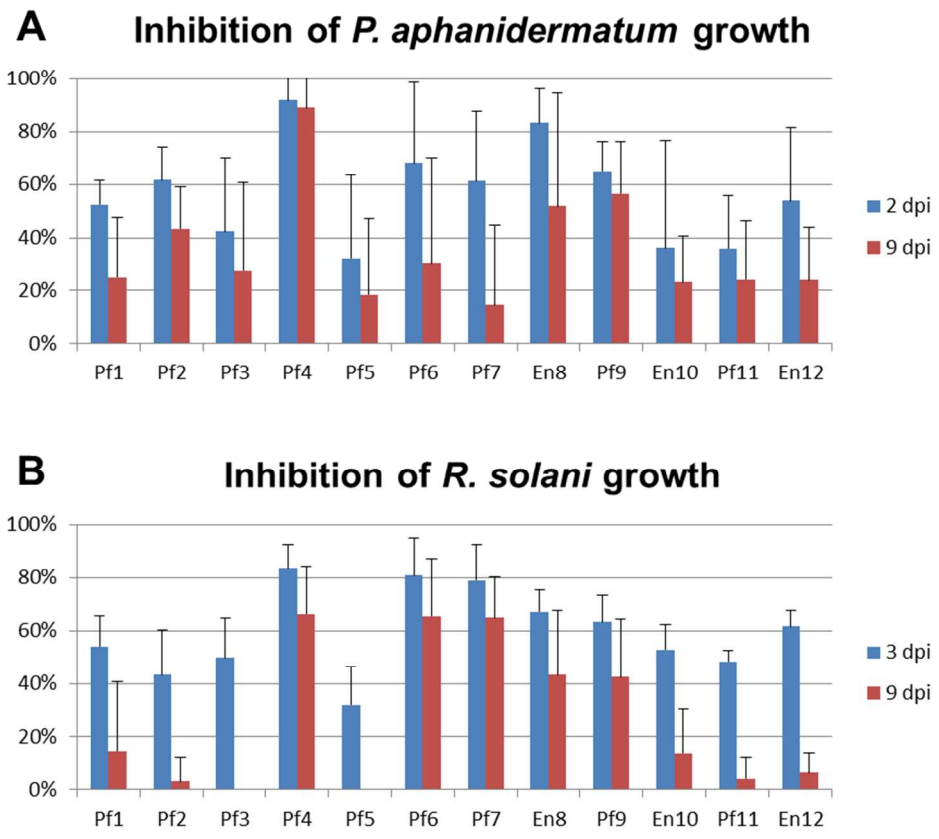

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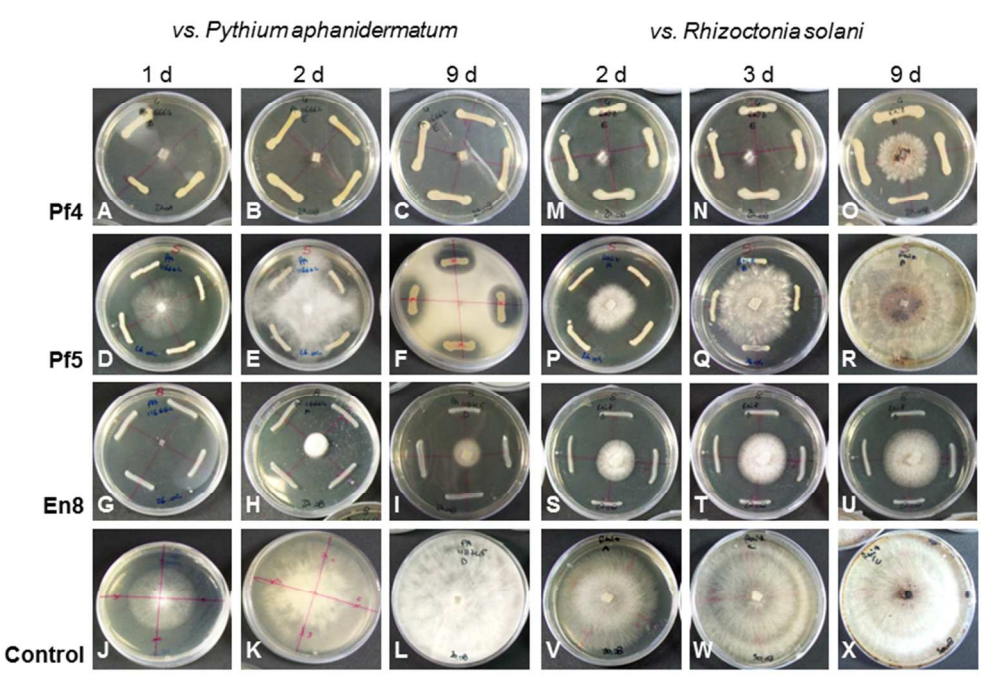

