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Comparative Genomic Analysis of *Pseudomonas chlororaphis* PCL1606 Reveals New Insight into Antifungal Compounds Involved in Biocontrol

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***Pseudomonas chlororaphis* PCL1606 is a rhizobacterium that has biocontrol activity against many soilborne phytopathogenic fungi. The whole genome sequence of this strain was obtained using the Illumina Hiseq 2000 sequencing platform and was assembled using SOAP denovo software. The resulting 6.66-Mb complete sequence of the PCL1606 genome was further analyzed. A comparative genomic analysis using 10 plant-associated strains within the fluorescent *Pseudomonas* group, including the complete genome of *P. chlororaphis* PCL1606, revealed a diverse spectrum of traits involved in multitrophic interactions with plants and microbes as well as biological control. Phylogenetic analysis of these strains using eight housekeeping genes clearly placed strain PCL1606 into the *P. chlororaphis* group. The genome sequence of *P. chlororaphis* PCL1606 revealed the presence of sequences that were homologous to biosynthetic genes for the antifungal compounds 2-hexyl, 5-propyl resorcinol (HPR), hydrogen cyanide, and pyrrolnitrin; this is the first report of pyrrolnitrin encoding genes in this *P. chlororaphis* strain. Single-, double-, and triple-insertional mutants in the biosynthetic genes of each antifungal compound were used to test their roles in the production of these antifungal compounds and in antagonism and biocontrol of two fungal pathogens. The results confirmed the function of HPR in the antagonistic phenotype and in the biocontrol activity of *P. chlororaphis* PCL1606.**

Pseudomonas is a large genus within the γ subclass of Proteobacteria known for its ubiquity in the environment, utilization of a striking variety of organic compounds as energy sources (Lessie and Phibbs 1984; Wu et al. 2010), and production of an array of secondary metabolites (Bender et al. 1999; Gross and Loper 2009; Raaijmakers et al. 2002). Certain strains live in a commensal relationship with plants, protecting them from infection by pathogens that would otherwise cause disease (Haas and Defago 2005; Lugtenberg and Kamilova 2009; Weller 1988). For example, rhizospheric *Pseudomonas* spp. can function as key components of ecological processes that suppress plant soil disease in agricultural and natural environments (Mazzola 2004; Mendes et al. 2011; Weller et al. 2002).

Due to the spectrum of ecological, metabolic, and biochemical characteristics of the genus *Pseudomonas*, it is not surprising that diversity among *Pseudomonas* spp. extends to the genomic sequence level. As additional genomic information becomes available, developments in genomic technologies can provide further insight into essential life processes (Collins et al. 2003). Comparative genomics has emerged as a powerful tool to identify functionally important genomic elements (Rodriguez-Palenzuela et al. 2010; Silby et al. 2009; Wu et al. 2010). A comparison of genomes within the *Pseudomonas fluorescens* group provided ample evidence that the tremendous ecological and physiological diversity of these bacteria extends to the genomic level (Loper et al. 2012).

The complete genomes of several species from the fluorescent group of *Pseudomonas* have now been sequenced (Baltrus et al. 2011; Loper et al. 2012; Paulsen et al. 2005; Redondo-Nieto et al. 2012; Shen et al. 2013; Silby et al. 2009, 2011) and only 25 to 35% of the genome of each strain is composed of core genes shared by all members of the genus (Loper et al. 2012). The genomes of *Pseudomonas* spp., like those of many other bacteria, display a highly mosaic structure, being composed of relatively stable core regions interspersed with regions that vary among the strains (Kimbrel et al. 2010; Paulsen et al. 2005; Silby et al. 2009, 2011). Regions that are unique to a specific strain are thought to shape that strain's distinctive characteristics, including its interactions with plant pathogens that are targets of biological control. Many of the unique genomic regions have features of horizontally acquired DNA (i.e., transposons, prophages, or genomic islands). Therefore, these features may be exploited as markers of genomic regions that define the distinctive attributes of an individual strain. Consequently, novel compounds, including antimicrobials, have been discovered using genomic-guided approaches (Loper et al. 2012).

Many biological control rhizobacterial strains have certain characteristics in common: the capacity to colonize plant surfaces, specifically the infection site of target pathogens; and the production of antibiotics toxic to target pathogens or the

Whole-genome DNA sequence of *Pseudomonas chlororaphis* PCL1606 is deposited in the GenBank database under accession Bioproject ID PRJNA263136.

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induction of systemic resistance responses in the plant (Haas and Defago 2005; Haas and Keel 2003). Antibiotics, which function as major determinants of biological control, fall into diverse classes, including the phenazines (Mavrodi et al. 2006; Pierson and Pierson 2010), polyketides (Gross and Loper 2009; Weller et al. 2007), cyclic lipopeptide (CLP) biosurfactants (Raaijmakers et al. 2010), and many others. Some strains of *Pseudomonas* spp. also produce phytohormones (Kang et al. 2006; Loper and Schroth 1986; Spaepen et al. 2007) or metabolites that alter plant hormone levels (Leveau and Gerards 2008; Xiao and Xu 2007), which directly influence the growth and development of their plant associates (Arshad and Frankenberger 1998). Other strains induce resistance responses in plants against disease (Bakker et al. 2007; Han et al. 2006). Plant-commensal strains of *Pseudomonas* spp. are intricately enmeshed in plant and soil biology through all of these diverse activities, and their functions as biological control agents have distinguished them as microorganisms with significant effects on agricultural productivity.

P. chlororaphis PCL1606 was isolated from the roots of healthy avocado trees in an area affected by the soilborne pathogenic *Rosellinia necatrix*, causing avocado white root rot (Pliego et al. 2012). This strain has broad antagonistic activity (Cazorla et al. 2006) and suppresses the phytopathogenic activity of *Rosellinia necatrix* and *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Previous experiments have demonstrated that *P. chlororaphis* PCL1606 produces the antifungal antibiotic 2-hexyl, 5-propyl resorcinol (HPR) and the antifungal volatile compound hydrogen cyanide (HCN) (Cazorla et al. 2006). Recently, HPR has been reported to be crucial for biological control and root colonization by *P. chlororaphis* PCL1606 (Calderón et al. 2013, 2014a). Here, we provide a comparative analysis of the genomic sequence of *P. chlororaphis* PCL1606 with nine other genomes from previously sequenced plant-associated *Pseudomonas* spp. strains isolated from various habitats, including soil and plant root and leaf surfaces, which exhibit biological control activities against bacterial, fungal, and oomycete pathogens through varied mechanisms (Table 1). Some of the strains used for the comparative analysis were obtained from disease-suppressive soils that exhibit natural processes of biological control due to the presence of indigenous microbiota that are antagonistic to soilborne plant-pathogenic fungi or nematodes. Genomes of many *P. chlororaphis* associated with herbaceous plants have been previously sequenced (Loper et al. 2012) but, as far as we know, this is the first report of the genome sequencing of a biocontrol *P. chlororaphis* strain isolated from the roots of a woody tree. Additionally, in those previously sequenced *P. chlororaphis* strains, the presence of genes encoding for production of antifungal compounds has been confirmed (phenazines, pyrrolnitrin, cyanhydric acid, and 2-hexyl, 5-propilresorcinol); however, *P. chlororaphis* PCL1606 does not show those genes associated with phenazine production, which are considered not essential for biocontrol of *Sclerotinia sclerotiorum* (Selin et al. 2010) but crucial for biocontrol of *F. oxysporum* (Chin-A-Woeng et al. 1998). Thus, elucidation of the role of the different antifungal compounds in biocontrol must be deciphered. For this, we complemented our genomic analysis with a phenotypic characterization of the antifungal antibiotics produced by *P. chlororaphis* PCL1606 to determine the role of these different compounds in the biocontrol ability of this strain.

