Res. Microbiol. 150 (1999) 45–59

Isolation and characterisation of a new antagonistic *Burkholderia* strain from the rhizosphere of healthy tomato plants

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(Submitted 5 March 1998; accepted 7 October 1998)

Abstract — A new *Burkholderia* strain (PVFi5A) which exhibits antagonism towards many bacterial and fungal plant pathogens has been partially characterised. This strain was isolated from the rhizosphere of tomato plants and was referred to the *Burkholderia cepacia* complex on the basis of cultural, morphological and biochemical characteristics, including determination of the 16S ribosomal DNA sequence and fatty acid profile. Strain PVFi5A is a Gram-negative, aerobic bacterium, oxidase- and catalase-positive, motile with a polar tuft of flagella, able to grow on a variety of media without producing diffusible pigments; it is avirulent to onion, able to grow at 41 °C and resistant to several antibiotic substances. Its fatty acid profile contains the hydroxy acids 18:1 2OH, 14:0 3OH and 16:0 3OH, but not the hydroxy acids 16:0 2OH and 16:1 2OH. The antagonistic activity of strain PVFi5A is due to its production of various, as yet unidentified, antimicrobial compounds, one or more of which may differ from those reported previously for certain '*B. cepacia*' strains. The ability of PVFi5A to suppress the growth of important bacterial and fungal phytopathogens makes this strain a potential biocontrol agent. © Elsevier, Paris

***Burkholderia cepacia* complex / tomato / biological control / rhizosphere**

1. Introduction

Burkholderia cepacia [69] is a non-fermentative, Gram-negative, motile, non-fluorescent, ubiquitous, soil-borne bacterium. It occurs as an opportunistic pathogen in plants, animals and man. The common host plant of *B. cepacia* is *Allium cepa*, but the bacterium also causes soft-rot symptoms in *Allium sativum*, *Lycopersicon esculentum* and *Agaricus bitorquis*, and a leaf-spot disease on a number of orchids (*Cymbidium* spp., *Dendrobium* sp. and *Paphiopedilum* spp.) [44]. Other strains of *B. cepacia*, which is also found in a wide range of clinical sources [19], may be the cause of pulmonary infections in children and young adults with cystic fibrosis [58].

Because of the ubiquity of *B. cepacia*, and also because a hypothetical protein coded by the DNA sequence of an insertion sequence (IS) element in *Mycobacterium tuberculosis* is homologous to proteins encoded by IS elements of *Agrobacterium tumefaciens* (IS*427* and IS*869*), *B. cepacia* (IS*402*), *Streptomyces lividans* (Tn*4811*) and *Rhizobium meliloti* (ISRm4), it has been hypothesized that *B. cepacia* plays an important role in the exchange of genetic material between plant pathogenic bacteria and bacteria which are pathogenic to man and animals [35].

Other noteworthy characteristics of *B. cepacia* strains are their broad resistance to antibiot-

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Abbreviations: ARDRA, amplified rDNA restriction analysis; CFU, colony-forming unit; DT, duplication time; IS, insertion sequence; KA, King's A (medium); KB, King's B (medium); LB, Luria Bertani; NA, nutrient agar; NSA, nutrient agar containing sucrose; SNS, Schwyn and Neilands medium; PDA, potato dextrose agar; RDP, Ribosomal Database Project; IAA, indole 3-acetic acid; Ta, temperature (annealing); WM, Woolley's solid medium; WMP, WM plus 0.1% peptone.

ics [23, 51]; their ability to synthesize new compounds [29, 38, 41, 42]; to bring about the biodegradation of recalcitrant molecules [6, 12, 16, 24, 61, 65]; and to cause the regression of some pathogens in soil and the rhizosphere of crop plants by the production of antibiotics, volatile antifungal compounds and siderophores [1, 4, 11, 17, 18, 20, 22, 25, 28, 32, 37, 40, 48, 50, 52].

There are thus many reports which demonstrate the potential value of *B. cepacia* in the biological control of plant diseases generally. On the other hand, few reports exist which specifically examine the role of this bacterium in the control of bacterial and fungal pathogens of vegetable crops.

The purpose of the present study was initially to isolate new antagonistic bacterial strains that would protect tomato and other vegetable crops (pepper, pumpkin, lettuce, etc.) at commercially acceptable levels from diseases caused by soilborne pathogens and that would promote the growth of the crops so protected. The strain we selected has been characterized and is referred to as the *Burkholderia cepacia* complex.

2. Materials and methods

2.1. Isolation of rhizosphere bacteria

Tomato, lettuce, chicory and pumpkin plants, 30- to 45-day-old, were collected in 1993 from two sites in Tuscany, Italy. Plants were lifted together with adherent soil into plastic bags, transported to the laboratory within 3 h, and analysed the same day. Plant roots were removed from the soil, shaken vigorously to remove excess soil, weighed, placed in 30 mL of phosphate buffer (0.05 M, pH 7) and agitated for 35 min on a wrist-action shaker. Soil suspensions were serially diluted (1:10 dilution). Aliquots of 100 mL from the 10^{-2} to 10^{-5} dilutions were spread onto four different media: Difco nutrient agar (NA); Schwyn and Neilands medium for the detection of siderophores (SNS) [47]; King's B medium (KB) [26]; and Sands and Rovira's agar medium for the isolation of fluorescent pseudomonads (NPC) [45].

Root sections (0.5 to 2 cm) were gently washed in sterile distilled water to remove adhering soil, weighed, surface-disinfected (95% ethanol for 1 min), rinsed in sterile distilled water (30 s), and 3-g samples macerated in 5 mL of phosphate buffer with a mortar and pestle. Serial 10-fold dilutions of the homogenates were prepared, and 0.1-mL aliquots were plated onto the media described. All plates were incubated at 28 °C.

