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Pseudomonas fluorescens UP61 isolated from birdsfoot trefoil rhizosphere produces multiple antibiotics and exerts a broad spectrum of biocontrol activity

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Abstract

The efficient use of rhizospheric microorganisms to control plant pathogens has been reported worldwide in different plants. *Pseudomonas fluorescens* UP61 is a biocontrol strain isolated from the rhizosphere of *Lotus corniculatus* (birdsfoot trefoil) from Uruguayan soils. This strain exhibited *in vitro* antagonistic activity against a broad spectrum of fungal and bacterial phytopathogens. It was an effective biocontrol agent in different hosts, reducing the disease incidence caused by *Sclerotium rolfsii* in beans and *Rhizoctonia solani* in tomato. *P. fluorescens* UP61 produced three antibiotics possibly involved in its biocontrol activity: 2,4-diacetylphloroglucinol, pyrrolnitrin and pyoluteorin. Molecular techniques such as 16S rDNA RFLP, RAPD and rep-PCR, and partial sequence of the *phlD* gene, revealed the similarity of UP61 with other biocontrol strains isolated worldwide that are able to produce these antibiotics.

Abbreviations: DAPG – 2,4-diacetylphloroglucinol; MAPG – monoacetylphloroglucinol; PCA – phenazine-1-carboxylic acid; Phz – phenazine; Plt – pyoluteorin; Prn – pyrrolnitrin.

Introduction

Rhizospheric fluorescent *Pseudomonas* spp. have been described as biological control agents that reduce the incidence of different plant pathogens (O'Sullivan and O'Gara, 1992). Root diseases have been the main research focus, although in the last decade attention has been drawn to the control by single rhizospheric strains of multiple pathogens affecting different plant organs (Kloepper et al., 1993). To minimize the inconsistency in the biocontrol activity of bacterial strains, it is appropriate to search for strains adapted to the soil and climate conditions where they will be applied (Handelsman and Stabb, 1996).

The most commonly reported mechanisms of biocontrol by fluorescent *Pseudomonas* spp. include production of antibiotics, hydrogen cyanide (HCN), lytic exoenzymes, competition for iron mediated by siderophores, competition for carbon,

and induced systemic resistance (Thomashow and Weller, 1996). The antibiotics 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (Plt), pyrrolnitrin (Prn) and different phenazine (Phz) derivatives, have been described in biocontrol *Pseudomonas* spp. as the main cause of their antagonistic activity (Thomashow and Weller, 1996; Ligon et al., 2000).

Multiple mechanisms of biocontrol have been described in some fluorescent *Pseudomonas* strains. An example is *P. fluorescens* CHA0 (Voisard et al., 1994), whose production of DAPG, Plt and HCN was shown to play a role in disease control. This strain is also able to induce systemic resistance in tobacco plants against the disease caused by *Tobacco necrosis* virus (Maurhofer et al., 1994). These different mechanisms allow CHA0 to act as a biocontrol agent in different host plants and against different pathogens. Although other multiple antibiotic producers have been reported (Keel et al., 1996), some biocontrol fluorescent pseudomonads usually have the ability to produce a single type of antibiotic (Mavrodi et al., 2001).

Pseudomonas fluorescens UP61 was isolated from the rhizosphere of *Lotus corniculatus* (birdsfoot trefoil) healthy plants from Colonia, Uruguay and reduced the incidence of *Rhizoctonia solani* and *Pythium ultimum* infection in this host. HCN and fluorescent siderophore production was detected among the factors possibly involved in its biocontrol activity (Bagnasco et al., 1998). Co-inoculation assays showed that its presence on birdsfoot trefoil, alfalfa and white clover seeds does not affect the growth promotion effect of rhizobia strains used locally as commercial inoculants (De La Fuente et al., 2001).

In the present work, the efficacy of *P. fluorescens* UP61 as a biological control agent in different host plants was studied. Antibiotic production by this strain was assessed and its genetic and phenotypic properties were compared to other biocontrol strains isolated worldwide.

