Full Paper

Candidate nematocidal proteins in a new *Pseudomonas veronii* isolate identified by its antagonistic properties against *Xiphinema index*

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The nematode *Xiphinema index* affects grape vines and transmits important viruses associated with fanleaf degeneration. *Pseudomonas* spp. are an extensive bacterial group in which important biodegradation and/or biocontrol properties can occur for several strains in the group. The aim of this study was to identify new *Pseudomonas* isolates with antagonist activity against *X. index*. Forty bacterial isolates were obtained from soil and root samples from Chilean vineyards. Thirteen new fluorescent pseudomonads were found and assessed for their antagonistic capability. The nematocide *Pseudomonas protegens* CHA0 was used as a control. Challenges of nematode individuals in King's B semi-solid agar Petri dishes facilitated the identification of the *Pseudomonas veronii* isolate R4, as determined by a 16S rRNA sequence comparison. This isolate was as effective as CHA0 as an antagonist of *X. index*, although it had a different lethality kinetic. Milk-induced R4 cultures exhibited protease and lipase activities in cell supernatants using both gelatin/tributyrin Petri dish assays and zymograms. Three proteins with these activities were isolated and subjected to mass spectrometry. Amino acid partial sequences enabled the identification of a 49-kDa protease similar to

metalloprotease AprA and two lipases of 50 kDa and 69 kDa similar to LipA and ExoU, respectively. Electron microscopy analyses of challenged nematodes revealed degraded cuticle after R4 supernatant treatment. These results represent a new and unexplored property in this species associated with the presence of secretable lipases and protease, similar to characterized enzymes present in biocontrol pseudomonads.

Key Words: biocontrol; exolipases; exoproteases; *Pseudomonas veronii*; *Vitis vinifera*; *Xiphinema index*

Introduction

Wine and table grape cultivar production strongly depends on plant root health and physiology. Soil-borne pathogens that affect these systems prevent water and nutrient uptake and lead to a number of physiological disorders, such as root rot and blackening, and plant wilt and stunting. In Chile, grape production is mostly carried out on clay and mineral-rich soils due to the strong Andean influence in the northern and central areas of the country (Ortega et al., 2003). However, the occurrence of several genera of plant-parasitic nematodes is a limiting factor

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for grape production (Aballay et al., 2011). One of the most damaging nematodes is the dagger *Xiphinema index* (Aballay et al., 2011), a natural vector of the *Grape fan leaf virus*, which is a widespread disease that affects these productive areas of the country (Fiore et al., 2008).

More than 120 species comprise the *Pseudomonas* spp. group, which includes bacteria with a broad spectrum of characteristics, including biocontrol (Mulet et al., 2010; Peix et al., 2009). Despite this, *Pseudomonas* spp. with this ability do not include *P. veronii* (Haas and Défago, 2005), and this species has been characterized by its biosorption/bioremediation properties. Different *P. veronii* isolates are highlighted by their metabolic biodegradation capability of aromatic compounds (Junca and Pieper, 2004; Witzig et al., 2006) or their biosorption of heavy metals (Vullo et al., 2008). *Pseudomonas veronii* was first defined by screening different pseudomonads subjected to differential DNA hybridization, and through the use of S1 nuclease protection assays of DNA-DNA hybrids (Elomari et al., 1996). In addition, this species exhibited a special ability to grow on α -aminobutyrate, sucrose, L-tryptophan, and others, as sole carbon sources (Elomari et al., 1996). A draft genome sequence of the *P. veronii* 1YdBTEX2 strain was recently released (de Lima-Morales et al., 2013), revealing several of the catabolic pathways involved in the degradation of soil pollutants.

In the present work, a new *P. veronii* isolate (i.e., R4), obtained from surveys conducted in grape vineyards and farms in central Chile, was identified using an *X. index* biocontrol panel. This new isolate exhibited a nematocidal activity that was as effective as that of the renowned *P. protegens* CHA0 strain (Stutz et al., 1986), which mediates its nematocidal activity through the generation of secondary metabolites, such as 2,4-diacetylphloroglucinol (2,4 DAPG), HCN, and extracellular lytic enzymes (Siddiqui et al., 2005). The R4 cell supernatants resulted in nematode disruption, and three candidate proteins responsible for this activity were isolated, partially sequenced, and identified in these extracts. The relevance of these findings in *P. veronii* is discussed in terms of the biotechnological tools of this species.

Materials and Methods

Isolation of native fluorescent pseudomonads. A survey of root samples from different vineyards located in the central region of Chile (between Valparaíso and O'Higgins administrative regions) was performed between September and December, 2009. Forty root and soil samples were cleaned manually by washing with tap water. The samples were placed in Erlenmeyer flasks containing 50 mL of washing solution (0.01% Tween 40 in sterile distilled water) and were shaken for 10 min at 200 rpm. The roots were washed with PBS buffer (10 mM K_2HPO_4 - KH_2PO_4 , 0.14 M NaCl, pH 7.2), sectioned into 2-cm pieces and incubated in the same type of flasks containing King's B liquid medium (20 g/L of peptone; 15 mL/L of glycerol; 1.5 g/L of K_2HPO_4 ; 1.5 g/L of $MgSO_4 \times 7H_2O$ and distilled water at pH 7.2 (King et al., 1954)) supplemented with 1 g/L of benomyl, 40 μ g/mL of carbenillicin and 13 μ g/mL of chloramphenicol for 24 h at 28°C and 200 rpm.