From each colony type on each medium, one colony was isolated and purified on Difco nutrient agar containing 5% sucrose (NSA). Lastly, one colony of each colony type on NSA was used to produce cultures that were maintained in Difco nutrient broth plus 10% glycerol (w/v) at –80 °C.

2.2. In vitro screening of potential antagonists

One-hundred and twenty-five potential antagonist bacterial isolates were selected and initially tested for their antagonism against a range of phytopathogenic bacteria and fungi as follows. Twenty-μL drops (5/dish) of a bacterial suspension (10^9CFU/mL) were placed equidistant from one another and 25 mm from the edge on 90-mm-diameter Petri dishes containing NSA or KB. After 96 h of incubation at 28 °C, a suspension (10^7 CFU/mL) of one of the phytopathogens used for the test (*Erwinia carotovora* pv. *carotovora* NCPPB2577, *Pseudomonas syringae* pv. *phaseolicola* B1962B and *P. syringae* pv. *tomato* NCPPB3310) was spread over the entire surface of the dish on top of the antagonist. Antibacterial activity was determined by measuring the zones of bacterial growth inhibition after 24 h of incubation of the dual cultures at 28 °C.

Ninety isolates that inhibited all three phytopathogens were then tested against the fungi *Sclerotinia sclerotiorum* PVFiL13, *Sclerotium rolfsii* PVFiL12, *Pythium debarianum* PVFiL9, *Fusarium oxysporum* f. sp. *lycopersici* PVPe-AZ and *Verticillium dahliae* PVNa424. Four rhizobacteria isolates were streaked about 15 mm from the edge of a Petri dish containing PDA. After 24 h at 28 °C, a 5-mm agar plug with mycelium was placed in the center of the dish. The dishes were incubated and the increase in radial growth of the fungus toward each bacterial streak was measured at 24-h intervals. A zone of inhibition

Table I. Inhibition (%)[a] of bacterial pathogens by strain PVFi5A.

Bacteria tested	*Strain identification*	*Antagonist*		
		PVFi5A	*Pss*[b]	*Pf*[c]
Pseudomonas syringae pv. *syringae*	Y32	40 (0.35)[d]	31 (0.45)	32 (0.47)
P. syringae pv. *phaseolicola*	B1962B	40 (0.30)	0	45 (0.33)
P. syringae pv. *glycinea*	NCPPB2753	40 (0.40)	24 (0.58)	35 (0.43)
P. syringae pv. *tabaci*	NCPPB2730	40 (0.35)	0	35 (0.40)
P. syringae pv. *maculicola*	NCPPB1777	40 (0.37)	0	27 (0.48)
P syringae pv. *tomato*	NCPPB3310	36 (0.33)	0	30 (0.43)
Pseudomonas corrugata	PC12	34 (0.38)	0	24 (0.58)
Erwinia carotovora pv. *carotovora*	NCPPB2577	44 (0.25)	24 (0.54)	28 (0.43)
Agrobacterium tumefaciens	CG634	40 (0.42)	0	21 (0.52)
Clavibacter michiganensis subsp. *michiganensis*	NCPPB2979	38 (0.34)	0	35 (0.37)
Xanthomonas campestris pv. *vesicatoria*	NCPPB402	45 (0.31)	27 (0.59)	33 (0.42)
Bacillus subtilis	MFi24	34 (0.38)	32 (0.41)	25 (0.52)

[a]Numbers in the table represent the halo of bacterial inhibition in mm.
[b]*Pseudomonas. syringae* pv. *syringae* strain B359, syringomycin producer.
[c]*Pseudomonas fluorescens* strain PVFi121.
[d]Values in parentheses are the ratio between the diameter of the bacterial colony and the diameter of the inhibition halo.

indicated antifungal activity, and the isolates were ranked according to the width of the clear zone. The top-performing isolates were tested for: i) production of siderophores in liquid culture with the method of Schwyn and Neilands [47]; ii) production of fluorescent pigments on KB; and iii) antibacterial and antifungal activities against *S. sclerotiorum* and the other microorganisms mentioned above. The production of indole 3-acetic acid (IAA) in liquid culture [14] and the ability of isolates to colonize tomato roots with the seedling bioassay chamber of Randawa and Schaad [43] were also assayed.

At the end of these experiments, the isolate PVFi5A, obtained from the rhizosphere soil of a tomato plant, was chosen for further characterisation. PVFi5A was non-fluorescent, non-IAA producing, siderophore-producing, an antagonist of phytopathogenic bacteria and fungi, and was able to colonize tomato roots in vitro.

2.3. Determination of antimicrobial activities of strain PVFI5A

2.3.1. Antibacterial activity

To assay the antibiotic effectiveness of PVFi5A against phytopathogenic bacteria, a 100-µL drop of a 10^9 CFU/mL suspension of this strain was spotted centrally onto NSA plates and incubated for 48 h at 28 °C. A 10^7 CFU/mL suspension of each test bacterium *(table I)* was then sprayed over the NSA plates, and after incubating for a further 24 to 72 h at 28 °C, the size of the inhibition zone was measured. A local strain of *Pseudomonas fluorescens* (PVFi121) and the syringomycin producer strain B359 of *P. syringae* pv. *syringae* [3] were used as control antagonists.

2.3.2. Antifungal activity

PVFi5A was streaked in the centre of 90-mm Petri dishes containing PDA, and 5-mm plugs of active mycelium were placed 20 mm from the edge of the bacterial streaks on either side. The fungi *(table II)* were inoculated onto the plates either 2–3 days before, or concurrently with the bacterium, depending on whether they were slow- or fast-growing. Every 24 h, the radii of fungal colony growth towards the bacterial streak and away from the streak were measured, and the percentage of fungal growth inhibition was calculated using the formula: A-B/A × 100, where A is the distance of myce-

Table II. Inhibition (%)[a] of fungal pathogens by strain PVFi5A.