RESULTS AND DISCUSSION

General genome features.

The genome features for each of the 10 plant-associated *Pseudomonas* spp. strains analyzed are summarized in Table 2. The comparative analysis of these *Pseudomonas* spp. strains was performed using the online tools from the CLgenomics program. The characteristics (size, GC content, number of protein-coding genes, and number of rRNA, tRNA, and other RNA genes) are within the range of previously sequenced *Pseudomonas* spp. genomes (Silby et al. 2011). The 10 selected genomes had a wide range of genome sizes, ranging from 5.59 to 7.07 M, with the number of protein-coding genes ranging from 5,597 to 6,223, indicating substantial strain-to-strain variation (Table 2). The assembled genome of *P. chlororaphis* PCL1606 had approximately 180-fold coverage, and this sequence is 6,662,896 bp in length, arranged in two contigs (6,646,309 and 16,587 bp), which is assembled as a single circular chromosome for analysis (Fig. 1; Supplementary Fig. S1). Among the studied *Pseudomonas* strains, *P. chlororaphis* PCL1606 has the highest value for G+C content (64.01%), followed by *P. protegens* Pf-5 (63.30%), and then the remaining *P. chlororaphis* strains (62.93 to 63.15%) (Table 2).

Table 1. *Pseudomonas* strains used in this study

Strain	Source	Target diseases for biological control	Genome reference
Pseudomonas chlororaphis			
PCL1606	Avocado rhizosphere, Spain	Avocado white root rot, tomato foot, and crown rot	This study
GP72	Rhizosphere of green pepper, China	Phytopathogens suppression	Shen et al. 2012
O6	Soil, Utah, U.S.A.	Wildfire of tobacco, spot of cucumber	Loper et al. 2012
P. chlororaphis subsp. *aureofaciens*			
30-84	Wheat rhizosphere, Washington, U.S.A.	Take-all of wheat	Loper et al. 2012
P. protegens			
Pf-5	Soil, Texas, U.S.A.	Seedling emergence	Paulsen et al. 2005
P. brassicacearum			
Q8r1-96	Wheat rhizosphere, Washington, U.S.A.	Take-all of wheat	Loper et al. 2012
P. fluorescens			
Pf0-1	Soil, Massachusetts, U.S.A.	Not detailed[a]	Silby et al. 2009
Q2-87	Wheat rhizosphere, Washington, U.S.A.	Take-all of wheat	Loper et al. 2012
SBW25	Sugar beet phyllosphere, Oxfordshire, U.K.	Seedling emergence	Silby et al. 2009
F113	Sugar beet rhizosphere	*Pythium ultimun, Phytophthora cactorum*, and *Fusarium oxysporum*	Redondo-Nieto et al. 2012

[a] Potentially important for biocontrol (Silby and Levy 2004).

In general, the genome features of *P. chlororaphis* strains PCL1606, O6, and 30-84 are very similar. The average genome size of *Pseudomonas* spp. isolated from the plant rhizosphere is lower (average of 6.63 Mbp) than those isolated from soil (average of 6.83 Mbp) and from phyllosphere (only one representative strain of 7.15 Mbp). *P. chlororaphis* GP72 has a lower number of RNA genes when compared with the other *P. chlororaphis* (Table 2).

Table 2. General genomes features of the 10 studied *Pseudomonas* spp.

Features	PCL1606	GP72	O6	30-84	Pf-5	Pf0-1	Q8r1-96	Q2-87	SBW25	F113
Chromosome size (Mbp)	6.66	6.66	6.98	6.67	7.07	6.44	6.60	6.37	6.77	6.85
G+C content (%)	64.01	63.13	62.93	62.95	63.3	60.52	60.96	60.63	60.50	60.78
RNA genes	157	80	143	160	115	119	279	241	97	90
rRNA genes	16	4	7	19	16	19	16	19	16	16
tRNA genes	71	56	60	74	71	73	65	68	66	66
Other RNA genes	70	20	72	67	28	27	198	154	15	8
Protein-coding genes	6,107	6,005	6,223	5,848	6,108	5,722	5,715	5,597	5,921	5,862
Contigs	2	347	30	13	1	1	5	2	1	1

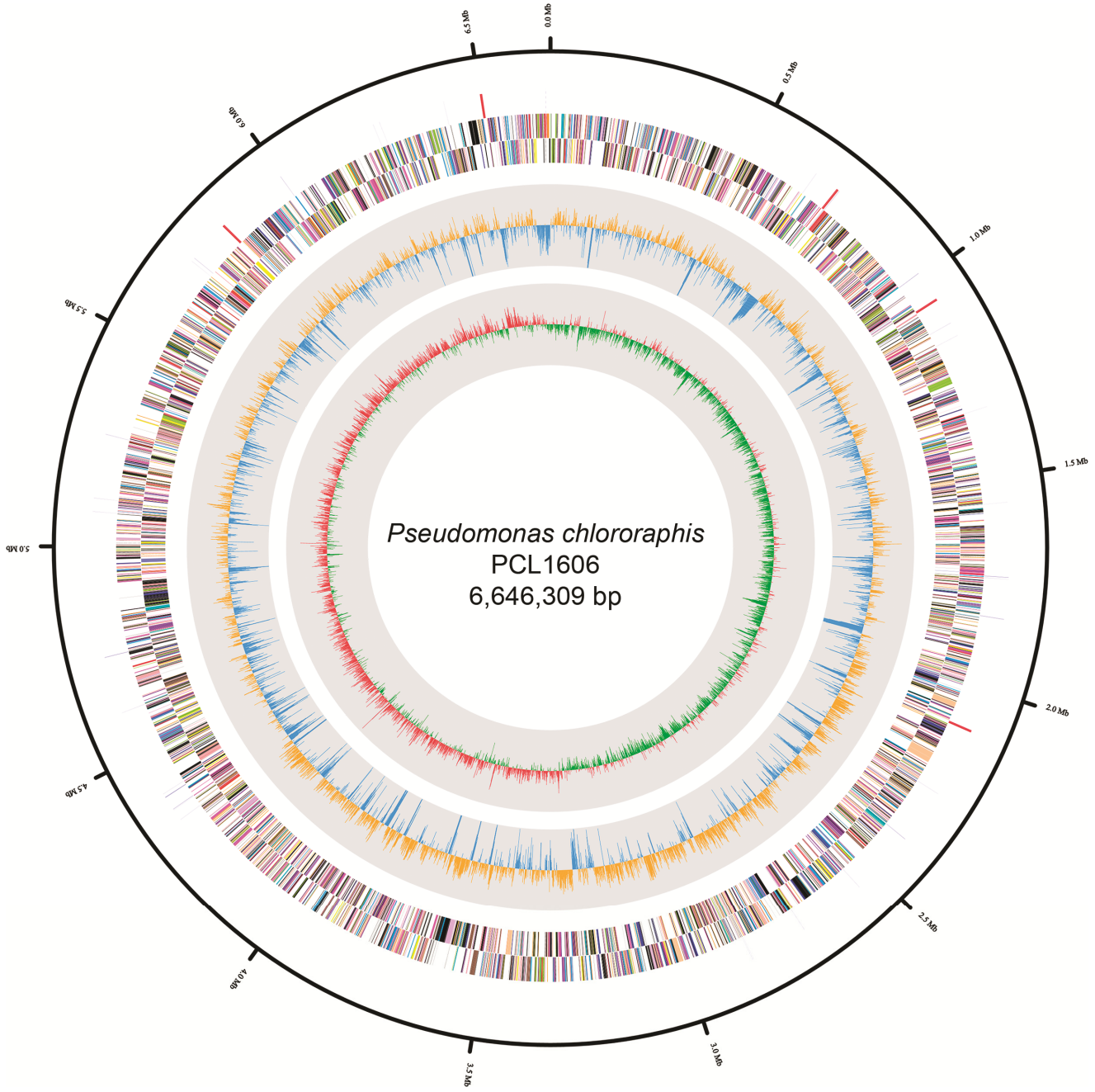

Fig. 1. Circularized genome map of *Pseudomonas chlororaphis* PCL1606. The 6.65-Mbp contig was merged at the 3′ end with the 5′ end of the 16.6-Kbp contig, generating circularized sequence. The first nucleotide of the map corresponds to the first nucleotide of the 6.65-Mbp contig. Each ring of the circle has a different genome information: rRNA/tRNA, reverse coding sequence (CDS), forward CDS, GC skew, and GC ration from the outermost ring moving in. Each color corresponds to a different cluster of orthologous group functional annotation.