Materials and methods

Microorganisms

Pseudomonas fluorescens UP61 and a collection of 24 isolates of fluorescent *Pseudomonas* spp. were isolated from the rhizosphere of healthy birdsfoot trefoil plants from two regions of Uruguay: Colonia and Tacuarembó. *Pseudomonas fluorescens* Q2-87, 2-79, CHA0, Pf-5 (Thomashow and Weller, 1996), *P. fluorescens* Q8r1-96 (Raaijmakers and Weller, 1998) and *Pantoea agglomerans* IC1270 (Chernin et al., 1995) were used for comparison. *Escherichia coli* JM109 was used for cloning procedures. Phytopathogenic bacteria used included *Agrobacterium tumefaciens, Clavibacter michiganensis* subsp. *michiganensis*, *Corynebacterium insidiosum, Erwinia carotovora*, *Erwinia herbicola*, *Pseudomonas phaseolicola*, *Pseudomonas syringae* pvs. *atrofaciens*, *lachrymans* and *syringae*, *Xanthomonas campestris* pvs. *vesicatoria*, *malvacearum* and *cucurbitae*. Bacterial cultures were stored frozen in KB or NB broth plus 25% glycerol at −80 °C. Fungal phytopathogens used included *Rhizoctonia solani* RB1 (INTA Balcarce Collection, Argentina), *Fusarium oxysporum* f. sp. *melonis*, *Sclerotium rolfsii*, *Botrytis cinerea* and *Gaeumannomyces graminis* var. *tritici* and the oomycete *Pythium ultimum*. Fungi were kept in PDA plates at room temperature or 4 °C, and replicated periodically.

Media and culture conditions

Bacterial strains were grown on: King's B medium (KB; King et al., 1954), Bacto *Pseudomonas* agar F (PSF, Difco®), PSF modified by the addition of 15 g of glycerol per litre and 50 μM $FeCl_3$ (PSFM), tryptic soy broth (TS) and tryptic soy agar (TSA) (Difco®), yeast malt (YM, Bangera and Thomashow, 1996), Luria-Bertani (LB, Sambrook et al., 1989), nutrient broth or agar (NB, NA, Difco®). *Pseudomonas* and phytopathogenic strains were routinely grown at 25 °C and *E. coli* at 37 °C, either on agar plates or in a rotary shaker (200 rpm). Fungi were grown on potato dextrose agar (PDA, Difco®) or Czapek (Difco®, for *F. oxysporum* f. sp. *melonis*) and incubated at 25 °C.

Fungal and bacterial inhibition assays

Antagonistic assays against phytopathogenic fungi were performed as described by Geels and Schippers (1983). *P. fluorescens* UP61 was inoculated near the edge of KB, PDA, PSF, PSFM, TSA or YM media plates using sterile a cotton swab. Czapek medium was used with *F. oxysporum* f. sp.

melonis instead of KB, PSF and PSFM, since the fungus grew poorly on these media. A 0.5-cm-diameter plug containing actively growing mycelium was placed in the center of the plate 24 h later. Plates were incubated at 28 °C until the mycelium in a control plate containing exclusively the phytopathogenic fungus reached the borders of the plate (3–20 days). The presence of a zone of fungal growth inhibition was considered as antagonism. Antibacterial activity was assessed on plates according to Homma et al. (1989). *P. fluorescens* UP61 was grown as spots on NA plates for 12–18 h and exposed to chloroform vapors for 30 min. After aeration, plates were covered with a suspension of the phytopathogenic bacteria, obtained by mixing 5 ml of diluted NA (0.6% agar) with 200 µl of a stationary phase culture in NB. Plates were incubated at 30 °C until inhibition halos were detected. Fungal and bacterial inhibition assays were repeated independently at least twice.

Disease protection bioassays

Sclerotium rolfsii – *beans*

One thousand-cubic-centimeter pots were filled up to 2/3 of their volume with sandy loam soil (pH 7.2, Rehovot, Israel) and 10 bean (*Phaseolus vulgaris* L.) seeds were sown. The remaining volume was filled with sieved soil infected with *S. rolfsii* sclerotia (70–80 mg kg^{-1} of soil). Bacteria were inoculated as a water suspension (10^9 CFU kg^{-1} of soil approximately) twice, at the beginning of the experiment and 7 days later. The disease control pots did not include bacterial inoculation, and a germination control was set without any bacterial or fungal inocula. Pots were kept in a greenhouse (28–30 °C) and watered daily. Disease incidence was evaluated after 15–20 days as the percentage of seedlings showing symptoms of stem rot.

Rhizoctonia solani – *tomato*

One plate containing a two-day-old *P. fluorescens* UP61 culture was used to bacterise 1 g of tomato seeds (*Lycopersicon esculentum* cv. San Pedro), and the *R. solani* RB1 inoculum was prepared using oat-kernels (Weller and Cook, 1983). Assays were performed in 400 cm^3 pots containing a mixture of sand and soil (1:3, Jardín Pocitos S.R.L., Montevideo, Uruguay), which were supplemented with 9 oat seeds inoculated with *R. solani* RB1, and 10 tomato seeds were sown per pot. Each experiment included the same controls as described above. Pots were placed in a growth chamber set for a 16-h photoperiod and 20 °C. Plants were harvested 14–17 days after sowing, and disease incidence was determined by scoring the number of emerged plants.

Experimental design and statistical analysis

Disease protection assays were designed in blocks. Each treatment was replicated six times and assays were repeated independently three times. Data were analysed by the analysis of variance procedure (ANOVA) with Statistica 5.0 (StatSoft, Inc.). Significant effects ($P < 0.05$) were subject to contrasts using Duncan's test.