Fungi tested	*Strain identification*	*Antagonistic bacteria used*		
		PVFi5A	*Pss*[b]	*Pf*[c]
Alternaria tenuis	PVFiL1081	80.0	n.d.[d]	n.d.
Armillaria mellea	IMI2024	80.9	n.d.	n.d.
Botrytis cinerea	PVFiL1063	57.1	n.d.	n.d.
Ceratocystis fimbriata	PVFiL584	84.3	n.d.	n.d.
Colletotrichum gloeosporioides	PVFiL28	77.7	n.d.	n.d.
Cryphonectria parasitica	PVFiL74	78.3	n.d.	n.d.
Epicoccum purpurescens	PVFiL1077	60.2	n.d.	n.d.
Fusarium oxysporum f. sp. *dianthii*	PVTOAG	75.6	n.d.	n.d.
Fusarium oxysporum f. sp. *lycopersici*	PVPeAZ	37.1	22.8	24.0
Fusarium eumartii	PVFiR1	84.2	n.d.	n.d.
Fusarium javanicum	PVFiR2	90.9	n.d.	n.d.
Fusarium solani	ISP1	80.0	n.d.	n.d.
Fusicoccum amygdali	PVBaSF	12.5	7.5	18.7
Geotrichum candidum	PVFiL11	42.8	57.9	8.1
Heterobasidion annosum	PVFiC1	70.0	n.d.	n.d.
Hypoxylon nummularium	PVFiL101	75.0	n.d.	n.d.
Leptographium serpens	PVFiCV 2	73.0	n.d.	n.d.
Monilia laxa	PVFiL26	78.7	n.d.	n.d.
Mucor sp.	PVFiL1046	90.0	n.d.	n.d.
Ophiostoma novo-ulmi	H328	54.0	65.6	28.0
Ophiostoma novo-ulmi	H322	92.5	n.d.	n.d.
Penicillium expansum	PVFiL1072	58.3	n.d.	n.d.
Peniophora gigantea	PVFiC32	91.8	n.d.	n.d.
Pestalotia sp.	PVFiP1	75.6	n.d.	n.d.
Phoma sp.	PVFiL1001	90.0	n.d.	n.d.
Phomopsis phoeniculi	PVFiL5	42.8	58.3	43.7
Phytophthora cambivora	PVFiT1	37.0	37.1	68
Phytophthora infestans	ATCC13196	85.0	n.d.	n.d.
Pythium debarianum	PVFiL9	78.7	n.d.	n.d.
Rhizopus oryzae	PVFiL1038	75.8	n.d.	n.d.
Rhizosphaera piceae	PVFiL102	83.3	n.d.	n.d.
Saccharomyces cerevisiae	MFi1	40.0	47.0	n.d.
Sclerotinia sclerotiorum	PVFiL13	92.3	n.d.	n.d.
Sclerotium rolfsii	PVFiL12	75.0	n.d.	n.d.
Seiridium cardinale	ATCC 38654	75.8	n.d.	n.d.
Seiridium cardinale	ATCC 2051	78.5	n.d.	n.d.
Taphrina deformans	PVFiL41	38.0	40.0	n.d.
Verticillium dahliae	PVNa424	51.4	33.3	56.0

[a]Percentage of fungal growth inhibition was calculated with the formula B-A/A × 100, where B is the distance of mycelial growth toward the bacterium and A the distance of mycelial growth away from the bacterium.
[b]*Pseudomonas syringae* pv. *syringae* strain B359.
[c]*Pseudomonas fluorescens* strain PVFi121.
[d]Not determined.

lial growth away from the bacterium, and B the distance of mycelial growth toward the bacterium.

To test the effect of nutritional and environmental conditions on the antagonist activity of PVFi5A, its antifungal and antibacterial inhibition was also assayed on Woolley's solid medium (WM) [68] modified according to Upadhyay et al. [60]: WM plus 0.1% peptone (WMP); WMP with NH_4NO_3 instead of $NaNO_3$ (WMP-N); WMP plus thiamine-HCl (0.5%) and either trehalose (0.2%) (WMP-TT) or xylose (0.2%) (WMP-TX) instead of sucrose, pH 5.0.

2.4. Preparation of active culture filtrate

Erlenmeyer flasks containing 200 mL of Woolley's liquid medium were inoculated with 100 mL of a PVFi5A suspension (10^8 CFU/mL) and incubated on a rotary shaker (100 rpm) at 28 °C for 4 days. The bacterial suspension was then centrifuged (10^4 g for 15 min), the supernatant passed through a millipore membrane (0.45 mm) and lyophilized. The lyophilised material was dissolved in a volume of distilled water equal to 10% and 50% of the bacterial suspension and filter-sterilised. The solution was assayed for inhibitory activity against *E. carotovora* pv.*carotovora*, *Ophiostoma novo-ulmi* (H322) and *Fusicoccum amygdali* by placing 100-µL aliquots of solution in 5-mm diameter wells cut in PDA plates. Plates were inoculated simultaneously on the surface with agar plugs of the fungi or were sprayed with the test bacterium after the antagonist solution had diffused into the agar. After 2 to 7 days of incubation, the plates were examined for mycelium-free or bacteria-free zones around the wells.

2.5. Morphological and biochemical characterisation

To assess the taxonomic position of strain PVFi5A, a total of 78 phenotypic features were examined. Cultures of *B. cepacia* ICMP5982 and ICMP5796, isolated from tomato and onion respectively, were examined for comparison. Cells and flagella were examined by phase-contrast light microscopy and electron microscopy. Colony characteristics were determined by using streaked cultures on nutrient agar with 5% sucrose (NSA). Pigment production was evaluated on tryptone soy agar (TSA), on King's media A and B (KA, KB) and on Difco nutrient agar (NA) after 2 and 5 days of growth at 27 °C. All other tests were performed by the methods of Smibert and Krieg [49] and Lelliott and Stead [30]. Media were inoculated with exponential-phase cells grown on NA containing 2% glycerol and incubated at 27 °C.