Phylogenetic analysis.

The phylogenetic relationship of *P. chlororaphis* PCL1606 with the other *Pseudomonas* spp. strains was further analyzed by comparing a set of eight protein-coding housekeeping genes (*rpoB*, *rpoD*, *gyrB*, *recA*, *ascA*, *aroE*, *mutL*, and *guaA*) (Fig. 2). The resulting phylogenetic tree showed that the 10 selected *Pseudomonas* strains grouped into a single large clade composed of three subclades. The four strains of *P. chlororaphis* were allocated into subclade 1. In this subclade, *P. chlororaphis* PCL1606 grouped together with the other sequenced *P. chlororaphis* strains, which is in agreement with previous results (Calderón et al. 2013). The biocontrol strain *P. protegens* Pf-5 (Table 1) is distantly associated with this group. Our analysis is also consistent with a recent report that assigned strain Pf-5 to the new species *P. protegens*, which is related to *P. chlororaphis* but also has distinct differential properties (Ramette et al. 2011).

Subclade 2 is composed of three *P. fluorescens* strains (Table 1) and the *P. brassicacearum* Q8r1-96 strain (previously classified as *P. fluorescens*). In this subclade, *P. fluorescens* Pf0-1 is also distantly associated with the remaining strains inside this cluster. This fact has been previously reported for this strain and the *P. protegens* Pf-5 strains, supporting the hypothesis that these strains reside in distinct lineages that will become more defined as the genomes of sister strains become available in the future (Loper et al. 2012). Subclade 3 included *P. fluorescens* biocontrol strain SBW25 along with other different *Pseudomonas* strains.

All of the strains in subclades 1 and 2 were isolated from plant roots or soil (Table 1). In subclade 3, strain SBW25, isolated from the leaves of sugar beet in England (Table 1), appears along with other two *P. fluorescens* strains: A506, which was isolated from leaf surfaces (Wilson and Lindow 1993), and SS101 isolated from wheat roots (Mazzola et al. 2007). Subclade 3 also includes the previously sequenced strain *P. synxantha* BG33R, which was isolated from the root of a peach tree (Kluepfel et al. 1993). These results are consistent with the results previously described in other studies (Loper et al. 2012), and these phylogenies are also congruent with those from a recent report in which a large number of strains representing many species of *Pseudomonas* were evaluated (Mulet et al. 2010). In this study, *P. fluorescens* and *P. chlororaphis* strains were also allocated in different clades from the other *Pseudomonas* spp.

Comparison among *P. chlororaphis* genomes.

In total, 6,107, 6,223, 5,848, and 6,005 protein-coding genes (including hypothetical proteins) of the *P. chlororaphis* strains PCL1606, O6, 30-84, and GP72, respectively, were reported. The analysis of the *P. chlororaphis* genomes identified 4,598 predicted protein-coding genes in a core genome for this group (Fig. 3). *P. chlororaphis* PCL1606 had the highest number of specific genes (19.4% of the genes, 1,186 genes) (Fig. 3; Supplementary Table S2), which included genes related to the type IV secretory pathway, fimbria genes, and the pyochelin gene cluster. The other *Pseudomonas* strains present a smaller num-

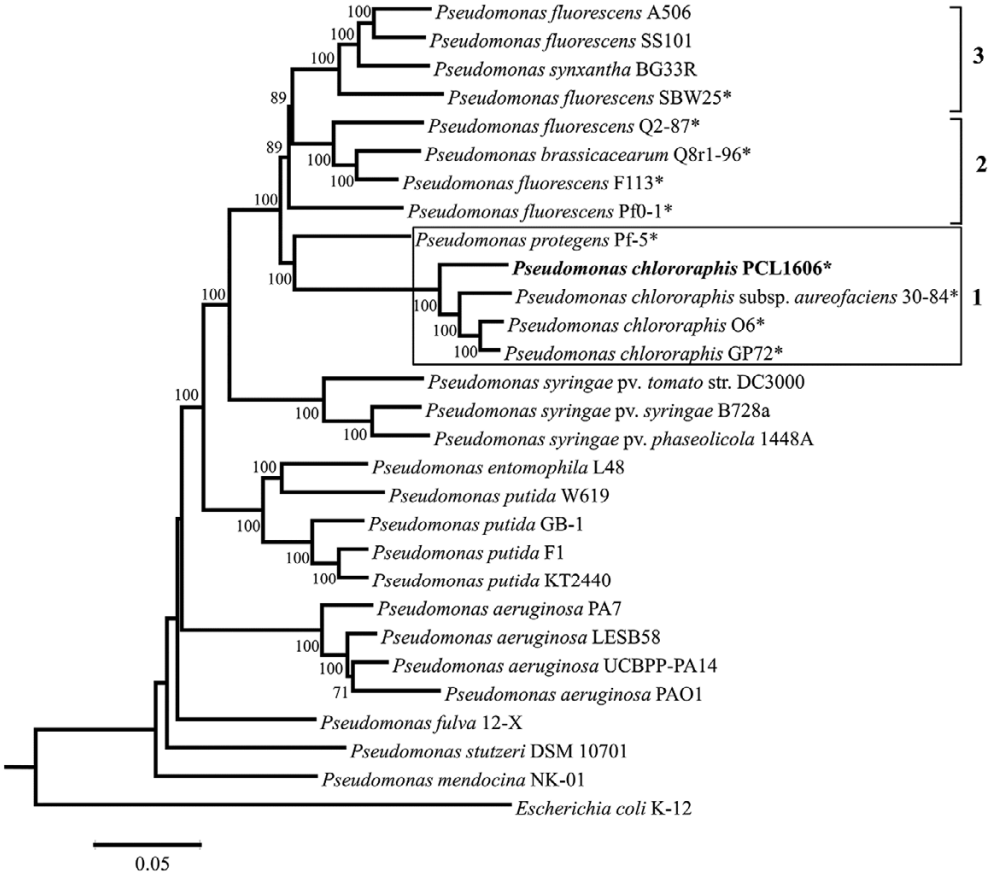

Fig. 2. Phylogenetic tree showing the relationship of the sequenced *Pseudomonas* spp. strains. The tree is based on the concatenated alignments of eight core housekeeping genes—*rpoB*, *rpoD*, *gyrB*, *recA*, *acsA*, *aroE*, *mutL*, and *guaA* (approximately 15 Mb)—and was generated using MEGA 5 (Tamura et al. 2011). *Escherichia coli* K-12 was used as an outgroup. The bar indicates sequence divergence. Percent bootstrap values of more than 70% (from 1,000 replicates) are indicated at the nodes. Strains of the *Pseudomonas fluorescens* group congregated within a single clade consisted of three subclades (lines showed), which are numbered 3 to 1. The *P. chlororaphis* groups analyzed in this study and *P. protegens* Pf-5 are in a box. *P. chlororaphis* PCL1606, sequenced in this study, is marked in bold.

ber of specific genes: 408, 418, and 328 protein-coding genes for O6, 30-84, and CP72 strains, respectively.