Detection of antibiotic biosynthetic loci

The presence of biosynthetic loci involved in the production of the four most common antibiotics found in fluorescent *Pseudomonas* strains with biocontrol activity (Thomashow and Weller, 1996) was screened by PCR. For the four different reactions, samples (5 µl) of the amplification products were electrophoresed in 0.8% agarose gels in 1X Tris-borate-EDTA (TBE) buffer at 100 V for 1 h at room temperature. Ethidium bromide was included in the gel at 0.5 µg ml^{-1}. All experiments were repeated three times independently, and reactions without DNA template were included.

DAPG. Primers Phl2a and Phl2b, which amplify a 745-bp segment, were developed from sequences within *phlD* of *P. fluorescens* Q2-87 (Raaijmakers et al., 1997). The reaction was performed according to Raaijmakers et al. (1997) except for the use of 1.5 mM $MgCl_2$, and the use of 4 µl of a diluted heat-lysed cell suspension as template. The PCR program consisted of an initial denaturation at 94 °C for 2 min followed by 24 cycles of 94 °C for 60 s, 46 °C for 45 s, and 72 °C for 45 s; and a final extension at 72 °C for 60 s.

Phz. Primers Phz1 and Phz2 were developed from sequences within *phzC* and *phzD* and allow the amplification of a 1.4-kb segment (D.V. Mavrodi,

Washington State University, USA, pers. comm.). The reaction was carried out as described for DAPG but with the addition of 3.2% DMSO, and a one-day-old colony was used as DNA template. Samples were exposed to a denaturation step at 94 °C for 120 s, followed by 25 cycles of 94 °C for 60 s, 56 °C for 45 s, and 72 °C for 105 s, finishing with 72 °C for 60 s.

Prn and Plt. Primers PrnCf and PrnCr detect a 719-bp segment from *prnC*, and PltBf and PltBr a 773-bp segment from *pltB*, genes involved in the biosynthesis of Prn and Plt, respectively. Prn and Plt primers were developed from *P. fluorescens* BL915 and Pf-5 sequences, respectively (Mavrodi et al., 2001). Reactions mixture were set as described for DAPG, but a one-day-old colony was used as DNA template. PCR cycling for both sets of reactions was performed according to Mavrodi et al. (2001).

Isolation and detection of antibiotics

Bacteria were grown in 5 ml of KB, YM, TS or PSFM media for 2 days at 27 °C in a rotary shaker at 200 rpm. Dilutions 1:100 in fresh broth were done and incubated under the same conditions for 3–4 days. 4 ml aliquots of cultures were extracted twice with ethyl acetate as described by Bonsall et al. (1997). The organic phase containing antibiotics was evaporated to dryness in a fume hood, suspended in 1 ml of 35% acetonitrile with 0.1% TFA, and spun at 14,000 rpm for 10 min. The supernatant was used for HPLC analysis. The activity of the organic phase was checked against *R. solani*.

The production of antibiotics was detected by high performance liquid chromatography (HPLC) according to Bonsall et al. (1997) with a 30-µl sample injection. HPLC gradient profiles were monitored at the spectral peak maxima of 250, 255, 270 and 308 nm, characteristic of phenazine-1-carboxylic acid (PCA), Prn, DAPG and Plt, respectively, in the designated solvent system.

An extract obtained from *P. aureofaciens* 30–84 grown in LB was used as standard for three Phz derivatives: PCA, 2-hydroxyphenazine-1-carboxylic acid, and 2-hydroxyphenazine. Monoacetylphloroglucinol (MAPG, Sigma), and purified DAPG, Plt and Prn were used as standards.

16S rDNA RFLP (restriction fragment length polymorphism) analyses

For 16S rDNA amplification, the PCR reaction included 2 pmol of each primer 8F and 1492R (McSpadden Gardener et al., 2000), 1.65 U Taq polymerase (Promega), 1X buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µg RNAse, 3 µl of thawed template (corresponding to 10^4–10^6 bacteria), and enough water (HPLC quality) to complete 25 µl. PCR cycling was performed according to McSpadden Gardener et al. (2000) but using 70 °C and 50 °C as extension and annealing temperatures, respectively.

Restriction digestion of the amplified 400-bp fragment was performed with 5 µl of 16S rDNA amplification product and 10 U of each enzyme *Msp*I, *Hae*III or *Rsa*I (New England Biolabs). Samples were incubated for 4 h 30 min at 37 °C, stored at –20 °C and electrophoresed in 1.5% agarose gels in 0.5% TBE at 140 V for 3–4 h at room temperature. Banding patterns were visualised by ethidium bromide staining and scored by comparison to a 100 bp DNA ladder.