2.6. Cellular fatty acid analysis

A qualitative and quantitative analysis of the cellular fatty acid composition of strain PVFi5A was performed at the Central Science Laboratory (CSL) at Hatching Green (Harpenden, UK) by the method of Stead [53]. The fatty acids were identified and quantified using the Microbial Identification System software (MIDI, Newark, DE, USA). The profiles obtained were compared with profiles in a commercial library which includes most bacteria of medical, environmental, veterinary and general interest, and a self-generated library composed entirely of plant pathogenic bacteria. A similar analysis was performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Mascheroder Weg (Braunschweig, Germany).

2.7. Analysis of plasmid content

Plasmid DNA was isolated by the methods described by Lennon and DeCicco [31]. *Agrobacterium tumefaciens* C58 and *Pseudomonas savastanoi* pv. *savastanoi* ITM317, harbouring plasmid species of 200, 88, 73, 63, 48, 42, 22, and 10 kb, were used as the source of size-reference plasmids [7, 21].

2.8. Amplification of 16S rDNA

The 16S rDNA was amplified from a cell lysate made as follows: a colony of approximately 1-mm diameter was resuspended in 20 mL of sterile distilled water, the suspension was heated to 95 °C for 10 min and then cooled in ice for 2 min.

Amplification of 16S rDNA was performed using 2 mL of cell lysate in 20 mL of 1 × Amplitaq buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin) with 150 ng each of the primers 27f and 1495r *(table III)*, 250 µM each of dNTPs and 1 U of Amplitaq (Perkin-Elmer). The reaction mixtures were incubated at 95 °C for 1 min and 30 s and then cycled 35 times through the following temperature profile: 95 °C for 30 s; annealing temperature (Ta) for 30 s; 72 °C for 4 min. The Ta was 60 °C for the first five cycles, 55 °C for the next five cycles and 50 °C for the last 25 cycles. Finally, the mixtures were incubated at 72 °C for 10 min and at 60 °C for 10 min. Two mL of each amplifica-

Table III. Oligonucleotides used for amplification and/or sequencing of 16S rDNA.

Position	*Length (nt)*	*Sequence*
27f	21	5'-GAGAGTTTGATCCTGGCTCAG
342r	16	5'-CTGCTGCCTCCCGTAG
559r	17	5'-CTTTACGCCCAGTAATT
575f	17	5'-AATTACTGGGCGTAAAG
704f	20	5'-GTAGCGGTGAAATGCGTAGA
765r	21	5'-CTGTTTGCTCCCCACGCTTTC
930f	17	5'-AAGGAATTGACGGGGGC
1495r	20	5'-CTACGGCTACCTTGTTACGA
519r	18	5'-GTATTACCGCGGCTGCTG
1114f	16	5'-GCAACGAGCGCAACCC

The position corresponds to the number of the *E. coli* 16S rDNA position where the 3' end of the primer anneals in forward (f) or reverse (r) orientation.

tion mixture was analysed by agarose gel (1.2% w/v) electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) containing 0.5 mg/mL (w/v) ethidium bromide.

Primers 27f and 1495r *(table III)* were designed on the basis of the conserved eubacterial sequences and were located at the 5' and 3' ends of 16S rDNA, allowing amplification of nearly the entire gene. The primers were synthesized by standard phosphoramidite chemistry, deprotected, dried, dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and used without further purification.

2.9. Restriction analysis of amplified 16S rDNA (ARDRA)

Approximately 1.5 mg of 16S rDNA, amplified by PCR, was treated with three units of the restriction enzyme *AluI* (Boehringer Mannheim) in a total volume of 20 mL at 37 °C for 3 h. The enzyme was then inactivated by heating the reaction mixture to 65 °C for 10 min. The reaction products were analysed by agarose gel (2.5% w/v) electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) containing 0.5 μg/mL (w/v) ethidium bromide.

2.10. Sequencing of 16S rDNA

The determination of the 16S rDNA nucleotide sequence was performed by an enzymatic method [46] using a Promega *f*molTM (thermal cycle) DNA Sequencing Kit and dATP 35S to label the synthesized DNA. The reactions were performed for 30 thermal cycles according to the instruction manual. The amplified 16S rDNA was purified from the reaction mix by agarose gel (1.2% w/v) electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) with 0.5 mg/mL (w/v) of ethidium bromide. A small agarose slice containing the band of interest (observed under long UV, 312 nm) was excised from the gel and purified using a QIAquick Gel Extraction kit (Quiagen) according to instructions.

The reactions were performed using the primers listed in *table III*, which were designed on the basis of conserved eubacterial sequences. Primers which did not show palindromic sequences longer than four bases, or which had complementarity at the 3' end, were denatured at 90 °C for 2 min before use; a Ta of 50 °C was used in cycle sequencing.

2.11. Analysis of sequence data

The 16S rDNA nucleotide sequence obtained was aligned with the most similar sequences *(table IV)* of the Ribosomal Database Project (RDP) [34] and GenBank using RDP utilities. The alignment was checked manually, corrected and then analysed using the neighbour-joining method according to the models of Jukes and Cantor with TREECON 2.2 [62]. The robustness of the inferred trees was evaluated by bootstrap resampling.

The 16S rDNA nucleotide sequence was recorded in GenBank under accession number AF042161.

2.12. Growth and sensitivity to antibiotics

To assess the growth characteristics of strain PVFi5A, 1 mL of an overnight culture of the bacterium was inoculated into 100 mL of Woolley's medium in 250 mL Erlenmeyer flasks. The number of viable bacterial cells in the growing culture was recorded at 1, 2, 3, 4, 5 and 7 days after inoculation.

The sensitivity of strain PVFi5A to various antibiotics was determined either by plating a

Table IV. 16S rDNA sequences used to construct phylogenetic tree.