A comparison of the functional categories based on clusters of orthologous groups was performed with the four *P. chlororaphis* genomes (Table 3). These results confirmed those obtained from the Venn diagram, where the *P. chlororaphis* strains are very similar, with a number of genes involved in different functions.

Analysis of genes encoding for antifungal related compounds.

Antimicrobial compounds toxic to phytopathogenic fungi, oomycetes, and bacteria are important contributors to biological control, including secondary metabolites, such as antimicrobial antibiotics, lipopeptides, siderophores, and toxins (Raaijmakers et al. 2002). Due to our interest in antifungal compounds, the presence of genes for well-known antifungal compounds was identified in the genome sequences of each selected strain (Table 4).

The analysis of the selected genomes revealed that the *P. chlororaphis* group displayed the consistent presence of HPR, pyrrolnitrin (PRN), HCN, and phenazine (PHZ). However, *P. chlororaphis* PCL1606 does not have PHZ genes in its genome. Along with these strains, Pf-5 is another strain from subclade 1 that harbors genes for the biosynthesis of antimicrobial antibiotics, such as 2,4-diacetylphloroglucinol (DAPG), PRN, HCN, and pyoluteorin genes (Paulsen et al. 2005) and rhizoxin genes (Gross and Loper 2009). The other biocontrol strains displayed genes for only one antimicrobial compound, such as Pf0-1 that has an HCN gene; or two compounds, such as the strains Q8r1-96, Q2-87, SBW25, and F113 that harbor genes for DAPG and HCN production. The cluster for HCN production is observed in the genome for all the strains, except in *P. fluorescens* SBW25. However, *P. fluorescens* SBW25 did not have any antifungal antibiotic genes in its genome. The gene cluster for prodigiosine and oocydin A were not present in this *Pseudomonas* strain.

CLP are a class of compounds produced by many strains of *Pseudomonas* spp. that exhibit surfactant, antimicrobial, antipredation, and cytotoxic properties (Gross et al. 2007; Mazzola et al. 2009; Raaijmakers et al. 2006, 2010). No genes coding for CLP have been detected in either *P. chlororaphis* PCL1606 or any other sequenced *P. chlororaphis* strain. Genes coding for production of the CLP orfamide A are present in the Pf-5

Table 3. Comparative analysis of the functional categories bases on COG of the protein-coding genes of the *P. chlororaphis* strains analyzed[a]

Functional categories based on COG (%)	PCL1606	GP72	06	30-84
Translation, ribosomal structure and biogenesis	242 (3.96)	243 (4.05)	245 (3.94)	240 (4.10)
Transcription	589 (9.64)	559 (9.31)	577 (9.27)	546 (9.34)
Replication, recombination and repair	170 (2.78)	169 (2.81)	201 (3.23)	168 (2.87)
Cell cycle control, cell division, chromosome partitioning	51 (0.83)	53 (0.88)	53 (0.85)	56 (0.96)
Posttranslational modification, protein turnover, chaperones	227 (3.72)	236 (3.93)	236 (3.79)	224 (3.83)
Cell wall/membrane/envelope biogenesis	349 (5.72)	363 (6.04)	395 (6.34)	355 (6.07)
Cell motility	217 (3.55)	186 (3.10)	192 (3.09)	187 (3.20)
Inorganic ion transport and metabolism	385 (6.30)	362 (6.03)	362 (5.82)	349 (5.97)
Signal transduction mechanisms	321 (5.26)	312 (5.20)	338 (5.43)	318 (5.44)
Energy production and conversion	371 (6.07)	374 (6.23)	377 (6.06)	365 (6.24)
Carbohydrate transport and metabolism	325 (5.32)	341 (5.68)	333 (5.35)	316 (5.40)
Amino acid transport and metabolism	653 (10.69)	652 (10.86)	650 (10.44)	620 (10.60)
Nucleotide transport and metabolism	134 (2.19)	131 (2.18)	140 (2.24)	133 (2.27)
Coenzyme transport and metabolism	228 (3.73)	247 (4.11)	244 (3.92)	226 (3.86)
Lipid transport and metabolism	307 (5.03)	302 (5.03)	309 (4.97)	284 (4.86)
Secondary metabolites biosynthesis, transport and catabolism	123 (2.01)	130 (2.16)	130 (2.08)	120 (2.05)
General function prediction only	743 (12.17)	706 (11.76)	743 (11.94)	694 (11.87)
Function unknown	672 (11.00)	639 (10.64)	698 (11.22)	647 (11.06)

[a] These results were generated using CLGenomics software. Number of genes and percentage (in parentheses) are shown. In each column, total = 100%.

Table 4. Detection of genes of compound present in the analyzed sequences of selected *Pseudomonas* spp.

	Antimicrobial antibiotic volatiles[a]							Cyclic-lipopeptides		Siderophores				Toxins
Strain	HPR	DAPG	PRN	HCN	PHZ	PLT	Rhizoxins	Orfamide	Viscosin	Pyoverdine	Pyochelin	Achromobactin	Hemophore	FitD
Subclade 1														
PCL1606	+	–	+	+	–	–	–	–	–	+	+	+	–	+
GP72	+	–	+	+	+	–	–	–	–	+	–	+	–	+
06	+	–	+	+	+	–	–	–	–	+	–	+	–	+
30-84	+	–	+	+	+	–	–	–	–	+	–	+	–	+
Pf-5	–	+	+	+	–	+	+	+	–	+	+	–	+	+
Subclade 2														
Pf0-1	–	–	–	+	–	–	–	–	–	+	–	–	–	–
Q8r1-96	–	+	–	+	–	–	–	–	–	+	–	–	–	–
Q2-87	–	+	–	+	–	–	–	–	–	+	–	–	–	–
F113	–	+	–	+	–	–	–	–	+	+	–	–	+	–
Subclade 3														
SBW25	–	–	–	–	–	–	–	–	+	+	–	–	+	–

[a] HPR = 2-hexyl, 5-propyl resorcinol, DAPG = 2,4-diacetylphloroglucinol, PRN = pyrrolnitrin, HCN = hydrogen cyanide, PHZ = phenazine, and PLT = pyoluteorin.

genome, in which orfamide A has been demonstrated to have a role in bacterial motility and to exhibit antimicrobial activity (Gross et al. 2007). Genes coding for production of the CLP viscosin were localized in the genome of *P. fluorescens* SBW25 (de Bruijn et al. 2007) and *P. fluorescens* F113. The gene clusters for arthrofactin, massetolide, putisolvin, amphisin, and entolysin were not present in any of the *Pseudomonas* strains analyzed.

The fluorescent pseudomonads are characterized by their production of fluorescent pigments in the large and diverse pyoverdine class (Visca et al. 2007), which function as siderophores for iron acquisition by the bacterial cell. Many genes are involved in the biosynthesis, utilization, and regulation of the pyoverdine iron-acquisition system (Cornelis et al. 2009), and all these *Pseudomonas* spp. have a full complement of pyoverdine genes, which can be present in three to seven clusters dispersed throughout the genome (Loper et al. 2012), improving the biocontrol ability of the *Pseudomonas* strains (Meyer 2000). Many *Pseudomonas* spp. produce secondary siderophores that also contribute to iron nutrition (Cornelis et al. 2009), such as pyochelin, which is only produced in the *P. protegens* Pf-5 (Youard et al. 2007) and *P. chlororaphis* PCL1606 strains. The gene clusters for the biosynthesis and transport of the siderophore achromobactin (Berti and Thomas 2009) are present in all *P. chlororaphis* strains (Table 4), suggesting specificity to this group, but the functional production of these secondary siderophores has not been confirmed.

In addition, the three genomes of Pf-5, SBW25, and F113 had a full set of the genes required for the biosynthesis and efflux of hemophore (Table 4), a protein that, when exported from the cell, can chelate heme with high affinity and then be bound and taken up by specific outer-membrane receptors (Wandersman and Delepelaire 2004).