Aliquots (100 μ L) of the bacterial suspensions were plated onto King's B agar medium (15 g of agar per 1000 mL of liquid medium). The proliferated colonies were monitored after 2 d of incubation at 28°C and observed under UV light for the identification of fluorescence using a Dual Intensity Transilluminator (UVP, Cambridge, UK).

Selection and identification of *Pseudomonas* spp. Fluorescent isolates were incubated in flasks containing 50 mL of liquid King's B medium and cultured for 12 h at 28°C and 150 rpm. Each bacterial culture (700 μ L) was centrifuged at $3,000 \times g$ for 5 min. The pellets were resuspended in 200 μ L of water for DNA extraction using the ZR Fungal/Bacterial DNA MiniPrep™ Kit (Zymo Research, Irvine, CA, USA) according to the procedures described by the manufacturer. PCR amplifications of the 16S rRNA genes of these isolates were performed using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACG GCTACCTGTTACGACTT-3') (Hernández et al., 2008). The thermal profile used for these reactions included an initial denaturation at 94°C for 3 min and 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 105 s. A final extension at 72°C for 3 min was applied. The expected amplification fragment was purified from agarose gels using the Zymoclean™ gel DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions and sequenced at Macrogen (Macrogen Inc., Seoul, Korea). The sequences were compared using GenBank/Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-blast_interface.cgi), with the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). The 16S rRNA gene phylogenetic tree was constructed using the neighbor joining method implemented in the MEGA6 program (Tamura et al., 2013) with the Kimura-2-parameter substitution model. Support for the hypotheses of relationships was assessed using 1000 bootstrap replicates. The resulting tree was visualized using TreeGraph 2 (Stöver and Müller, 2010).

Determination of the growth rates of fluorescent isolates.

The specific growth rates (μ) of the selected fluorescent isolates were determined in three independent experiments in triplicate by indirect cell counting during time course analyses using a growth liquid medium for 12 h at 28°C and 150 rpm. Data acquisition and calculation for relationships between OD₆₀₀ and cell number were obtained as indicated by Widdel (2007). Optical densities were determined using a Biochrom WPA Biowave II UV/Visible spectrophotometer (Biochrom Ltd., Cambridge, UK). Cell counts were performed using a Neubauer counting chamber (Brand, Wertheim, Germany) (0.1-mm depth $\times$ 0.0025-mm² counting surface) using an Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan). The generated μ values (h⁻¹) were subjected to ANOVA, and the average values were separated using Tukey standard deviation (SD). Statistical analyses were performed using Statgraphics Centurion XV (Manugistics, Inc., Rockville, MD, USA) at a significance level of 5%.

PCR detection of the polyketide synthase D gene. Bacterial DNA was isolated using the ZR Fungal/Bacterial DNA MiniPrep™ Kit (Zymo Research), and PCR assays were performed using the primers phl2a (5'GAG-

GACGTCGAAGACCACCA3') and ph12b (5'-ACCGCAGCATCGTGTATGAG3') and the amplification conditions reported by Raaijmakers et al. (1997). The amplification products of the expected size (745 base pairs) were purified from agarose gels using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research), cloned into pGEM®-T (Promega, Madison, WI, USA) and sequenced at Macrogen.

Xiphinema index population.

Isolation and multiplication system: Soil samples were collected from different vineyards located in the central area of Chile and processed using 850- μ m and 250- μ m sieves as described by Aballay and Insunza (2002). Hallmark morphological structures of adult nematode individuals were judged according to the protocol described by Luc and Cohn (1982) using a Mini Nikon 20X field microscope (Nikon Corporation, Tokyo, Japan). Four hundred nematodes were incubated in soil breeding containers (7-m length $\times$ 1.5-m width $\times$ 30-cm depth) filled with 300 kg of sterilized sand and leaf litter (1:1) and planted with eight 2-year-old *Ficus carica* plants. The system was maintained at $23 \pm 3^\circ\text{C}$.

Nematode sampling: Ten soil mix samples were randomly collected from the container, and nematodes were independently isolated. DNA was extracted from a single juvenile individual from each sample using thermal shock disruption methodology (Luc and Cohn, 1982). PCR amplifications were performed to target the intergenic region between the STI1/5,8S rRNA genes (accession number JF437918.1) specific to *Xiphinema index* (Wang et al., 2003) using the primers I27 (5'-GAGTCG-TAACGTTTCTCGTCTATCAGG-3') and X-G (5'-CAATTCGCACTTATTCTCGGGGCT-3'), which amplify a fragment of 385 bp. The identity of the amplified region was determined by cloning into a pGEM-T easy cloning vector (Promega) according to the manufacturer's procedures and sequencing (Macrogen). The nematodes' identity was confirmed after every reproduction cycle using these procedures.

In vitro challenge of *X. index* with *Pseudomonas* spp. isolates. Bacterial isolates were incubated in King's B liquid medium to a density of 1.5×10^9 cells per mL. A 500- μ L aliquot of each bacterial suspension was added to 12.5 mL of semi-solid King's B agar medium, transferred into 24-well cell culture plates (Series 662-160; Greiner Bio Cellstar, Kremsmünster, Austria) and incubated at 25°C for 16 h. *Pseudomonas protegens* CHA0 and *Escherichia coli* DH5 α were used as control bacteria. After incubation, 10 nematodes were plated into independent wells in triplicate. Challenges were carried out and recorded for up to three hours using a Sony Digital HyperHad model SSC-D104P camera recorder (Sony Corporation of America, New York, NY, USA) coupled to a model SMZ-1010X Nikon lens (Nikon Inc., Melville, NY, USA). The challenged individuals were transferred into a new 24-well plate containing 0.5 mL of sterile water per well and each individual was punctured with a single human hair as in Tsai (2000), to confirm nematode mortality. The data obtained from three independent *in vitro* challenges between controls, *Pseudomonas* spp. and *X. index* individuals were

subjected to ANOVA using a *P* value < 0.05 . Means were separated using a Tukey test at the 5% significance level using Statgraphics Centurion XV. Similar procedures were carried out for YM and LB media (Sambrook and Russell, 2001) experimentation.