RAPD and rep-PCR analyses

For RAPD (random amplified polymorphic DNA) analyses, primers M13 and D7 (Keel et al., 1996) were used. Reaction mixtures contained 3.2 mM of each primer, 1 U of *Taq* polymerase Stoffel fragment (Perkin–Elmer), 1X Stoffel fragment buffer (Perkin–Elmer), 4 mM $MgCl_2$, 160 µM dNTPs, 4 µl of genomic DNA (50 ng approximately) and enough water (HPLC quality) to complete 25 µl. Genomic DNA was purified by standard methods (Sambrook et al., 1989). PCR cycling was performed according to Mavrodi et al. (2001) but with a final extension at 72 °C for 10 min.

Repetitive extragenic palindromic-PCR (rep-PCR) reactions were performed using primers ERIC and BOX (de Bruijn, 1992). Reaction mixtures and cycling were performed according to McSpadden Gardener et al. (2000), using 25 pmol of each primer.

RAPD and rep-PCR analyses were repeated independently twice, and reactions without DNA template were included. Samples (6 µl) of the PCR products were electrophoresed in 1.5% agarose gels in 0.5X TBE at 140 V for 6h at 4 °C. Gels were stained in a solution of ethidium bromide.

Data were analysed by GelCompar 4.0 (Applied Maths, Kortrijk, Belgium).

Cloning of a phlD gene fragment

Cloning procedure was performed according to McSpadden Gardener et al. (2001) with some modifications. A fragment of *phlD* was amplified by PCR with primers Phl2a and Phl2b as described above. The specific 745-bp band amplified was separated in a 0.8% agarose gel, extracted with a gel extraction kit (QIAEX II, Qiagen), and ligated into pGEM®-T Easy vector (Promega) according to the manufacturer's protocol. Plasmids were introduced into competent cells of *E. coli* JM109 according to Sambrook et al. (1989). Recombinant clones were selected on LB containing Amp (100 μg ml^{-1}), X-Gal (20 μg ml^{-1}) and IPTG (23 μg ml^{-1}). Plasmid DNA was isolated by miniprep (Sambrook et al., 1989), checked for the presence of the insert by digestion with *Eco*RI (GIBCO BRL Technologies), and used for sequencing.

DNA sequencing

The nucleotide sequences were determined by the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin–Elmer) according to the manufacturer's instructions. The fragment of about 400 bp of 16S rDNA was purified by QIAEX II (Qiagen), cycle sequenced with primer 519R (Lane et al., 1985), and used for sequencing. Data were compared to DNA databases using BLAST analysis (Altschul et al., 1997).

Two different clones containing *phlD* were analysed using primers M13 Universal and Reverse (McSpadden Gardener et al., 2001). Nucleotide sequence data were analysed with OMIGA 1.1.3 software (Oxford Molecular Ltd.) and ClustalW (European Bioinformatics Institute), and for amino acid sequences BLASTP (Altschul et al., 1997) analysis was used.

Results

Antagonistic activity in vitro

P. fluorescens UP61 showed a broad spectrum of antagonistic activity *in vitro* against different microbial pathogens affecting roots, stems, leaves, seeds or fruits of different crops. Antagonism against fungi was observed with little variation in all the growing media tested such as KB, PDA, PSF, PSFM, TSA, YM and Czapek. Notably, *R. solani* and *S. rolfsii* were not inhibited when tested on PDA, while an important activity was observed in KB. The addition of iron to PSF (PSFM) did not affect the antagonistic activity of UP61 against *P. ultimum* and *R. solani*.

When UP61 was tested against twelve bacterial plant pathogens, inhibition halos were observed on NA plates against *A. tumefaciens*, *C. michiganensis* subsp. *michiganensis*, *C. insidiosum*, *P. syringae* pv. *atrofaciens*, *P. syringae* pv. *syringae*, and *X. campestris* pv. *malvacearum*.

Biocontrol activity in different hosts

Disease incidence achieved by *S. rolfsii* in beans was about 48% in three independent experiments performed under greenhouse conditions. *P. fluorescens* UP61 efficiently decreased disease incidence ($P = 0.001$) to about 20% (Figure 1), *P. agglomerans* IC1270 achieved to about 28% reduction ($P = 0.017$). Treatment-assay interactions were not significant in bean ($P = 0.806$) or tomato ($P = 0.503$) experiments.

Bacterised tomato seeds contained an inoculum of about 5×10^9 CFU $seed^{-1}$. Growth chamber assays showed that inoculation with UP61 increased the emergence of tomato plants by 70% in the presence of *R. solani* compared to the disease control (Figure 1). Root rot was not detected in plants infected with the isolate used, for this reason only damping-off was evaluated.