Finally, certain strains in the *P. fluorescens* group can be toxic to insects and, in some cases, this toxicity is associated with the gene clusters encoding the Mcf (makes caterpillars floppy) toxin produced by the entomopathogen *Photorhabdus luminescens* (Daborn et al. 2002). Another gene, *fitD* (fluorescens insect toxin), which is closely related to *mcf*, is present in the genome of *Pseudomonas protegens* Pf-5 and is associated with that strain's lethality against the tobacco hornworm *Manduca sexta* (Pechy-Tarr et al. 2008). The *fit*ABCDEFGH locus is located in the genome of Pf-5 and the *P. chlororaphis* strains PCL1606, GP72, O6, and 30-84. Genes distantly related to *fitD* (27 to 28% identity) are present in the genomes of Q8r1-96, Q2-87, and Pf0-1 but the other genes of the *fit* locus are not present in these strains.

A comparative analysis of the three sets of genes encoding for antifungal compounds in the genome of *P. chlororaphis* PCL1606 (HPR, HCN, and PRN) demonstrated that the gene size and the synteny of the three gene clusters are very similar in the analyzed *Pseudomonas* strains.

The gene cluster for HCN production was detected in all the strains, except in *P. fluorescens* SBW25. The three genes involved in HCN biosynthesis displayed high similarity (more than 74%) at the amino acid level and high synteny in the nine compared *Pseudomonas* strains (Supplementary Fig. S2).

The other two antifungal compounds present in *P. chlororaphis* PCL1606 (PRN and HPR) are exclusive to the *P. chlororaphis* strains, except PRN, which is also present in *P. protegens* Pf-5. Genes encoding for both compounds displayed high similarity at the amino acid level among the *P. chlororaphis* sequences when compared with that of PCL1606 (91 to 96% for PRN [Supplementary Fig. S3] and 82 to 91% for HPR [Supplementary Fig. S4]). These percentages of homology suggest high similarity but a level of diversity among of the genes present in *P. chlororaphis* PCL1606 related to the other *P. chlororaphis* strains.

An important aspect in this study is that these three analyzed cluster genes are present in the *Pseudomonas* strains that belong to subclade 1 and all of these genes had high similarity at the amino acid level, which indicates the similar properties between these strains.

Role of antifungal antibiotic production in the biocontrol ability of *P. chlororaphis* PCL1606.

An analysis of the secondary metabolite encoding genes present in the genome of *P. chlororaphis* PCL1606 (Table 4) revealed the presence of biosynthetic genes that could be involved in the production of such antifungal compounds. These antifungal metabolites have been previously described as involved in the biocontrol ability in *Pseudomonas* spp., such as HCN (Michelsen and Stougaard 2012; Ramette et al. 2011), PRN (Burkhead et al. 1994; Hill et al. 1994) and HPR (Calderón

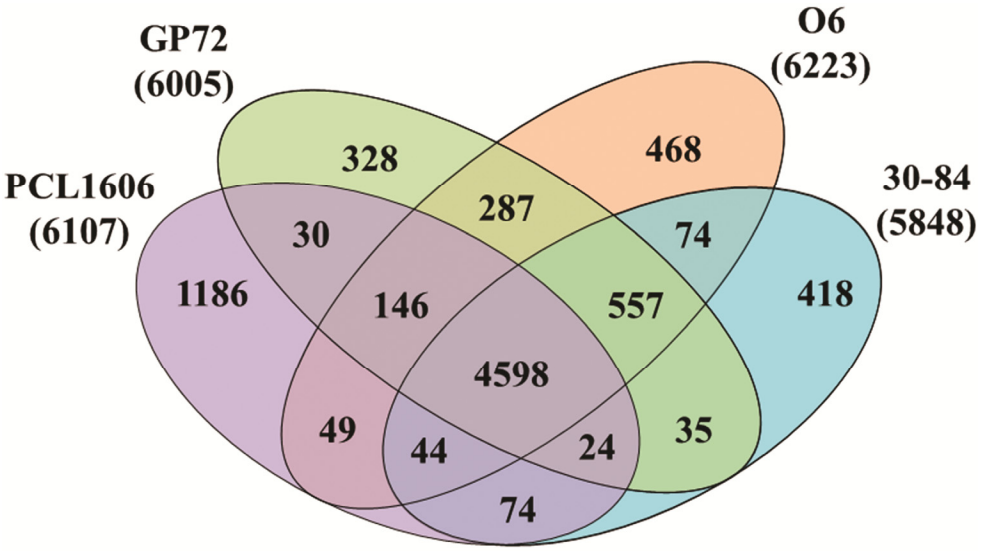

Fig. 3. Genomic diversity of *Pseudomonas chlororaphis* strains. Each strain is represented by an oval. The number of orthologous protein-coding genes shared by all strains (i. e., the core genome) is in the center. Overlapping regions show the number of coding sequences (CDS) conserved only within the specified genomes. Numbers in the nonoverlapping portions of each oval show the number of CDS unique to each strain. The total number of protein coding genes within each genome is listed below the strain name.

et al. 2013; Cazorla et al. 2006). The production of HPR and HCN was previously reported in *P. chlororaphis* PCL1606 (Cazorla et al. 2006). Therefore, this is the first report of PRN production in *P. chlororaphis* PCL1606. To confirm PRN production, after testing different media and conditions, organic extractions after 5 days of growth in King's B medium (KB) at 25°C revealed low PRN production that was not always consistent (Supplementary Fig. S5). Therefore, the functionality of the PRN genes was confirmed in this strain (Table 5); however, the production was much reduced and could only be detected under very specific conditions (5 days of growth on KB at 25°C).

To determine the potential role of these antifungal antibiotics in the biocontrol ability of *P. chlororaphis* PCL1606, site-directed insertional mutants were constructed in the biosynthetic genes of each antibiotic (HPR, HCN, and PRN). Mutagenesis has been previously used to demonstrate that antibiotics produced by *Pseudomonas* spp. play important roles in the biological control of plant disease (Anjaiah et al. 1998; Calderón et al. 2013; Chin-A-Woeng et al. 1998; Cronin et al. 1997; Hokeberg et al. 1998; Keel et al. 1990; Thomashow and Weller 1988; Vincent et al. 1991). Single, double, and triple insertional mutants within the different antifungal antibiotic genes were constructed (ΔHPR, ΔHCN, ΔPRN, ΔHPR-HCN, ΔHPR-PRN, ΔHCN-PRN, and ΔHPR-HCN-PRN) and phenotypic characterization of each derivative mutant was performed. The derivative strains did not have any antibiotic production, depending on the disrupted gene (Table 5). The phenotypic characterization of these derivative mutants does not reveal altered growth on rich (KB) or minimal medium (M9) (data not shown).

The insertional mutants were then tested for their antagonistic activity against *R. necatrix* CH53 and *F. oxysporum* f. sp. *radicis-lycopersici* ZUM2407 using an agar plate assay. The antagonistic activity of the derivative mutants that had a disrupted biosynthetic gene, *darB* (single, double, and the triple mutant), was markedly reduced. *darB* is a key gene involved in the biosynthesis of HPR in PCL1606 (Calderon et al. 2013). Disruption of this gene resulted in failure of HPR production and antagonism against fungi. Derivative mutants with biosynthetic HCN and PRN genes (single and double mutants) retained their antifungal activity and exhibited a phenotype consistent with the wild-type PCL1606 (Table 5). The degree of antagonism displayed by the mutants was clearly correlated with HPR production, based on the lack of fungal antagonism in the derivatives, which confirms the crucial role of HPR production in biological control (Calderón et al. 2013, 2014b; Cazorla et al. 2006).