Protease and lipase activity analyses.

Petri dish assays: Direct evaluation of cells was carried out using overnight cultures of R4 and CHA0 grown in King's B medium. Bacterial cultures (10 μ L) were used to inoculate gelatin (Smith and Goodner, 1958) and Spirit blue agar (Starr, 1941) Petri dishes for proteolytic and lipase direct colony analyses, respectively. The dishes were incubated at 25°C for 48 h for protease and 12 h for lipase analyses, and evaluated for visible degrading activity as judged by the formation of clear haloes around the inoculated area. For supernatant evaluation, cell cultures were grown at 20°C in 50 mL of King's B medium with and without skim milk powder (1 g/L, Oxoid, Hants, UK; Rajmohan et al., 2002) for 72 h and centrifuged for 15 min at 4°C and $23,000 \times g$. The cell supernatants were collected, filtered using a 0.45- μ m filter unit (Millipore, Massachusetts, USA), and incubated with three volumes of cold acetone for 1 h at -20°C . The aqueous phase from each tube was removed by centrifugation at $13,000 \times g$ for 15 min, and the corresponding pellets were resuspended in 1 mL of 50 mM phosphate buffer (pH 7.4). Total proteins (20 μ g) were sown on gelatin and tributyrin Petri dishes, incubated at 4°C and 25°C , respectively, and evaluated visually as indicated. Proteins were determined using the Bradford method (Bradford, 1976).

Electrophoretic assays: The total proteins (20 μ g) from each milk-induced cell supernatant were separated using 12% (w/v) denaturing polyacrylamide gel electrophoresis, as described by Sambrook and Russell (2001).

Protease activity on gelatin gels: Acrylamide gels were renatured four times by immersion in a 0.25% Triton X-100 solution (50 mM Tris-HCl, pH 7.4) and placed over gelatin gels. The gelatin gels were prepared using 12% acrylamide and 0.2% gelatin. Protein electrotransference from the acrylamide to gelatin gels was carried out at 20 V for 20 min using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA). After transfer, the gelatin gels were incubated overnight in developing buffer (30 mM Tris-HCl, 200 mM NaCl, 0.005 mM ZnCl_2 , 5 mM CaCl_2 and 0.3 mM NaN_3 ; pH 7.4) at 4°C and stained with Coomassie Blue. Protease activities were judged as clear zones on a blue background.

Lipase activity on tributyrin gels: Acrylamide gels were renatured with a 0.25% Triton X-100 solution and placed directly on tributyrin gels that were prepared with 12% acrylamide and 2.0% tributyrin. Protein transfer was carried out by capillary transfer using developing buffer [30 mM Tris-HCl, 200 mM NaCl, and 0.02 % w/v NaN_3 ; pH 7.4] for 16 h at 25°C . Lipase activities were judged as clear zones in the gels.

Cell supernatant nematicide trial and scanning electron microscopy: The protein extract (23 μ g) obtained from the milk-induced R4 cell supernatants was added to a well of a 96-well microplate (CulturePlate-96, Perkin Elmer Inc., Waltham, MS, USA) containing 30 μ L of buffer (Tris 25 mM, NaCl 150 mM, pH 7.5) and 30 nematodes. The

Fig. 1. Phylogenetic relationships of new grapevine-associated isolates based on the pseudomonad 16S rRNA gene.

Distances were calculated using Kimura 2-parameters. R, root isolates; S, soil isolates; NF, *Delftia acidovorans* isolates. Circles indicate isolates from this study; rhombuses indicate type strains in the species.

plates were incubated at $24 \pm 1^\circ\text{C}$ for 3 h and the samples were analysed using scanning electron microscopy. Three experiments were performed in triplicate; nematodes were incubated with buffer as a control. Scanning electron microscopy of the challenged samples was prepared by fixation of the samples on 3% glutaraldehyde in 0.268 M sodium cacodylate buffer (pH = 7.0), followed by dehydration and critical point drying, and gold-coating on a 0.22- μm polycarbonate membrane. The samples were visualized using a TM 3000 SEM (Hitachi, Tokyo, Japan).

Mass spectrometry analysis of purified proteins. The total proteins (10 μg) obtained from the milk-induced R4 cell supernatants were subjected to denaturing 12% polyacrylamide gel electrophoresis, after which the bands were cut and the proteins were isolated and processed as indicated by Fernandez et al. (1998). Peptide samples (1 μL) were deposited onto MSP 96 target polished steel micro Scout target plates (Bruker Daltonics, Wissembourg, France), processed as indicated in Fotso et al. (2014) and analysed using a MALDI-TOF Microflex mass spectrometer (Bruker Daltonik GmbH, Germany) controlled by the flexControl 3.0 software (Bruker Daltonik GmbH, Germany). Spectra were recorded under positive ion reflector mode (ion acceleration voltage was 20 kV for MS acquisition), and spectra analyses were performed using the

Fig. 2. Comparative morphology of *X. index* individuals after co-culture with different *Pseudomonas* isolates.