Biosynthetic genes involved in antibiotic production

A collection of 25 native fluorescent *Pseudomonas* spp. strains selected for their antagonism *in vitro* against *P. ultimum* (Bagnasco et al., 1998) was used in all the PCR reactions aimed to detect biosynthetic genes for antibiotics. Genes involved in DAPG production were detected only in strain *P. fluorescens* UP61, and in two isolates from Tacuarembó, which amplified a band of the same size as that from *P. fluorescens* Q2-87. Both Tacuarembó isolates proved to be genetically close when analysed by RAPD-PCR with primers

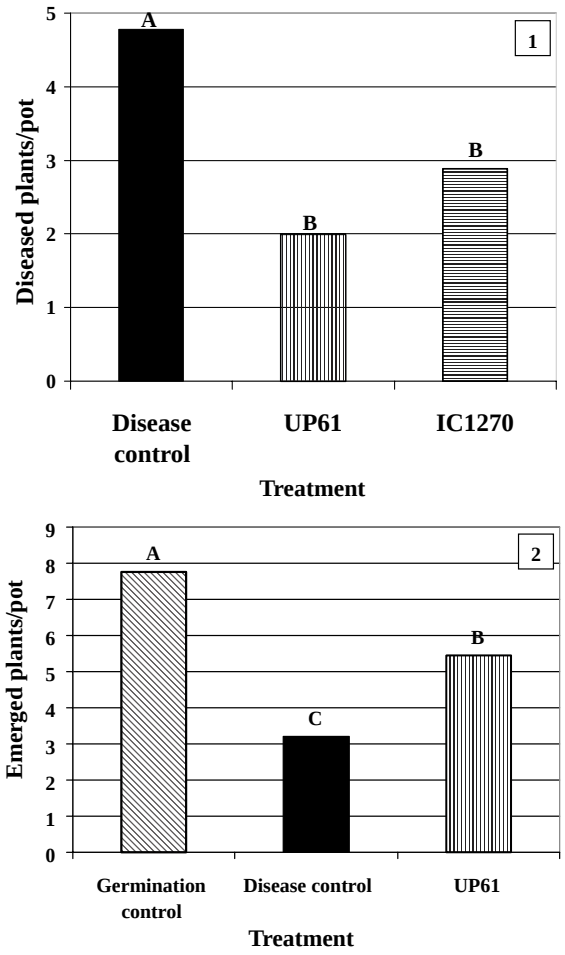

Figure 1. Biocontrol of *S. rolfsii* in beans (1) and *R. solani* in tomato (2) by antagonistic bacteria under controlled conditions. In (1) the experiment was carried out in the greenhouse. *P. fluorescens* UP61 and *P. agglomerans* IC1270 were inoculated as a water suspension in the soil. No diseased plants were found in the germination control (data not shown). In (2), the experiment was performed under growth chamber conditions. Seeds were inoculated with *P. fluorescens* UP61 using methylcellulose as adherent (5×10^9 CFU seed^{-1}). Values represent means from three independent experiments. Data were analysed by ANOVA. Different letters mean significant difference between treatments ($P = 0.004$ (1), $P = 1.4 \times 10^{-7}$ (2)).

M13 and D7, but both differed from UP61 (data not shown).

Using primers Phz1 and Phz2, a band of 1.4 kb was amplified from *P. fluorescens* 2–79, a strain bearing the genes for PCA production (Mavrodi et al., 1998). Nevertheless, these genes were not detected in UP61 nor in the other strains from the collection.

When the collection was screened for biosynthetic genes for Prn and Plt, only UP61 amplified the specific bands obtained with *P. fluorescens* Pf-5 and CHA0 (strains that produce both antibiotics, Thomashow and Weller, 1996) at 719 bp and 773 bp for Prn and Plt, respectively.

Production of antibiotics in broth

The production of antibiotics depended on the media in which *P. fluorescens* UP61 was grown. When analysed on PSFM, it was possible to identify by HPLC the production of Prn and Plt (Figure 2). The spectra and the retention times of the compounds produced by UP61 (19.06 min for Prn and 14.8 min for Plt) were the same as those observed for the standards.

When the strain was grown on YM the production of DAPG (Figure 2) and its precursor MAPG was detected, with retention times of 16.7 and 9.78 min, respectively. The spectra were identical to those observed for the standards.

Spectra of the peaks detected in HPLC elution profiles of *P. fluorescens* UP61 extracts grown in KB and TS media did not match with any of the standards (data not shown). Nevertheless, the antagonistic activity of UP61 on these media was demonstrated against *P. ultimum* and *R. solani* (see above).