The effectiveness of producing antibiotics in biocontrol and, more generally, in microbial interactions has often been questioned due to the indirect nature of the supporting evidence and the perceived constraints to antibiotic production in rhizosphere environments (Raaijmakers et al. 2002). However, antibiotic compounds produced by root-colonizing fluorescent pseudomonads may play a significant role in suppressing soilborne diseases in crops (Dowling and O'Gara 1994).

Biocontrol assays against avocado white root rot and tomato foot and root rot were conducted as previously described (Cazorla et al. 2006). When no bacteria were applied to the avocado roots, more than 70% of the plants infected with *R. necatrix* showed disease (Fig. 4). However, under our experimental conditions, root inoculation with the wild-type *P. chlororaphis* PCL1606 strain reduced the disease index (DI) to 42.6%. The derivative mutants ΔHCN, ΔPRN, and ΔHCN-PRN showed disease indices of 42.6, 40.7, and 46.3%, respectively, which were not significantly different from the control PCL1606 strain. In contrast, all the derivative mutants where the biosynthetic gene for the production of HPR was interrupted (ΔHPR, ΔHPR-HCN,

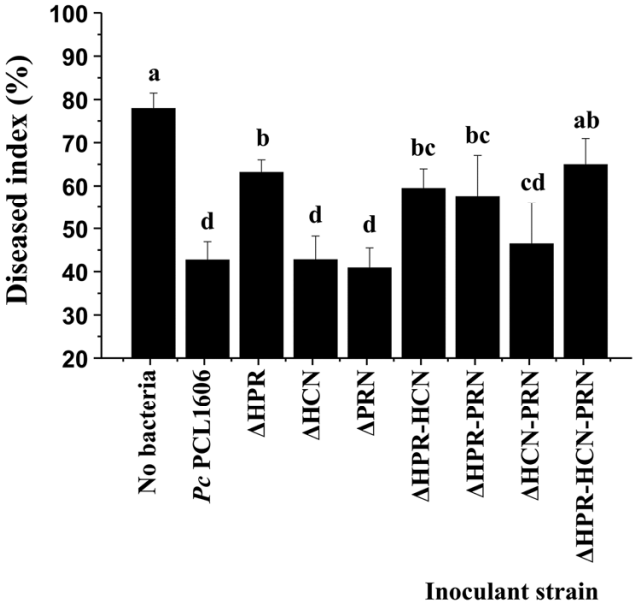

Fig. 4. Biocontrol of *Rosellinia necatrix*-induced white root rot in avocado plants resulting from *Pseudomonas chlororaphis* PCL1606 and its derivative mutants on the three different antibiotics produced. Single, double, and triple derivative mutants in 2-hexyl, 5-propyl resorcinol (ΔHPR), hydrogen cyanide (ΔHCN), and pyrrolnitrin (ΔPRN) were assayed. Roots from commercial 6-month-old avocado plants were inoculated with the derivative strains before being transferred to potting soil that was infected with *R. necatrix*. Plants were scored as sick or healthy 21 days after bacterization. Data were analyzed for significance using an arcsine square root transformation with analysis of variance followed by Fisher's least significant difference test (P = 0.05). Values with different letter indications denote a statistically significant difference.

Table 5. Main characteristics of the derivative strains of *Pseudomonas chlororaphis* PCL1606 used in this study

	Antibiotics production[a]			Antagonism to[b]	
Strain	**HPR**	**HCN**	**PRN**	***Rosellinia necatrix***	***Fusarium oxysporum***
P. chlororaphis PCL1606	+	+	+	+	+
ΔHPR	–	+	+	–	–
ΔHCN	+	–	+	+	+
ΔPRN	+	+	–	+	+
ΔHPR-HCN	–	–	+	–	–
ΔHPR-PRN	–	+	–	–	–
ΔHCN-PRN	+	–	–	+	+
ΔHPR-HCN-PRN	–	–	–	–	–

[a] Production of the antibiotics 2-hexyl, 5 propyl resorcinol (HPR) and pyrrolnitrin (PRN), detected by think-layer chromatography analysis, and the volatile compound hydrogen cyanide (HCN), detected by a colorimetric assay.
[b] Bacterial strains showing a mycelial inhibition zone after 5 days of growth were considered antagonistic.

ΔHPR-PR, and ΔHPR-HCN-PRN) were shown to have lost significant biocontrol activity when using the avocado–*R. necatrix* test system, with disease indices of 63, 59.3, 57.4, and 64.8%, respectively, which are significantly higher disease indices than the wild-type strain (Fig. 4).

The site-directed mutants were also tested in a tomato–*F. oxysporum* f. sp. *radicis-lycopersici* biocontrol system (Fig. 5), which produced results similar to those observed in the avocado–*R. necatrix* test system. When no bacteria were added, 94.4% of the plants showed typical disease symptoms after 21 days of growth in soil that was infested with *F. oxysporum* spores, whereas the introduction of the wild-type *P. chlororaphis* PCL1606 strain reduced the disease incidence to 43%. The ΔHCN, ΔPRN, and ΔHCN-PRN derivative mutants had disease indices of 55, 55, and 51.3%, respectively, which is not significantly different from the control PCL1606 strain. However, we observed a decrease in the biocontrol activity of these derivative mutants. In contrast, as occurs in avocado–*R. necatrix* system, all the derivative mutants where the biosynthetic gene for the production of HPR was mutated (ΔHPR, ΔHPR-HCN, ΔHPR-PR, and ΔHPR-HCN-PRN) showed no significant biocontrol activity in the avocado–*R. necatrix* test system, with disease indices of 83.8, 78.7, 74.5, and 86.5%, respectively, similar to the diseased plants when no bacteria were added and significantly different from the wild-type strain (Fig. 5).

Both biocontrol test systems (avocado–*R. necatrix* and tomato–*F. oxysporum*) confirmed that HPR production is crucial in the biocontrol ability of *P. chlororaphis* strain PCL1606, as was previously demonstrated (Calderón et al. 2013). Thus, a secondary role for HCN and PRN production can be assigned during the biocontrol interaction under our experimental conditions.

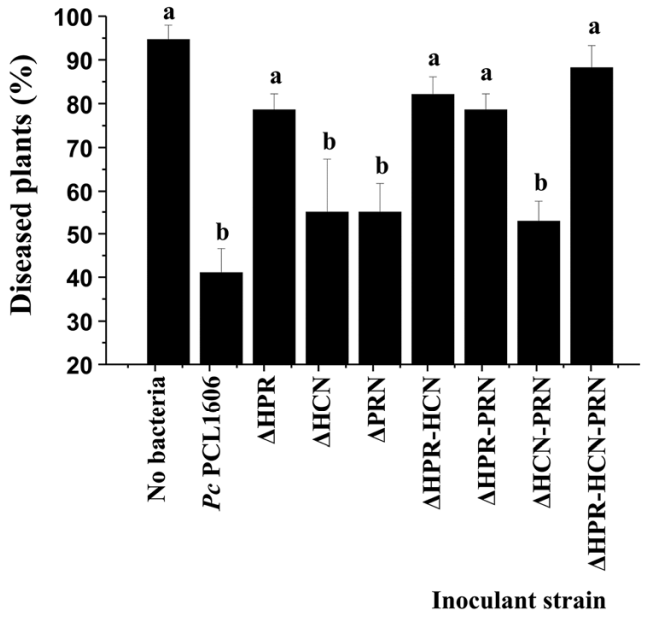

Fig. 5. Biocontrol of *Fusarium oxysporum* f. sp. *radicis-lycopersici*-induced tomato foot and root rot resulting from *Pseudomonas chlororaphis* PCL1606 and its derivative mutants on the three different antibiotics produced. Single, double, and triple derivative mutants in 2-hexyl, 5-propyl resorcinol (ΔHPR), hydrogen cyanide (ΔHCN), and pyrrolnitrin (ΔPRN) were assayed. Tomato seed were coated with bacteria prior to being grown in soil infected with *Fusarium* spores. Plants were scored as healthy or sick after 21 days of growth. Data were analyzed for significance using an arcsine square root transformation with analysis of variance followed by Fisher's least significant difference test (P = 0.05). Values of bars with different letter designations represent a statistically significant difference.