The typical morphology of *X. index* individuals recruited from propagation soil pools indicated a long and wavy body (a), in which a short mucro on the tail (a, bottom right) and a stylet (a, top right) were observed. Challenges between these nematodes and the putative biocontrol isolates were carried out using semi-solid agar dishes of King's B medium in which nematode mobility was judged in co-culture time courses. Semi-solid plates that were pre-incubated for 16 h with 5.8×10^7 bacterial cells were used to challenge ten *X. index* nematodes. Challenges were recorded for three hours using a digital camera. Nematode death was confirmed by transferring the individuals into new plates containing sterile water (b) and puncturing each individual with a single human hair (lethality results are shown in Table 1). While the viable and active nematodes were judged as wavy individuals with constant motion (b, left picture), the non-viable nematodes became rigid and straight (b, right picture).

flexAnalysis version 2.2 software (Bruker Daltonik GmbH). Peptide mass fingerprint and MS/MS profiles were processed for database searches using the Mascot Server tool (Matrix Science Limited, London, England).

Results

Primary characterization of grape-associated isolates

Fluorescence assays, 16S rRNA gene cloning and sequencing enabled a primary selection of 13 out of the 40 isolates collected from the different grapevine yards. Seven of these isolates were obtained from roots (i.e., R1 to R7), and six isolates were obtained directly from soil (i.e., S1 to S6), samples from the areas surrounding the grapevine yards (Table 1). A 1,200 base pair (bp) fragment of the corresponding 16S rRNA gene from each isolate was used for BLAST-N alignments and phylogenetic relationships (Fig. 1). The phylogenetic tree clustered these grape-associated isolates into two major *Pseudomonas* spp. groups; isolates R1 to R3 and S8 to S13 were clustered with *P. putida* strains (branch support between 70 and 90%), and isolates R4 to R7 were clustered with *P. veronii* strains (branch support between 56 and 66%). Isolates R4 to R7 were grouped into a major cluster in which some

Table 1. Primary characterization of the collected isolates.

Isolate	Fluorescence	Source	μ (h ⁻¹)*	First challenge: (Lethality over <i>X. index</i> at 1 h (%))**	Second challenge: (Lethality over <i>X. index</i> at 3 h (%))**	phlD PCR (745 bp amplicon)
R1	+	Root	0.25 ± 0.04 a	43 ± 5.8 bcd	nd	–
R2	+	Root	0.55 ± 0.02 cd	67 ± 5.8 def	90 ± 5.8 a	–
R3	+	Root	0.62 ± 0.01 d	77 ± 15.3 ef	93 ± 5.8 a	–
R4	+	Root	0.46 ± 0.03 b	73 ± 5.8 def	100 [†]	–
R5	+	Root	0.49 ± 0.01 bc	43 ± 15.3 bcd	nd	–
R6	+	Root	0.46 ± 0.01 b	23 ± 11.6 abc	nd	–
R7	+	Root	0.48 ± 0.03 b	27 ± 15.3 abc	nd	–
S8	+	Soil	0.45 ± 0.04 b	17 ± 11.5 ab	nd	–
S9	+	Soil	0.71 ± 0.04 e	33 ± 23.1 abc	nd	–
S10	+	Soil	0.73 ± 0.02 e	27 ± 5.8 abc	nd	–
S11	+	Soil	0.72 ± 0.02 e	53 ± 11.6 cde	nd	–
S12	+	Soil	0.62 ± 0.01 d	50 ± 10.0 bcde	nd	–
S13	+	Soil	0.51 ± 0.02 bc	17 ± 5.8 ab	nd	–
NF15	–	Soil	0.61 ± 0.02 d	7 ± 5.8 a	nd	–
<i>P. simiae</i> WCS417	–	Arabidopsis	0.20 ± 0.03 a	30 ± 5.8 abc	nd	–
<i>P. protegens</i> CHA0	+	Tobacco	0.53 ± 0.03 bc	97 ± 5.8 f	100 [†]	+
<i>E. coli</i> DH5 α	–	commercial	nd	0 [†]	0 [†]	–
No bacteria	nd		nd	nd	0 [†]	–

+ and –, positive and negative analysis result, respectively.

nd, not determined.

*Results obtained using King's B medium for bacterial growth. Values are expressed as the mean ±SD of three experiments performed in triplicate. Means with different letters are significantly different ($p < 0.05$, ANOVA, Tukey-SD).

**Results obtained using King's B medium for bacterial growth and nematode challenge. Values are expressed as the mean ±SD of three experiments performed in triplicate. Means with different letters (a–e) are significantly different ($p < 0.05$, ANOVA, Tukey-SD).

[†]All experiments and trials had this experimental value.

clades were formed by 2,4 DAPG non-producing *P. simiae* WCS417 (isolated from wheat (Van Peer et al., 1990)), *P. fluorescens* SBW25 (isolated from sugar beet (Thompson et al., 1995)), and *P. veronii* CIP104663 (isolated from activated sludge (Ajithkumar et al., 2003)) and 1YdBTEX2 (isolated from a benzene-contaminated site (de Lima-Morales et al., 2013)). The 2,4-DAPG-producing *P. protegens* strains CHA0 and Pf-5 were clustered in a separate branch of the same group. Isolates R1 to R3 and S8 to S13 formed the second group, which included different *P. putida* strains and a group formed by *P. mendocina*, *P. stutzeri* and *P. aeruginosa* strains. One non-fluorescent (NF) isolate was included in this phylogenetic comparison (isolate NF15); this isolate clustered into a different group close to *Delftia acidovorans* IAM12409, which is a soil bacterium that is unrelated to pseudomonads (isolated from soil enriched with acetamide (Den Dooren de Jong, 1926)).