Organism	*Strain*	*Accession no.*
Burkholderia andropogonis	ATCC23061	X67037
Burkholderia caribensis	MWAP64	Y17009
Burkholderia caryophylli	ATCC25418	X67039
Burkholderia cepacia	G4	L28675
Burkholderia cepacia	PHP7	X80287
Burkholderia cepacia	DSM50181	X87275
Burkholderia cepacia	ATCC25416 (T)	M22518
Burkholderia cocovenenans	LMG11626 (T)	U96934
Burkholderia gladioli	ATCC10248	X67038
Burkholderia glathei	LMG14190 (T)	U96935
Burkholderia glumae	LMG2196 (T)	U96931
Burkholderia graminis	C4D1M	U96939
Burkholderia graminis	AUS35	U96941
Burkholderia phenazinium	LMG2247 (T)	U96936
Burkholderia plantarii	LMG9035 (T)	U96933
Burkholderia sp.	CRE7	U37340
Burkholderia sp.	C3B1M	U96938
Burkholderia thailandensis	E264	U91838
Burkholderia vandii	LMG16020 (T)	U96932
Burkholderia vietnamiensis	TVV70	U96929
Burkholderia vietnamiensis	TVV75	U96928
Pseudomonas aeruginosa	DSM500071 (T)	X06684

loopful of cells onto Luria-Bertani (LB) [33] agar plates containing an antibiotic (see below) at a concentration of 25 mg/L, or by the use of an agar diffusion method. The antimicrobial substances tested and the concentrations per disc applied in the agar diffusion test were as follows: chloramphenicol (30 μg), erythromycin (15 μg), carbenicillin (100 μg), gentamicin (10 μg), kanamycin (50 μg), nalidixic acid (30 μg), neomycin (30 μg), novobiocin (30 μg), polymyxin B (37.5 μg), rifampin (5 μg), spectinomycin (30 μg), streptomycin (30 μg), tetracycline (30 μg). Ampicillin and penicillin G were tested only by the first method.

2.13. Pathogenicity tests

Strains PVFi5A, ICMP5982 and ICMP5796 were examined for their ability to macerate onion tissue of the cvs. 'Bianca agostana' and 'Dorata di Parma'. Onion slices were streaked with single colonies from NSA cultures incubated for 48 h at 28 °C. Maceration was assessed after incubation in a moist chamber for 96 h at 28 °C.

2.14. Extraction of antimicrobial compounds

Ninety-mm-diameter Petri dishes containing PDA or NSA were seeded with cells of strain PVFi5A and, for comparison, strain ICPM5796. After 5 days of growth at 27 °C, dish cultures were cut into 2-cm squares and extracted in a Waring Blendor four times with 250 mL ethyl acetate at room temperature. The extract was centrifuged ($8 \times 10^4 \times 45$ min) at 15 °C, the supernatant collected, filtered on anhydrous sodium sulphate and evaporated under reduced pressure to give an oily residue (39 mg from 15 Petri dishes). Twenty mg of crude extract was dissolved in 10 mL methanol and the soluble material fractioned on a silica gel column (1 × 27 cm; Merck, Kieselgel 60, 0.063–0.20 mm). The column was eluted initially with chloroform:methanol (4:1, v/v), then with ethyl acetate:methanol:water (8.5:2:1, v/v/v) and finally with 200 mL methanol. A total of 107 4-mL fractions (36 from the first column elution, 70 from the second, and the methanolic fraction) were collected, pooled into 11 groups of homogeneous fractions and evaporated to dryness: 1–4 (2.6 mg); 5–7 (1.3 mg); 8–15 (2 mg); 16–26 (1.5 mg); 27–35 (0.5 mg); 36–50 (0.4 mg); 51–66 (0.8 mg); 67–71 (1 mg); 72–87 (3.9 mg); 88–107 (2.5 mg) and the methanolic fraction (7.3 mg). For thin-layer chromatography (TLC) analyses of all fractions and crude extracts from Petri dishes, 0.25-mm-thick silica gel plates (Merck, Kieselgel, 60 F254) were used. The plates were spotted with samples in methanol and developed with chloroform:methanol (4:1, v/v). The separated compounds were visualized by exposure to UV (253 and 365 nm) and/or spraying with a 5% H_2SO_4 solution in methanol followed by a 5% phosphomolybdic acid solution in methanol, and then heating to 110 °C for 10 min.

Aliquots of the crude extracts and the pooled fractions were redissolved in a small volume of distilled water and assayed for antimicrobial activity *against O. novo-ulmi* (H322), *F. amygdali*, *E. carotovora* pv. *carotovora* and *P. syringae* pv. *tomato* by the agar diffusion method (concentration per disc: 0.2–0.5 μg). To score the degree of

inhibition, the following arbitrary scale was used: 1 = slight inhibition; 2 = moderate inhibition; 3 = strong inhibition; 4 = complete inhibition.

3. Results

3.1. Antagonism

Strain PVFi5A showed strong antagonism against all 38 fungi *(table II)* and 12 bacteria tested *(table I)*. The antagonistic effect was most pronounced against the fungi *Mucor* sp., *Fusarium javanicum, O. novo-ulmi, S. sclerotiorum, Peniophora gigantea,* and *Phoma* sp. and among the bacteria, against *E. carotovora* pv. *carotovora* and *Xanthomonas campestris* pv. *vesicatoria*. The fungi *F. amygdali, Phytophthora cambivora, F. oxysporum f. sp. lycopersici, Taphrina deformans, Saccharomyces cerevisiae, Phomopsis foeniculi* and *Geotrichum candidum,* and the bacteria *P. corrugata* and *Bacillus subtilis* were less sensitive to the antagonistic effect of strain PVFi5A.

Overall, the inhibition in growth of the fungi as well as the width of the bacterial inhibition halo were greater with PVFi5A than with B359 and/or PVFi121, the control antagonists.