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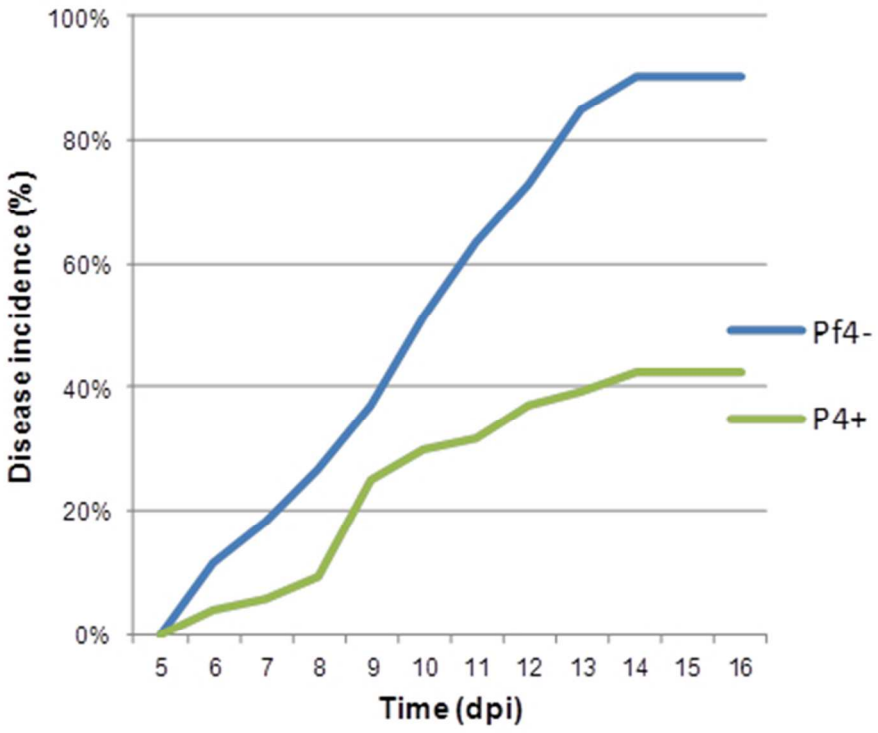

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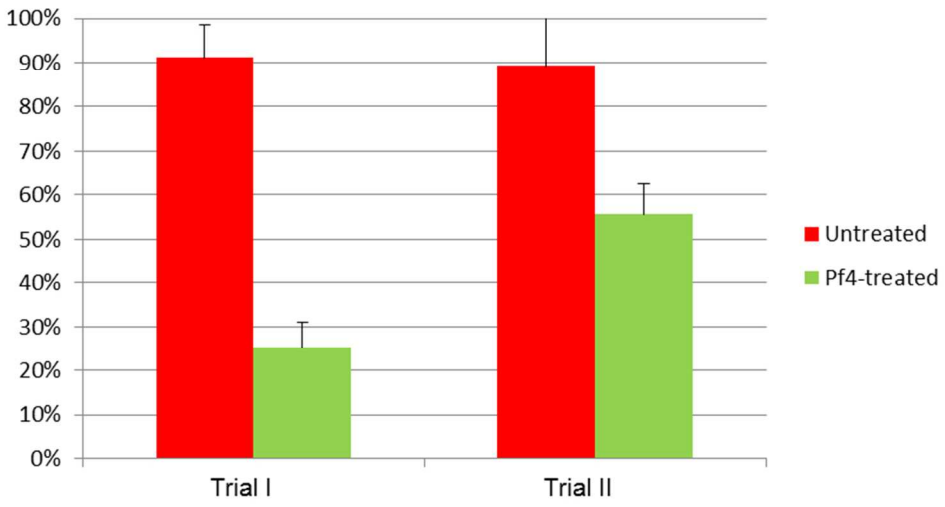