X. index antagonistic activity

Time-course analysis of each isolate allowed for the establishment of specific growth rates based on King's B medium (Table 1). From these data, an evaluation of the nematicidal activity of 5.8×10^7 cells/mL was performed using a one-hour co-culture with *X. index* individuals in semi-solid agar-nutrient dishes (Table 1). A typical morphological structure of a female *X. index* individual under culture is shown in Fig. 2a, in which a cylindrical body (up to 5 mm in length), a stylet on the head (top right) and the presence of a mucro on the tail (bottom right) were observed. The comparative results obtained using semi-solid dishes to co-culture isolates and strains with *X. index* individuals are shown in Fig. 2b; while active nema-

Table 2. Effect of the culture medium on R4 nematicidal activity.

Strain + treatment	μ (h ⁻¹)*	Lethality over <i>X. index</i> at 3 h (%)**
R4 + King's B	0.46 ± 0.03 a	100 [†]
CHA0 + King's B	0.52 ± 0.04 ab	100 [†]
R4 + LB	0.60 ± 0.03 bc	83 ± 5.8 a
CHA0 + LB	0.63 ± 0.09 c	87 ± 5.8 a
R4 + YM	0.61 ± 0.02 c	100 [†]
CHA0 + YM	0.65 ± 0.05 c	100 [†]

*Results obtained using the indicated medium for bacterial growth. Values are expressed as the mean ±SD of three experiments performed in triplicate. Means with different letters are significantly different ($p < 0.05$, ANOVA, Tukey-SD).

**Results obtained using the indicated medium for bacterial growth and nematode challenge. Values are expressed as the mean ±SD of three experiments performed in triplicate. Means with different letters (a–c) are significantly different ($p < 0.05$, ANOVA, Tukey-SD).

[†]All experiments and trials had this experimental value.

todes were wavy individuals in constant motion (Fig. 2b, left picture), non-viable nematodes became rigid and straight (Fig. 2b, right picture). The highest average lethality was obtained with the R2 (67%), R3 (77%), and R4 (73%) isolates (Table 1). After challenging with *Pseudomonas protegens* CHA0 and *P. simiae* WCS417, 97% and 30% of the challenged nematodes, respectively, lost their viability. In general terms, the other new isolates had lethality rates lower than 53%. A second challenge between the three strongest antagonists (i.e., lethality at 1 h > 65%) and *X. index* individuals was evaluated after 3 h of co-culture under the same conditions (Table 1). From these results, the R4 isolate showed the same antagonistic capability as the 2,4-DAPG-producing strain CHA0; how-

Fig. 3. Proteolytic activities in R4 cells and supernatant.

Direct protease assays of R4 and CHA0 cells (a) were visualized by plating the bacteria that were grown overnight in King's B medium onto gelatin-agar Petri dishes and incubating the dishes for 48 h at 25°C (*E. coli* DH5 α was used as a negative control). Protease activity in the cell supernatants (b) was evaluated by inoculating 20 μ g of total proteins extracted from the bacterial supernatants of 72-h cultures grown in King's B medium (R4 and CHA0) or King's B medium supplemented with 1 g/L of milk (R4+M and CHA0+M), followed by plating onto agar-gelatin Petri dishes and incubation at 25°C for 72 h (b). Protease zymograms of these cell supernatants (c) were obtained using the same cell supernatants as before; 20 μ g of protein extracts were subjected to gelatin-acrylamide gel electrophoresis, after which the gels were incubated overnight at 4°C and stained with Coomassie blue.

ever, the close phylogenetic relationship of the R4 isolate to *P. fluorescens* or *P. veronii* suggests that the R4 isolate would potentially differ from strain CHA0 in genetic traits. In addition, the negative controls (i.e., *E. coli* DH5 α and no bacteria) showed no effect on the *X. index* individuals viability after 3 h of challenge. PCR amplification of the 2,4-DAPG-synthesis marker gene, polyketide synthase D (*phlD*) gene (Picard and Bosco, 2003), in new isolates and CHA0 demonstrated that genomic DNA extracted from these strains, including R4, lacked the expected 745-bp fragment which was only seen in the CHA0 sample (Table 1). The possible antagonistic dependence of R4 and the CHA0 strain on the culture medium was assayed by adding LB and YM media to both the growth and challenge conditions (Table 2). Under these additional culture conditions, improved μ values compared with King's B medium (0.46 ± 0.03 (h $^{-1}$) and 0.52 ± 0.04 (h $^{-1}$) for R4 and CHA0, respectively) were obtained, although lower lethality under LB conditions were also observed. In contrast, the μ value for R4 was 0.60 ± 0.03 (h $^{-1}$) in LB, and this isolate showed a lethality of $83 \pm 5.8\%$. In YM, the μ value for R4 was 0.61 ± 0.02 (h $^{-1}$) and the lethality of this isolate against *X. index* individuals reached 100%. *Pseudomonas protegens* CHA0 had μ values of 0.63 ± 0.09 (h $^{-1}$) in LB and 0.65 ± 0.05 (h $^{-1}$) in YM, resulting in $87 \pm 5.8\%$ and 100% lethality, respectively.