The antagonism achieved by strain PVFi5A varied with the nutrient medium tested: PDA, NSA or Woolley's agar medium. Good growth was obtained on PDA, NSA, WMP-N and WM without supplement or supplemented with 0.1% peptone (WMP), while on WMP modified according to Upadhyay et al. [60] (= WMP-TT and WMP-TX) strain PVFi5A had only scanty growth. Antagonistic activity was displayed on all media on which the bacterium had good growth, in particular PDA, NSA and WMP, with lower activity on WM without peptone. No antagonistic activity at all was recorded on WMP-TT or WMP-TX.

Since strain PVFi5A did not inhibit fungi and bacteria by producing volatile inhibitory factors, its inhibitory effect was probably due to antibiotics and/or siderophores. To ascertain the involvement of siderophores in the antagonistic effect, NSA was supplemented with 10^{-5} M $FeCl_3$ and inoculated with PVFi5A; after 3 days of incubation at 28 °C, plates were sprayed with *A. tumefaciens* C58 and *P. syringae* pv. *syringae* Y32 to test for antagonism. PVFi5A inhibited the growth of both bacteria in the presence of iron, which suggested that siderophores were not involved in the antagonism. The other possibility, that this strain produced antibiotics, was investigated by examining whether concentrated culture filtrates and crude extracts inhibited the growth of fungi and bacteria.

3.2. Bioassay of fractions tested for inhibitory activity against fungi and bacteria in vitro

PVFi5A concentrated culture broth and ethyl acetate crude extracts from PDA and NSA bacterial growth were highly inhibitory to *O. novo-ulmi, F. amygdali, E. carotovora* pv. *carotovora* and *P. syringae* pv. *tomato,* with an average inhibition score of 3. Further assays utilised partially purified fractions from organic soluble extracts. The results showed that in the case of strain PVFi5A, high inhibitory activity was associated with the most markedly polar metabolites present in fractions 36–50 (fraction A), 51–66 (fraction B) and 72–87 (fraction C), all eluted with chloroform:methanol. By contrast, in the case of strain ICPM5796, antimicrobial activity was associated with a less strongly polar fraction (7–8 (fraction A1), eluted with chloroform:methanol) and two other polar fractions (11–66 (fraction B1) and 67–76 (fraction C1) eluted with ethyl acetate:methanol:water). The average inhibition score was 2 for fractions B and A1 and 1 for fractions A, C and C1. There was no inhibition of the tested organisms with any other fractions.

The chromatographic patterns of the fractions obtained from the cultures of strains PVFi5A and ICPM5796 were significantly different and complex, showing in both cases the presence of several secondary metabolites with different polarity. (However, the chromatographic pattern of PDA and NSA extracts was substantially the same.) Each active fraction contained more than one individual compound; this was visualized by spraying the TLC plates with sulphuric and phosphomolybdic acid. The isolation

and the chemical and biological characterisation of isolated compounds of the active fractions are under way.

3.3. Morphological and biochemical characterisation

The cells of PVFi5A were straight, non-spore-forming rods, 0.64–0.95 × 1.1–1.7 μm, occurring singly or in pairs, Gram-negative, aerobic and motile by means of long wavy polar flagella. Two distinct colony types were occasionally observed on NSA. Colonies of one variant (PVFi5A) were whitish, circular, entire and convex, often surrounded by an opaque halo, averaging 1.5 to 2 mm in diameter after 72 h at 27 °C, and with a smooth opaque appearance, while colonies of the other variant (PVFi5B) were circular and rugose with microundulate margins. No diffusible pigments were produced on NSA, TSC, or KA.

In the biochemical tests *(table V)*, strain PVFi5A tested positive for catalase and oxidase activities, oxidative metabolism of carbohydrates, growth in the presence of 5% NaCl, reduction of nitrate to nitrite, hydrolysis of urea, utilisation of citrate, malonate, alanine, asparagine, glutamine, proline, citraconate, gluconate, azelate, pimelate, suberate, and growth at 41 °C. Strain PVFi5A produced acid from arabinose, galactose, glycerol, inositol, lactose, maltose, mannitol, mannose, salicin, sucrose, tartrate, trehalose, and xylose; no acid was produced from erythritol, melezitose, melibiose or raffinose. Strain PVFi5A tested negative for production of indole, H_2S, fluorescent pigments and acetoin, denitrification, arginine dihydrolase, methyl red, aesculin, gelatin and tyrosine hydrolysis, levan formation from sucrose, phenylalanine deaminase, β-galactosidase, pectolytic activity, growth on minimal medium and a hypersensitivity reaction on tobacco.

The two variants showed only minor differences, mainly in colony morphology, and were considered to belong to the same species.

3.4. Growth and sensitivity to antibiotics

In Woolley's medium at 27 °C, strain PVFi5A initiated logarithmic growth after a lag phase of 24 h, with a duplication time (DT) of approximately 97 h. Cells increased from 3.8×10^5 to 3.3×10^9 CFU/mL in 48 h (DT = 3 h and 40 min). The maximum stationary phase remained at levels of about 5.0×10^9 CFU/mL (DT = 74 h and 10 min) for 72 h.

In antibiotic sensitivity tests, strain PVFi5A showed resistance to erythromycin, carbenicillin, gentamicin, kanamycin, neomycin, polymyxin B, rifampin, spectinomycin, streptomycin, tetracycline, ampicillin and penicillin G, and was sensitive to chloramphenicol, nalidixic acid and novobiocin. Both methods used to test the susceptibility of strain PVFi5A to antimicrobial substances gave the same results.