It is important to remark on the dual effect of HPR production in biocontrol, which has a direct effect on the fungal strain and is involved in the efficient colonization of the avocado root (Calderón et al. 2014b).

In conclusion, the genome sequencing of the biocontrol strain *P. chlororaphis* PCL160 provided a phylogenetic study that grouped this strain into the fluorescent group of *Pseudomonas*, being placed close to the *P. chlororaphis* clade. However, bioinformatics analysis of this genome revealed clear differences with the genome from other representative *P. chlororaphis* strains already sequenced. Some remarkable aspects are the absence of genes involved in phenazine biosynthesis and the presence of genes involved in pyochelin biosynthesis. Moreover, the roles in biocontrol of the antifungal compounds present in PCL1606 have been elucidated, revealing a minor role of HCN and PRN but a major role of HPR when controlling phytopathogenic soilborne fungal pathogens under our experimental conditions.

MATERIALS AND METHODS

Genome sequencing and annotation.

Genomic DNA from *P. chlororaphis* PCL1606 was extracted from bacterial cells growing in an overnight culture in tryptone-peptone-glycerol (TPG) medium at 25°C and 150 rpm using the Genomic DNA Purification JETFLEX kit (Genomed GmbH, Löhne, Germany). To verify DNA quality, polymerase chain reaction (PCR) amplifications were performed in a final volume of 25 µl, which contained 10 µM each primer, 2.5 U of GoTaq Flexi DNA polymerase (Promega Corp., Madison, WI U.S.A.), dNTP mixture (250 µM each dNTP), 2 mM $MgCl_2$, 1× GoTag Flexi buffer, and approximately 50 ng of template DNA. The PCR amplification conditions consisted of an initial denaturation for 2 min at 94°C; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1 min; and then a final extension at 72°C for 10 min. The PCR products were analyzed for size and quantity by electrophoresis on 1% agarose gels in Tris-acetate-EDTA buffer (40 mM Tris-HCl and 1 mM EDTA, pH 8). Ethidium bromide was used at a concentration of 1 µl/ml to visualize the DNA bands.

The genome of *P. chlororaphis* PCL1606 was sequenced at the BGI company (BGI Tech Solutions Co., Ltd., China) using the Illumina Hiseq 2000 sequencing platform and was assembled using SOAP denovo software (Li et al. 2008, 2010). To help during the assembly process, the sequence of *P. chlororaphis* O6, which has the higher chromosome size, was used.

The genome of *P. chlororaphis* PCL1606 was automatically annotated using the services of ChunLab Inc. (Seoul, Korea). Manual curation and the comparative analysis was performed using the CLgenomics v.1.1.2 software.

Genome comparisons.

The genome sequence of *P. chlororaphis* PCL1606 was aligned against the sequences of other *Pseudomonas* spp. genomes obtained from the National Center for Biotechnology Information's Entrez database and the EzGenome database. The accession numbers for the nine *Pseudomonas* spp. genomes used in the comparative analysis with *P. chlororaphis* PCL1606 were AHAY00000000 (*P. chlororaphis* GP72), AHOT00000000 (*P. chlororaphis* O6), AHHJ00000000 (*P. chlororaphis* subsp. *aureofaciens* 30-84), CP000076 (*P. protegens* Pf-5), CP000094 (*P. fluorescens* Pf0-1), AHPO00000000 (*P. brassicacearum* Q8r1-96), AGBM00000000 (*P. fluorescens* Q2-87), AM181176 (*P. fluorescens* SBW25), and CP003150 (*P. fluorescens* F113). These *Pseudomonas* strains were selected for further comparison purposes based on their characterized and distinctive bio-

logical control properties and their isolation from different habitats (bulk, rhizospheric soil, or aerial plant surfaces) Table 1).

Amino acid alignments to detect secondary metabolites were performed using the CLC Main Workbench (Aarhus). The four *chlororaphis* genomes (PCL1606, GP72, O6, and 30-84) were compared using a Venn diagram analysis software (VENNY, Computational Genomics). Core genes among compared strains were obtained using the "comparative genomics" tool of CLgenomics software.

Phylogenetic studies.

A phylogenetic analysis of *P. chlororaphis* PCL1606, including other strains belonging to the genus *Pseudomonas,* was performed using a multilocus sequence analysis of the concatenated alignments of eight highly conserved housekeeping genes: *rpoB*, *rpoD*, *gyrB*, *recA*, *acsA*, *aroE*, *mutL*, and *guaA*. Multiple alignments were performed using ClustalW (Larkin et al. 2007) and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa were clustered in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein 1985). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The concatenated sequences of the housekeeping genes yielded an alignment with approximately 15,000 nucleotides that could be compared among all selected strains. The phylogenetic analyzes were conducted using MEGA 5 (Tamura et al. 2011).

Phenotypic characterization of the antifungal antibiotics produced by *P. chlororaphis* PCL1606.

Microorganism cultures and conditions. The bacterial strains used for the phenotypic characterization of the antibiotics are listed in Table 6. TPG medium (triptone, peptone, and glycerol, each component at 10 g/liter) was routinely used to culture *Pseudomonas* strains at 25°C. Agar-agar (Difco Laboratories, Detroit) was added to a final concentration of 1.5% to produce solid media. Luria Bertani (LB) (Bertani 1951) medium was used to grow *Escherichia coli* strains at 37°C. The bacterial strains were stored at –80°C in LB with 10% dimethyl sulfoxide. When necessary, the media were supplemented with kanamycin (50 μg/ml), gentamicin (30 μg/ml), or spectinomycin (50 μg/ml).

The fungal strains used in this study (Table 6) were grown at 25°C on potato dextrose agar (PDA) (Difco Laboratories) or TPG plates. *R. necatrix* was stored at 4°C as previously described (Gutierrez-Barranquero et al. 2012) and *F. oxysporum* spores were stored in 10% glycerol at –80°C.

The plasmids used in this study for cloning purposes are also listed in Table 6.

Construction of P. chlororaphis *PCL1606 insertional mutants.* PCR, cloning, and plasmid purification were performed following standard procedures (Sambrook and Russell 2001). The selected biosynthetic genes of HPR, PRN, and HCN (*darB*, *prnC*, and *hcnB*, respectively) were inactivated by insertional mutagenesis as previously described (Calderón et al. 2013), and the resulting antibiotic production was measured on TPG and KB (King et al. 1954). To accomplish this inactivation, vectors were constructed via insertion to disrupt the various antibiotic biosynthesis genes using single-crossover homologous recombination. To construct the integrative plasmids, DNA fragments of 379, 743, and 596 bp from inside the open reading frame of the *darB*, *hcnB*, and *prnC* biosynthetic genes, respectively, were obtained using specific PCR primers and *P. chlororaphis* PCL1606 as template. The amplified DNA fragments were then cloned into the pCR2.1-TOPO, pJQ200SK, and pSW25T vectors (Table 6). These integrative plasmids were then transformed into wild-type *P. chlororaphis* PCL1606 using standard electroporation (Choi et al. 2006). Five colonies from each independent transformation assay were randomly selected and the correct insertion and orientation of the plasmid within the target gene were confirmed by PCR. The resulting derivative mutants were named ΔHPR, ΔHCN, and ΔPRN for the simple insertional mutants; ΔHPR-HCN, ΔHPR-PRN, and ΔHCN-PRN for the double insertional mutants; and ΔHPR-HCN-PRN for the triple insertional mutant. The insertional mutants were selected in the presence of kanamycin, gentamycin, or spectinomycin, depending on the vector. Additionally, the mutants were analyzed for appropriate insertion using PCR and then sequencing to confirm gene disruption. Growth on M9 minimal medium and TPG compared with the wild-type strain confirmed that these mutants did not have altered growth.