Fig. 4. Lipolytic activities in R4 cells and supernatant.

Direct lipase assays of R4 and CHA0 cells (a) were visualized by plating the bacteria that were grown overnight in King's B medium onto Spirit blue Petri dishes for 12 h at 25°C (*E. coli* DH5 α was used as a negative control). Lipase activity in the cell supernatants (b) was evaluated by inoculating 20 μ g of total proteins extracted from the bacterial supernatants of 72-h cultures grown in King's B medium (R4 and CHA0) or King's B medium supplemented with 1 g/L of milk (R4+M and CHA0+M), followed by plating onto tributyrin-agar Petri dishes for 72 h at 25°C. Lipase zymograms of these cell supernatants (c) were obtained using the same cell supernatants as before; the protein extracts were subjected to tributyrin-acrylamide gel electrophoresis and the gels were incubated at 25°C for 16 h.

R4 exoprotease and lipase activity characterization

Rapid screens performed on gelatin Petri dishes revealed that after 48 h of incubation, R4 cells produced degradation haloes with a higher activity exhibited by CHA0 (Fig. 3a). The supernatants from milk-induced cultures that were grown for 72 h and inoculated on Petri dishes for an additional 72 h revealed the induction of proteolytic activity (Fig. 3b), which was characterized by at least three bands at approximately 49, 40, and 30 kDa (Fig. 3c). Similar assays for the detection of lipase activities demonstrated that R4 bacterial cultures grown for 72 h in King's B medium and then plated for 12 h on tributyrin-agar Petri dishes exhibited an activity that was relatively higher than CHA0 lipase activity and the control (Fig. 4a). The same activities were observed in the zymograms (Fig. 4c) and the supernatants from the milk-induced cultures grown for 72 h and inoculated on Petri dishes for an additional 72 h (Fig. 4b). The zymograms indicated activity zones at 55 kDa for CHA0 and both 69 and 50 kDa for R4 (Fig. 4c).

Preliminary characterization of active proteins

The candidate nematocidal proteins (30, 40, 49, 50, and 69 kDa) in the R4 cell supernatant were gel extracted, partially sequenced, and identified (Fig. 5). Sequencing of the peptides (Fig. 5 left panels, bold residues) enabled

Fig. 5. Candidate proteins for nematicidal activity in R4.

Pseudomonas R4 cell supernatants from cultures grown under milk induction were subjected to electrophoretic separation, and proteins with putative nematicidal activity were gel extracted and sequenced using mass spectrometry. Integrated mass/charge profiles led to the identification of peptides (left panels, bold amino acids) that were assembled and assigned (using Mascot server tool) into three full-length proteins: AprA (a), LipA (b), and ExoU (c) (full-length sequences in left panels). The proteins corresponded to deduced amino acid sequences from the *P. veronii* strain 1YBTEX2 genome (whole-genome shotgun project deposited at DDBJ/EMBL/GenBank under the accession number AOUH000000000). Functional schemes for these proteins (right panels) were built using available information from protein descriptions in pseudomonads. Residues and/or motifs involved in protein activity (underlined residues), secretion (italic and underlined), calcium binding (boxed), and relevant residues (dotted) for these functions are shown (left panels) and represented (right panels). Accession numbers: AprA WP_017845712; LipA (b), WP_017845717; ExoU (c), WP_017844966.

Fig. 6. Scanning electron microscopy (SEM) of *X. index* individuals challenged with R4 cell supernatants.

Pseudomonas R4 cell supernatants from cultures grown with milk induction were pelleted with acetone and resuspended using phosphate buffer. Total proteins (20 μ g) were added to the wells of 96-well microplates containing 100 μ L of buffer and 30 nematodes. The plates were incubated at $24 \pm 1^\circ\text{C}$ for 3 h and the samples were analysed using SEM. Nematode morphology after individual microscopy immobilization without proteins (a) and with the addition of R4 proteins (b)–(d) is shown. All of the individuals were affected by the treatment; the effects shown vary from small cuticle perforations at specific areas (b, white arrow; detail shown in c) leading to the exposure of internal organs including digestive system (c), to pronounced degradation of vital areas (d) leading to dehydration of nematode bodies.

the recognition of assembled protein contexts (Fig. 5 left panels) with a high identity to deduce proteins based on the genome information of the *P. veronii* isolate 1YdBTEX2 (DDBJ/EMBL/GenBank genome project accession number AOUH00000000). The percentage of sequenced residues of the assigned *P. veronii* full-length protein (i.e., “coverage”) varied depending on the assignment. A 49-kDa protein (accession number WP_017845712 in 1YdBTEX2) with a 60% identity to *P. aeruginosa* PAO1 metalloprotease AprA (NCBI accession number NP_249940) was identified with a coverage of 14% (i.e., 69 sequenced/477 total residues; Fig. 5a bold font in the sequence panel). The extracted proteins of lower mass (i.e., 30 and 40 kDa) corresponded to this same protein. The isolated 50-kDa protein (WP_017845717 in 1YdBTEX2) was similar to LipA lipase from *P. fluorescens* SBW25 (90% identity; accession number WP_012724316.1) based on 198 experimentally sequenced amino acids (41% coverage; Fig. 5b, bold fonts). The 69-kDa protein (WP_017844966 in 1YdBTEX2) resulted in ExoU exophospholipase, with an identity of 46% to *P. aeruginosa* UCBPP-PA14 (accession number ABJ10150.1), as derived from 206 sequenced residues (i.e., coverage 32%; Fig. 5c bold font). The challenge of *X. index* individuals against this protein-enriched supernatant fraction, which contained at least these three identified proteins from R4, was conducted by *in vitro* co-cultures during a 3-h challenge. All of the treated individuals died after incubation, indicated by light microscopy and puncture of these samples (not shown). Electron microscopy analysis (Fig. 6) revealed that control individuals showed

a whole body integrity and rigid shape (Fig. 6a) under the fixation procedures used. On the contrary, challenged nematodes exhibited very few individuals with initial steps of degradation (Figs. 6b and c), and most of them formed clusters of degraded entities in which nematode cuticles, external pores, and striations across the body resulted in internal emptying of intestines and other structures (Fig. 6d).