Genetical comparison with antibiotic-producing strains

16S rDNA. Using primers 8F and 1492R, a fragment of 16S rDNA of approximately 1600 bp was amplified. Restriction analysis banding patterns with *Hae*III, *Msp*I and *Rsa*I clearly showed the similarity between *P. fluorescens* Pf-5 and UP61, which differed from 2-79 and Q8r1-96. Pf-5 and UP61 yielded bands at 130 bp, 180 bp, 220 bp and 900 bp (when restricted with *Hae*III); 120 bp, 140 bp, and 570 bp (with *Msp*I); and 140 bp, 350 bp and 850 bp (with *Rsa*I), approximately. The sequence of a 400 bp 16S rDNA fragment was studied after sequencing with primer 519R. The region between 30 and 332 bp was analysed for UP61, and showed 99% identity with strains *Pseudomonas* sp. PsK (Kuske et al., 1999) and *Pseudomonas* sp. HS2 (Hoffman et al., 1998) when compared to DNA databases by using BLAST analysis.

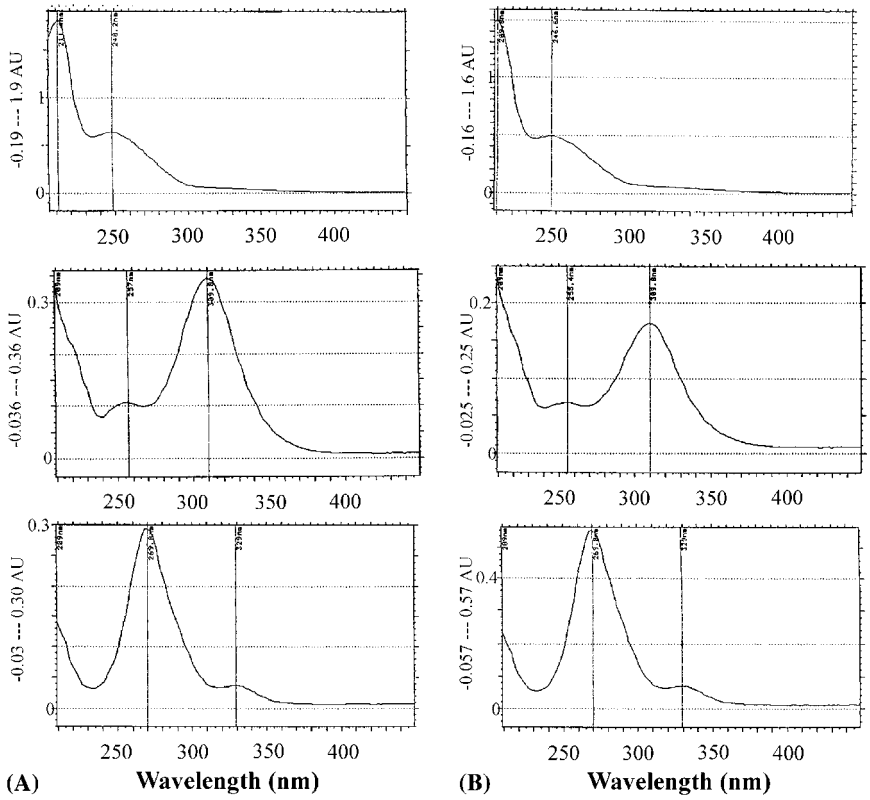

Figure 2. Spectrum analysis of antibiotics produced by *P. fluorescens* UP61 compared to standards. From top to bottom: pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol. A: standard compounds; B: compounds detected in *P. fluorescens* UP61 extracts. AU: absorbance units.

Genomic fingerprints. UP61 was compared by RAPD and rep-PCR with strains *P. fluorescens* CHA0 and Pf-5, which produce the antibiotics DAPG, Prn and Plt; and *P. fluorescens* Q2-87 that produces only DAPG (Thomashow and Weller, 1996). Each PCR reaction was repeated twice, rep-PCR results were reproducible while RAPD patterns were more variable (Figure 3). rep-PCR amplification yielded complex genomic fingerprints consisting of 10–15 amplification products ranging in size from 300 to 3000 bp. For RAPD analyses, 4–7 amplification products were found with sizes from 350 to 1200 bp. Results from the four different reactions showed the tendency of UP61, Pf-5 and CHA0 to form a cluster, which clearly differentiated them from Q2-87. Similarity of UP61 with Pf-5 and CHA0 was 45% for primers ERIC and 58% for BOX. On the other hand, these three strains presented less than 10% similarity with Q2-87 for both primers.

phlD sequence from UP61. The presence of the 745-bp insert from the UP61 *phlD* gene in pGEM®-T Easy vector was verified by restriction of the recombinant plasmids with *Eco*RI. DNA sequences with primers M13 Universal and M13 Reverse, using two different clones containing the recombinant plasmid, were identical. Sequences obtained with internal primers Pf-5 Int 1 and Pf-5 Int 2 confirmed these results. The DNA sequence of the *phlD* fragment from *P. fluorescens* UP61 is available at the National Centre for Biotechnology Information, Accession Number AF214108.