3.5. Cellular fatty acid profile

The cellular fatty acid composition of strain PVFi5A, as determined in the two laboratories (CSL and DSM), is shown in *table VI*. The two fatty acid profiles showed some quantitative and qualitative differences. With CSL, the major fatty acid components of strain PVFi5A were: summed feature 7 (62.62%), C16:0, and summed feature 3. Summed feature 3 (comprising 16:1 iso and/or 14:0 3OH) and summed feature 7 (comprising 18:1 w7c, 18:1 w9t and 18:w12t) could not be distinguished by the Microbial Identification System and were referred to 14:0 3OH and 18:1 w7c, respectively [63].

With DSM, the major components were C18:1 w7c (36.87%), C16:1 w7c, C16:0 and the hydroxy fatty acids 14:0 3OH and 16:0 3OH. The hydroxy fatty acid 18:1 2OH (3.67%) was found in the CSL profile but not in that supplied by the DSM. Moreover, the levels of most of the fatty acids, including those most often represented (C18:1 w7c, C16:0, C16:1 w7c), differed greatly in the two profiles. There were even greater differences between the fatty acid composition of strain PVFi5A and that of strains of *B. cepacia* examined by other authors [53, 63, 69]. These differences concerned both the quantity and the quality of the fatty acids with the most significant ones relating to the hydroxy fatty acids 16:0 2OH and 16:1 2OH, which are not found in strain PVFi5A at all. These data suggested that strain PVFi5A belonged to *Pseudomonas* rRNA

Table V. Physiological and biochemical characteristics of strain PVFi5A.

Test or characteristic	*Result*
Motility	+
Flagella	polar > 1
Acetoin production	–
Gram reaction	–
Spore formation	–
Methyl red test	–
Oxidase	+
Catalase	+
Diffusible pigments:	
fluorescent	–
pyocyanine	–
yellow	–
Production of:	
indole	–
H_2S	+
$NO_3 \rightarrow NO_2$	–
$NO_2 \rightarrow N_2$	–
IAA	–
siderophores	+
Hydrolysis of:	
aesculin	–
gelatin	–
starch	w
tyrosine	–
urea	+
Phenylalanine deaminase	–
Potato soft rot	–
Hypersensitive reaction on tobacco	–
Glucose fermentation	–
Levan formation from sucrose	–
ONPG	+
Growth on minimal medium	–
Growth at 41 °C	+
Growth in:	
2% NaCl	+
5% NaCl	+
Arginine dihydrolase	–
Utilisation of:	
citrate, malonate, alanine, asparagine, glutamine, proline, citraconate, gluconate, azelate, pimelate, suberate	+
Acid from:	
adonitol, dulcitol, fructose, geraniol, glucose, propylene glycol, rhamnose, sorbitol	w
arabinose, galactose, glycerol, inositol, lactose, maltose, mannitol, mannose, salicin, sucrose, tartrate, trehalose, xylose	+
erythritol, melezitose, melibiose, raffinose	–
Antibiotic sensitivity	
chloramphenicol, nalidixic acid, novobiocin,	sensitive
erythromycin, carbenicillin, gentamicin, kanamycin, neomycin, polimixin B, rifampin, spectinomycin, streptomycin, tetracycline, ampicillin, penicillin G	resistant
Antibiotic production (antifungal and antibacterial)	+
Volatile antagonistic activity	–
Onion maceration	–

Symbols: +, positive; –, negative; w, weak reaction.

Table VI. Cellular fatty acid composition (%) of strain PVFi5A and reference strains of *Burkholderia cepacia*.

Fatty acids	*PVFi5A*		*B. cepacia* [a]	
	CSL[b]	DSM[c]	EY645	Eight strains[d]
12:0	0.89	0.96	–	–
14:0	2.47	2.65	5	3.9
15:0	–	–	1	tr
16:0	11.96	22.37	29	22.4
16:1 w7c	1.34	23.57	3	14.9
17:0 Cyclo	0.56	2.74	21	9.1
17:0	–	–	–	tr
18:0	3.22	0.91	8	1.1
18:1	–	–	8	–
19:0 Cyclo w8c	3.15	0.96	15	5.6
16:0 2OH	–	–	3	1.0
16:1 2OH	–	–	1	1.1
18:1 2OH	3.67	–	1	2.3
19:0 Cyclo 2OH	–	–	2	–
14:0 3OH	5.4	4.97	4	4.8
16:0 3OH	4.7	4.0	6	4.5
18:1 w7c	62.62	36.87	–	28.6

[a]Data from [53, 69].
[b]Central Science Laboratory, Hatching Green, Harpenden, UK.
[c]Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Maschewder Weg, Braunschweig, Germany.
[d]NCPPB945, NCPPB946, NCPPB1962, NCPPB2993T, NCPPB3025, NCPPB3480, A3228a, A3328b.

group 2, which used to include a number of species that have now been transferred to the genus *Burkholderia*.

3.6. Onion maceration test

Over a period of 4 days, strain PVFi5A merely caused a slight brown-yellow discoloration of slices of cv. Dorata di Parma onion but did not cause any maceration or discoloration of cv. Bianca agostana onion slices. The behaviour of the two *B. cepacia* strains used as controls was different: strain NCPPB5796 caused, in 48 h, a complete maceration of the onion slices of both cultivars; strain NCPPB5982 caused the maceration of the cv. *Bianca agostana* onion slices but had no effect on the cv. Dorata di Parma slices except for a very slight brown-yellow discoloration which did not become apparent until 5 days after inoculation.

3.7. Isolation of plasmids

Under the conditions used in this study, the presence of plasmids was not detected in strain PVFi5A.

3.8. Amplification, restriction analysis and sequencing of 16S rDNA

When the 16S rDNA of strain PVFi5A was amplified by PCR, an amplification fragment of about 1450 bp (not shown) was observed. Restriction analysis (ARDRA; [64]) of the amplified DNA was performed with the enzyme *AluI* which in various recent works [8, 9, 10, 15] was used to generate species-specific restriction patterns. The restriction pattern obtained was identical to that of the reference strain *B. cepacia* LMG 11351, also named PHP7 [13, 57] suggesting that strain PVFi5A could be included in the *B. cepacia* complex. To confirm the results obtained from restriction analysis, we have also determined the almost complete nucleotide sequence of the 16S rDNA.