Table 6. Bacterial and fungal strains and plasmids used in this study

Strain	Relevant characteristics[a]	Reference or source[b]
Bacterial strain		
Pseudomonas chlororaphis		
PCL1606	Wild-type, isolated from Spanish avocado rhizosphere.	Cazorla et al. 2006
ΔHPR	PCL1606 derivative insertional mutant in *darB* gene, Km^r	Calderón et al. 2013
ΔHCN	PCL1606 derivative insertional mutant in *hcnB* gene, Km^r	This study
ΔPRN	PCL1606 derivative insertional mutant in *prnC* gene, Km^r	This study
ΔHPR-HCN	PCL1606 derivative insertional mutant in *darB* and *hcnB* genes, Km^r, Gm^r	This study
ΔHPR-PRN	PCL1606 derivative insertional mutant in *darB* and *prnC* genes, Km^r, Gm^r	This study
ΔHCN-PRN	PCL1606 derivative insertional mutant in *hcnB* and *prnC* genes, Km^r, Gm^r	This study
ΔHPR-HCN-PRN	PCL1606 derivative insertional mutant in *darB*, *hcnB*, and *prnC* genes, Km^r, Gm^r, Spe^r	This study
Escherichia coli		
DH5α	General-purpose host strain	Boyer and Roulland-Dussoiz 1969
Fungi		
Rosellinia necatrix CH53	Wild-type, isolated from avocado trees with symptoms of white root rot; high virulence	López-Herrera and Zea-Bonilla 2007
Fusarium oxysporum f. sp. *radicis-lycopersici* ZUM2407	Causes crown and foot rot of tomato	IPO-DLO
Plasmids		
pCR2.1-TOPO	TA cloning vector for polymerase chain reaction products, Amp^r, Km^r.	Invitrogen
pJQ200SK	Suicide vector, P15A *oriV sacB mob*, Gm^r	Quandt and Hynes 1993
pSW25T	pSW25::oriT_{RP4}; ori$V_{R6K\gamma}$, Spe^r	Demarre et al. 2005

[a] Km^r, Gm^r, Spe^r, and Amp^r = resistant to kanamycin, gentamycin, spectinomycin, and ampicillin, respectively.
[b] IPO-DLO = Institute for Plant Protection-Agriculture Research Department, Wageningen, The Netherlands.

Antagonism. The antagonistic activity of the wild-type and derivative strains was tested using the dual-plate test, as previously described (Cazorla et al. 2006; Geels and Schippers 1983). The initial screening for in vitro antifungal activity against the phytopathogenic fungi *R. necatrix* CH53 and *F. oxysporum* f. sp. *radicis-lycopersici* ZUM2407 (Table 6) was performed on TPG and PDA plates as follows. A 0.6-cm-diameter mycelium disk from a 2- to 5-day-old fungal culture was placed in the center of a petri dish and the bacterial strains were inoculated at a distance of approximately 3 cm from the fungus. Bacterial strains that inhibited mycelial growth after 5 days at 25°C, as judged by a growth inhibition zone, were considered antagonistic.

Antifungal extraction. The strains used in this study were tested for the production of HCN and the antifungal antibiotics HPR and PRN, as previously described (Cazorla et al. 2006). HCN detection was performed following the paper indicator procedure described by Castric (1975). To detect HPR and PRN, cell-free supernatants from 5-day-old liquid KB or TPG cultures of the corresponding strains were extracted with chloroform/methanol (2:1, vol/vol). The extracted material was fractionated in toluene via thin-layer chromatography (TLC) using silica RP-18F_{254S} TLC plates (Merck AG, Darmstadt, Germany). After drying, spots were detected on the chromatogram under UV light at 254 nm and *Rf* values were calculated. Antibiotic production was also determined by spraying these TLC plates with diazotized sulfanilic acid and watching for a characteristic color change (Whistler et al. 2000). Spots with an *Rf* value of approximately 0.9 that were brown to dark green in color were considered positive for HPR. Spots with an *Rf* value of approximately 0.8 that were maroon in color indicated the presence of PRN. The strain *P. chlororaphis* PCL1606 were used as the reference for antibiotic production.

Biocontrol assays against avocado white root rot. Biocontrol assays against avocado white root rot were performed using the avocado–*R. necatrix* system, as previously described (Cazorla et al. 2006). Six-month-old commercial avocado plants were obtained from Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain). The roots from the avocado plants were disinfected by immersion in 0.1% NaOCl for 20 min and then washed and bacterized following the method previously described (Cazorla et al. 2006), with slight modifications. The roots of the avocado plants were immersed in a suspension of the bacterial isolate (10^9 CFU/ml) or in sterile tap water for 20 min. Any excess bacterial suspension was allowed to drip off, after which the seedlings were placed into pots containing 30 g of wet potting soil (Jongkind Grond B.V., Aalsmeer, The Netherlands) and infected with *R. necatrix* using inoculated wheat grains (four infected grains per pot) as described previously (Freeman et al. 1986). Five sets of 10 avocado seedlings each were tested per treatment. The seedlings were grown in a chamber at 24°C and 70% relative humidity with 16 h of daylight and were watered twice per week.

Because it was difficult to monitor the symptoms on the avocado roots due to the overgrowth of *R. necatrix*, aerial symptoms were recorded on a scale of 0 to 3 and a normalized DI was calculated using a previously described formula (Cazorla et al. 2006). The DI was determined approximately 21 days after bacterization.

Biocontrol assays against tomato root rot. Biocontrol trials in the tomato–*F. oxysporum* f. sp. *radicis-lycopersici* system were conducted as previously described (Cazorla et al. 2006; Chin-A-Woeng et al. 1998). One-third of a 7-day-old PDA plate culture of the strain *F. oxysporum* f. sp. *radicis-lycopersici* ZUM2407 was homogenized and inoculated in an Erlenmeyer flask containing 200 ml of potato-dextrose broth. After 3 days of growth at 28°C under aeration (110 rev/min), the fungal material was placed on top of sterile glass wool and the filtrate was adjusted to 5×10^5 spores/ml. For soil inoculation, spore suspensions were mixed thoroughly with potting soil to a final concentration of 3×10^6 spores/kg.

Tomato seed (*Solanum lycopersicum* L. 'Moneymaker') were coated with bacteria by dipping the seed in a mixture of 1% (wt/vol) methylcellulose (Sigma-Aldrich, St. Louis) and 10^9 CFU/ml bacteria in phosphate-buffered saline buffer. Coated seed were dried in a sterile stream of air. One seed was sown in each pot that contained 25 g of soil approximately 1.5 cm deep. Ten sets of 10 plants each were included in each treatment. The seedlings were grown in a greenhouse at 24°C with 70% relative humidity and 16 h of daylight and were watered from the bottom. Diseased plants were counted only after a considerable fraction of the untreated control plants (above 60%) showed symptoms, which usually occurred 18 days after sowing. The plants were removed from the soil and washed and the plant roots were examined for tomato crown and root rot symptoms, which were detected by the presence of root browning and lesion formation. Roots without any disease symptoms were designated as healthy.

Statistical methods. The data were statistically analyzed using an analysis of variance (Sokal and Rohlf 1986) followed by Fisher's least significant difference test ($P = 0.05$) using SPSS 12 software (SPSS Inc., Chicago). All experiments were performed at least three times.

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EzGenome: www.ezbiocloud.net
National Center for Biotechnology Information: www.ncbi.nlm.nih.gov
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