Discussion

Although nematode antagonism by *Pseudomonas* spp. has received some attention, few studies have focused on the biocontrol of the grapevine nematode *X. index*. Recently, Aballay et al. (2011) described the collection of approximately 400 rhizobacterial isolates associated with *V. vinifera* L. roots. Using fatty acid methyl ester profile analysis, these authors identified 131 different species in their collection and challenged *X. index* individuals using pot assays (greenhouse) and direct immersion in bacterial filtrate (*in vitro*) assays. Two *P. fluorescens* isolates were identified among the most suppressive isolates, in addition to *Bacillus brevis*, *B. megaterium*, *Pseudomonas corrugata*, *Serratia plymuthica*, *Stenotrophomonas maltophilia*, *Pseudomonas putida*, and *Pseudomonas savastanoi* pv. *Fraxinus* isolates. We used 16S rRNA gene sequence comparisons as a preliminary analysis in the identification of different *Pseudomonas* spp. inhabiting the roots of grapevines. Native *P. putida* isolates (i.e., S8 to S13) that exhibited a high phylogenetic identity (>98%) to *P. putida* Arph1 and *P. putida* KT2440, which are known pyoverdine producers and soil isolates (Matthijs et al., 2009), were found in our collection. Another set of root isolates (i.e., R1, R2, and R3) exhibited a high identity (>98%) to *P. putida* IAM1236, whose native isolates from the roots of *Panicum antidotale* show nitrogen fixation and plant growth promotion (Mirza et al., 2006). The identified *P. veronii* R4 to R7 isolates showed closer phylogenetic relationships with *P. simiae* strain WCS417, *P. fluorescens* SBW25 and *P. veronii* CIP104663. The clustering results obtained from the 16S rRNA gene analysis in the present work were consistent with a previous *Pseudomonas* spp. phylogenetic analysis performed by Moynihan et al. (2009), in which genomic and sequence-based approaches were integrated to reconstruct the phylogeny of *P. fluorescens* and the 2,4-DAPG synthesis (*phlD*) gene cluster. In that work, three groups were characterized: a) the *P. syringae* group (i.e., DC3000, 1448A, and B728a), b) the *P. fluorescens* group (i.e., Pf-5, SBW25, F113, and Pf01), and c) the cluster formed by the *P. putida* KT2440 and *P. entomophila* L48 strains. Most of our grape root-associated bacteria were not predicted to be 2,4-DAPG producers. Brazelton et al. (2008) demonstrated that 2,4-DAPG production inhibits primary root formation and stimulates lateral root growth in tomato via an auxin-dependent signal transduction pathway. In addition, our results (Fig. 1) showed that the isolates R4 to R7 exhibit relationships with root growth-promoting rhizobacteria that are characterized by IAA production, efficient root colonization capability and antagonistic activity due to phenazine acid derivatives (Mavrodi et al., 2006).

The propagation system for *X. index* allowed a proper population of individuals, as judged by PCR (data not shown) and morphology (Fig. 2). Loss of mobility (i.e., viability) trials using different *in vitro* culture media were performed for adequate R4 *X. index in vitro* challenges. Tsai (2000) observed dead *Meloidogyne javanica* individuals in similar *in vitro* 24-h mobility tests, and Terefe et al. (2009) observed *M. incognita* deaths in 24-, 36- and 48-h challenges. Under our conditions, viability assays using King's B medium for *X. index* revealed that individuals did not survive more than 24 h using this semi-solid dish-culturing approach. Nevertheless, this time-course analysis using short-term cultures between 1 and 3 h was informative and revealed important differences in antagonistic features between strains that achieved 100% *X. index* mortality (i.e., isolate R4 and the CHA0 strain). The biocontrol strain *P. protegens* CHA0 required as little as one hour of interaction to achieve 100% effectiveness in King's B dishes; the R4 isolate was able to affect nematode viability at the same rate after 3 h of challenge (Table 1). In addition, the use of King's B and YM media resulted in the same maximal rates (100%) of lethality for both R4 and CHA0. These media have been described as strong promoters of secondary metabolite synthesis for *P. protegens* Pf-5 (Bangera and Thomashow, 1999) and CHA0 (Neidig et al., 2011).