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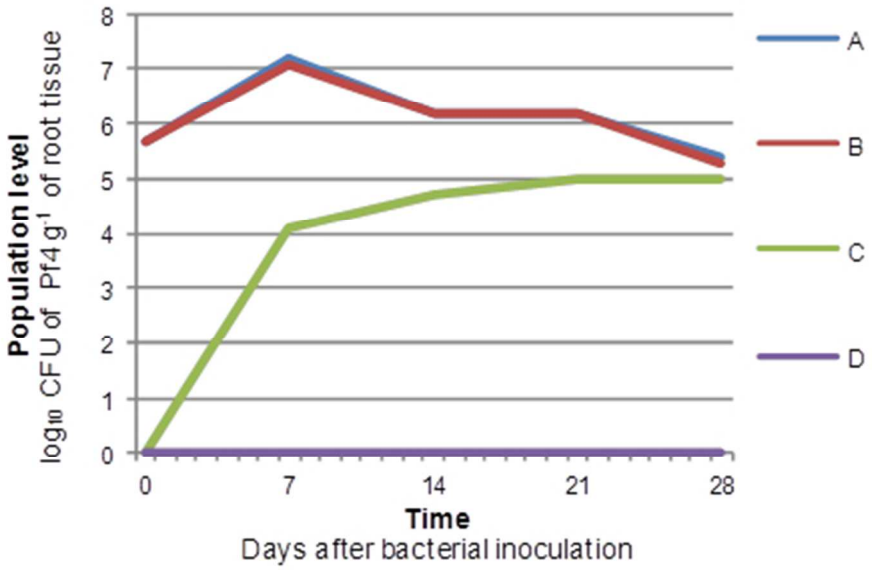

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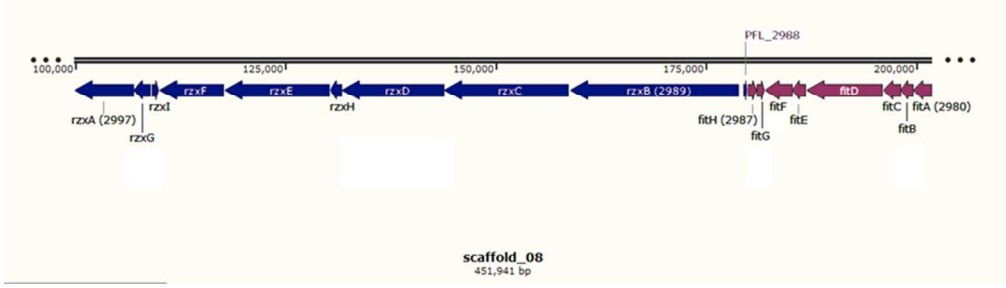

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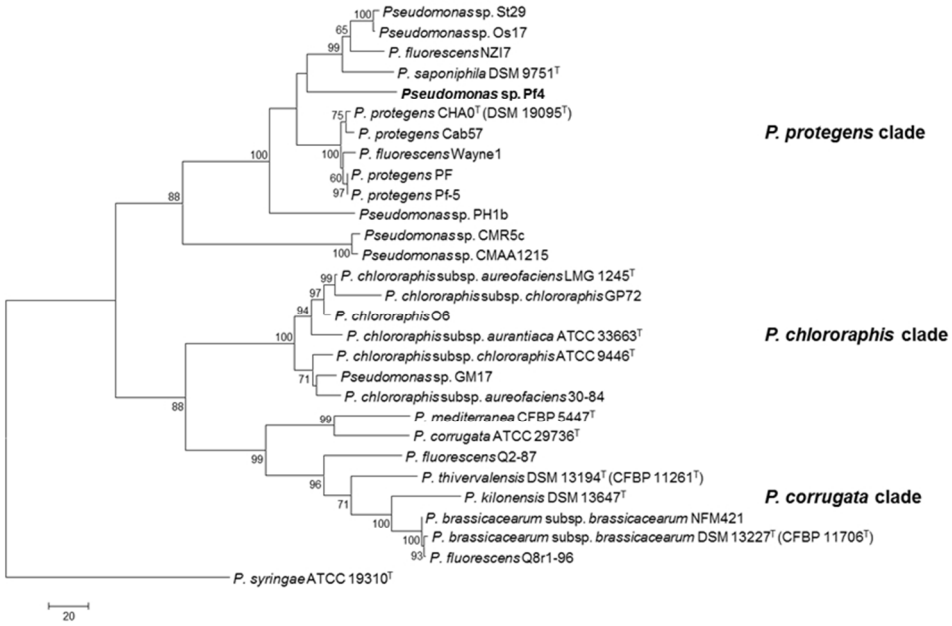

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