The sequence was compared with other DAPG producer *P. fluorescens* strains. The nucleotide sequence of UP61 was highly similar with other DAPG, Plt and Prn producer strains such as CHA0 (97% identity) and Pf-5 (98%). Again, a difference was observed with strains that only produce DAPG, such as *P. fluorescens* Q2-87,

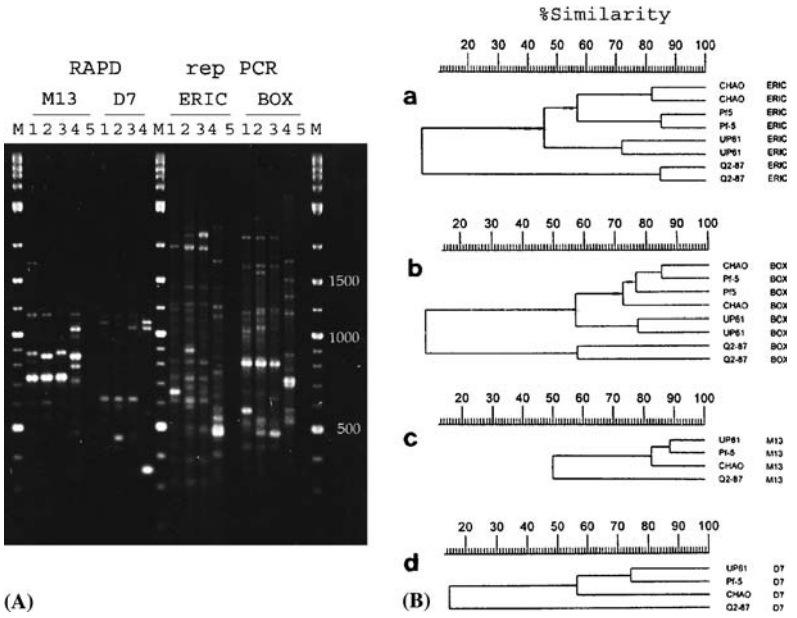

Figure 3. Genetic comparison by RAPD and rep-PCR of *P. fluorescens* UP61 with antibiotic producing strains. (A) Gel electrophoresis of PCR products amplified by RAPD-PCR (left) with primers M13 and D7, and rep-PCR (right) with primers ERIC and BOX. Lanes M: molecular weight marker, a mixture of 100-bp and 1-kb DNA ladders (New England Biolabs). On the right side molecular sizes are shown (bp). Lanes 1: *P. fluorescens* UP61, lanes 2: *P. fluorescens* CHA0, lanes 3: *P. fluorescens* Pf-5, lanes 4: *P. fluorescens* Q2-87, lanes 5: no DNA template. (B) Cluster dendogram of *P. fluorescens* strains based on rep-PCR with primers ERIC (a) and BOX (b), and RAPD with primers M13 (c) and D7 (d). In (a) and (b) results from two different experiments are shown.

Q8r1-96 and 1M1-96 which showed 76%, 78% and 78% identity, respectively.

The comparison of amino acid sequences deduced from nucleotides showed a 98% similarity between UP61 and CHA0, and 88% between UP61 and Q2-87.

Discussion

P. fluorescens UP61, a strain isolated from a forage legume rhizosphere from South American soils presents a broad spectrum of activity against phytopathogens *in vitro* and in different host plants.

The lack of influence of iron concentration in the culture media on the antagonistic activity of this strain had been previously observed (Bagnasco et al., 1998). The differential inhibition of *R. solani* and *S. rolfsii* observed in PDA and KB may be due to a poor production of antifungal factors and/or a strengthening of fungal growth.

The biocontrol activity of UP61 against *P. ultimum* and *R. solani* was previously demonstrated with birdsfoot trefoil under growth chamber conditions (Bagnasco et al., 1998). It is worth noticing that in experiments with beans and tomato presented in this work, different soils, temperatures, photoperiods and bacterial inoculation procedures were used. Studies performed with *P. fluorescens* CHA0 have shown the relative lack of specificity of this strain, which is able to act as a biocontrol agent in eight different plants and against six different phytopathogens (Voisard et al., 1994). The different composition of the root exudates could affect production of metabolites by rhizobacteria. This may explain the results obtained with CHA0 that showed that DAPG (but not Plt) was responsible for its biocontrol activity in wheat and cucumber, while Plt was active in cress and cotton (Maurhofer et al., 1994; Loper et al., 1997). Moreover, Kraus and Loper (1995) detected the differential expression of Plt genes in *P. fluorescens* Pf-5 in cotton and cucumber seeds.