Considering the suppressive performance described for CHA0, our results suggested that R4 utilizes different mechanisms for influencing nematode viability. The first difference between these strains arose from *phlD* gene amplification (Table 1); this gene is a marker for 2,4-DAPG producers (Keel et al., 1996). Regarding R4 selection, Hassan et al. (2011) isolated and identified the *P. putida* NH-50 strain from sugar cane and demonstrated that, although the same primers we used in this work did not lead to positive amplification in that strain, the high efficiency obtained for red rot disease control, compared with the CHA0 strain, was due to pyoluteorin synthesis and HCN production. In addition, 2,4-DAPG non-producer mutants of CHA0 (i.e., DAPG- and CHA631) did not differ from the native strain with respect to inhibiting *C. elegans* individuals (Neidig et al., 2011) under *in vitro* conditions. Nematicidal potential will depend on the actual sensitivity of nematode targets to 2,4-DAPG, as observed for *M. javanica* (Hamid et al., 2003) and *M. incognita* J2 or *C. elegans* J1 (Meyer et al., 2009).

Although the protease activity in R4 seemed not as relevant as in CHA0 (Fig. 3), the occurrence of two important lipase activities (i.e., 50 and 69 kDa) was identified in the R4 cell extracts (Fig. 4). From these observations, the immediate involvement of these activities in the nematicidal activity against *X. index* can be proposed. In CHA0, AprA has been described to inhibit *Meloidogyne incognita* egg hatching and to cause the death of young nematodes (Siddiqui et al., 2005). Consistent with the activity detected in the gels and the protein sequencing results, a metalloprotease (49 kDa) and a phospholipase (69 kDa), which were similar in size to the *P. aeruginosa* AprA and ExoU proteins, respectively, were observed. The latter corresponds to an effector protein of the type III secretory system in *P. aeruginosa* and is one of the most important

virulence factors (Abd et al., 2008). This virulence factor induces organelle cell membrane damage and vacuole fragmentation in yeast (Sato and Frank, 2004) and leads to irreversible damage and rapid cell necrosis in mammalian cells (Bleves et al., 2010).

Several findings suggest that low enzyme concentrations could cause cytotoxicity in cell membrane of nematodes. Bacterial charges found in the rhizosphere could reach 10^8 bacteria per gram of root tissue (Haas and Keel, 2003); consequently, the secretory enzymes produced by bacteria could lead to structural damage of nematodes sharing the same ecological niche. In addition, micro-wounds on the cuticle of nematodes could improve colonization by other agents naturally occurring in the same area, leading to the exposure of nematodes to further metabolites from the rhizosphere under a synergistic effect to R4. Also it has been estimated that just 300 to 600 ExoU molecules from *P. aeruginosa* are required for a cytotoxic effect (Phillips et al., 2003; Rabin et al., 2006). ExoU from R4 showed an identity of 45% to the *P. aeruginosa* UCBPP-PA14 enzyme and presented conserved domains for recognition and catalytic activities on the target cell (Fig. 5). Finally, R4 secreted enzymes present recognition domains for types I and III secretion systems, and the latter has been reported to make direct contact with the target (Phillips et al., 2003; Rabin et al., 2006) which could enable the direct secretion of ExoU to the cell membrane of the cuticle in the nematodes.

Several of the sequenced peptides (bold residues in left panels; Fig. 5) from these proteins are found in relevant functional motifs observed in characterized AprA (Zhang et al., 2012), LipA (Duong et al., 1994), and ExoU (Price-Whelan et al., 2007) proteins from model *Pseudomonas* strains (right panels; Fig. 5). The secreted nature of these proteins is consistent with the nematicidal effect observed in R4 cell supernatants, suggesting that several of these exoenzymes in R4 could be responsible for the nematicidal activity. At the C-terminus of AprA metalloprotease (Fig. 5a), which is secreted by the Type 1 Secretion System (T1SS; Duong et al., 1996), one out of three "repeat in toxin" (RTX) motifs (Fig. 5a, right panel) containing a GGXGXD arrangement (Linhartová et al., 2010) was identified (Fig. 5a, boxed amino acids). "Repeat in toxin" motifs have been described to coordinate Ca^{+2} -enabled protein folding and stability. In a similar way, LipA residues participating in Ca^{+2} coordination (Thr¹¹⁸, Gln¹²⁰, Ser¹⁴⁴; Fig. 5b) and catalytic activity (Asp²⁵⁵; Fig. 5b) were experimentally identified (Angkawidjaja et al., 2007; Duong et al., 1994). As in AprA, RTX motifs were experimentally determined in the C-terminal portion of the putative LipA. In the exophospholipase ExoU, a T3SS effector protein (Phillips et al., 2003), 61 N-terminal residues supporting this secretory classification (Hauser, 2009), and several sequenced amino acids linked to secretory (Thr²⁹ to Arg⁴⁴) and cell localization (Ala⁵⁶⁵ to Arg⁵⁷⁰; Ala⁵⁹³ to Arg⁶²⁰) motifs, were confirmed (Fig. 5c). These results could suggest ExoU capability for cell membrane recognition and targeting (Hauser, 2009; Rabin and Hauser, 2005) in R4.

Recently, the whole-genome shotgun project for R4 has been deposited in DDBJ/EMBL/GenBank under the ac-

cession JXWQ00000000 (version JXWQ02000000). The described characterization and identification of this new *P. veronii* isolate, and the functional assignments of these candidate proteins have been reinforced. The cuticle of nematodes is rigid and is composed of proteins and lipids that can be affected by hydrolytic enzymes from nematocidal bacteria (Lian et al., 2007). Our results suggested that at least these hydrolytic enzymes might be involved in the nematocidal activity via cuticle degradation of connective tissues in their digestive tracts (Yang et al., 2013). In particular, the lipolytic activity could be relevant for the observed nematocidal activity against *X. index*, and indicates the potential application of the R4 isolate as a source of biotechnological biocontrol tools in the future.

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