P. fluorescens UP61 produces three antibiotics possibly involved in biological control. Different studies using primers Phl2a-Phl2b dealt with the occurrence of DAPG producers in natural soils,

finding that about 10% of the rhizosphere fluorescent *Pseudomonas* of wheat grown in suppressive soils (Raaijmakers et al., 1997), and maize (Picard et al., 2000) have these genes. DAPG producers have been found in African, Asian, Australian, European and North American soils (Keel et al., 1996), but no studies had been conducted in South America. They were also found in the rhizosphere of different plants such as cotton (Howell and Stipanovic, 1979), tobacco (Stutz et al., 1986), sugar beet (Shanahan et al., 1992), wheat (Raaijmakers et al., 1997), maize (Picard et al., 2000), cucumber and tomato (Keel et al., 1996). Nevertheless there are no reports in forage legumes due to scarce research on biological control on these plants, except for a few reports on *Bacillus* (Handelsman et al., 1990) and *Streptomyces* (Jones and Samac, 1996). Raaijmakers et al. (1997) did not find strains with *phzC* and *phzD* genes in wheat roots growing in suppressive or conducive soils, when they screened the fluorescent pseudomonad populations by PCR and hybridisation techniques. Hammer et al. (1999) showed that *prnC* (target of primers PrnCup-PrnClow), *prnA*, *prnB* and *prnD* are highly conserved among different bacterial genera such as *Pseudomonas*, *Burkholderia* and *Myxococcus*. The gene detected by PCR, *pltB*, is known to be fundamental for Plt production in *P. fluorescens* Pf-5 (Nowak-Thompson et al., 1999) but it is still unknown if this gene is conserved in nature.

Studies on the influence of the culture media in the antibiotic production by biocontrol *Pseudomonas* spp have shown that glucose stimulates the production of DAPG by *P. fluorescens* Pf-5 (Nowak-Thompson et al., 1994) and CHA0 (Duffy and Défago, 1999), while it inhibits the production of Plt and Prn, which are stimulated by glycerol. The carbon source in YM is glucose, and glycerol in PSFM, so the observations of these authors might explain our results with *P. fluorescens* UP61. However, Shanahan et al. (1992) showed that production of DAPG in *P. fluorescens* F113 was higher when the carbon source was sucrose, fructose or mannitol, while glucose and sorbose yielded poor production. Therefore, influence of the culture media on antibiotic production depends on the strain and probably could be related to the composition of root exudates of the host plant (Duffy and Défago, 1999). That may explain why Plt was detected in KB cultures of Pf-5 and CHA0 (Keel et al., 1996), but not in UP61. Despite the fact that our studies show that the three strains are similar, they were isolated from different environmental conditions and host plants. Schnider-Keel et al. (2000) demonstrated that Plt, salicylate and fusaric acid repress the production of DAPG in *P. fluorescens* CHA0, which may explain why simultaneous production of DAPG and Plt was never detected with UP61. Further studies are necessary to determine the identity of the active compound(s) produced by UP61 in culture media where this strain presented antagonistic activity but none of the known antibiotics was detected.

Keel et al. (1996) described two different clusters among DAPG producer, separated by their ability to additionally produce Plt, when a collection of 45 strains isolated from Switzerland, United States, Ireland, Italy and Ghana was analysed. Similar results were obtained by other authors using different techniques: McSpadden Gardener et al. (2000) used rep-PCR with primer BOX, ARDRA and growth on different substrates; and Mavrodi et al. (2001) used *phlD* RFLP and RAPD with primer M13. Both authors defined 14 genotypes among 123 DAPG producer strains belonging to widely distributed soils. Later on, Landa et al. (2002) expanded this classification to 17 distinctive genotypes. Only strains belonging to a specific genotype (denominated A) presented the genes for Prn and Plt when they were screened by PCR and hybridisation using the same target genes used in our work. Results from genetic analyses showed strong similarities of *P. fluorescens* UP61 with these other isolates grouping it with A genotype strains of DAPG producers. Nevertheless the origins of these strains are very dissimilar: *P. fluorescens* CHA0 was isolated from tobacco plants in Switzerland, *P. fluorescens* UP61 from birdsfoot trefoil in Uruguay, and *P. fluorescens* Pf-5 from cotton in USA.

The putative active site of the polyketide synthase PhlD described in Q2-87 ($QLGC^{138}VAG$, Bangera and Thomashow, 1999) was conserved in UP61 ($QLGC^{65}VAG$). Nevertheless a difference of one amino acid was found in CHA0 ($HLGC^{65}VAG$), where glutamine is substituted by histidine in position 62, which might indicate that this amino acid does not play an important role in the putative active site. In all the other studied

strains 1M1-96, UP61, Pf-5 and Q8r1-96, these 7 amino acids were conserved. The site was described by Bangera and Thomashow (1999) as related to the gene encoding for chalcone and stilbene synthases from plants, to which PhlD shows 31% identity and 53% similarity. It remains to be proved if the putative active site really plays this function in PhlD.

The selection of *P. fluorescens* UP61 was based on its antagonistic activity against phytopathogens, and not following physiological or genetic patterns. Nonetheless our results suggest that it may be possible to use genetic comparisons to predict the broad activity of biocontrol strains.

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