The sequence obtained was aligned with that of various members of the main eubacterial lineages [67]. The alignment of the 16S rDNA sequence with that of several members of this group was used to draw a phylogenetic tree *(figure 1)*. The analysis of the phylogenetic tree clearly showed that strain PVFi5A belongs to

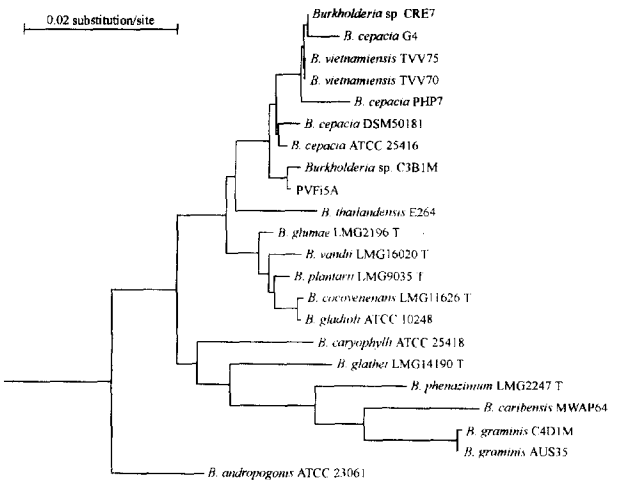

Figure 1. Phylogenetic neighbour joining tree obtained with the 16S rDNA sequences of members of genus *Burkholderia*. Isolate PVFi5A is located inside the *B. cepacia* complex [63].

the *B. cepacia* complex as defined in Vandamme et al. [63].

4. Discussion

Antagonism of *'B. cepacia'* against plant pathogenic fungi and the utility of this bacterium in the biological control of many plant and postharvest fungal diseases has been reported more than once. However, strains of *'B. cepacia'* have rarely been tested for their antagonism against bacterial phytopathogens [59], and this in spite of the fact that the control of bacterial diseases is generally considered very difficult.

This paper describes the isolation and characterisation of a new *Burkholderia* strain that displays antagonism toward a varied group of phytopathogenic bacteria as well as towards some pathogenic and non-pathogenic fungi. On the basis of the physiological and biochemical tests performed, we found that the characteristics indicated a member of the *B. cepacia* complex, although some of the tests described for the genomic species of this complex gave opposite results in our study. Strain PVFi5A had initially been assigned to an unknown species of *Pseudomonas*, on the basis of cellular fatty acid analysis (see below).

Differences between PVFi5A and other *'B. cepacia'* strains in some of the tests (colony colour, production of yellowish or greenish pigments, hydrolysis of starch, sensitivity to erythromycin) were, however, consistent with the high metabolic and physiologic diversity within collections of *'B. cepacia'* strains [36, 70]. Differences between strain PVFi5A and other *'B. cepacia'* strains in cellular fatty acid profiles were more significant. Data from the literature indicate that C16:0, C16:1 and C18:1 are the dominant components in *'B. cepacia'* strains generally, while the most characteristic are various 2OH and 3OH fatty acids: 16:0 2OH, 16:1 2OH, 18:1 2OH, 14:0 3OH and 16:0 3OH [53, 63, 69]. With strain PVFi5A, on the other hand, C18:1 accounted for more than 60% of the total fatty acid peak area (in other strains from 0 to ca. 29%), while the only hydroxy fatty acids detected were 18:1 2OH, 14:0 3OH and 16:0 3OH. Oyaizu and Komagata [39] and Stead [53], although they used different methods of saponification and methylation, stressed the importance of 3- and 2-hydroxy acids in grouping *Pseudomonas* strains (*B. cepacia* was formerly classified as belonging to the genus *Pseudomonas*, rRNA group 2). On the other hand, it has also been reported that the presence/absence of specific hydroxy acids is the most important feature of a fatty acid profile [56]. This would mean that, on the basis of the results obtained in the cellular fatty acid analysis, strain PVFi5A belonged either to an as yet undescribed *Burkholderia* species or to another genus altogether (for example, the profile of this strain bears some resemblance to *Oligella urethralis*, which contains 3 hydroxy acids, 14:0 3OH, 16:0 3OH and 18:1 2OH, or to species of the genus *Agrobacterium*, which contain 2 hydroxy acids, 14:0 3OH and 16:0 3OH). A third possibility would be that only the 3-hydroxy acids had taxonomic value for *'B. cepacia'*. This uncertainty was solved by sequencing the 16S rDNA of strain PVFi5A. The determination and analysis of this almost complete nucleotide sequence assigned this isolate to the *B. cepacia* complex [63] *(figure 1)*.

The ability of strain PVFi5A to inhibit the in vitro growth of several phytopathogenic bacte-

ria and fungi was investigated. The strain severely restricted the growth of most of these bacteria and fungi by the production of antimicrobial compounds, but probably not by the production of siderophores. Antimicrobial compounds were synthesized and secreted by strain PVFi5A in a number of culture media (PDA, NSA, KB, Woolley's medium) including an Fe(III)-rich medium (NSA supplemented with 100 mM $FeCl_3$).

The growth of strain PVFi5A on media routinely used in antimicrobial assays (PDA and NSA) was generally the same, and so were the antagonistic activities of this strain against the various fungi (on PDA) and bacteria (on NSA). Detectable differences in both growth and antagonism were only observed when the effect of environmental factors (carbon and nitrogen sources) on the antagonism of strain PVFi5A was tested, according to Upadhyay et al. [60]. However, we found that: i) an ammonium form of nitrogen (NH_4NO_3), irrespective of its nitrate form (KNO_3), did not enhance the antagonistic activity of strain PVFi5A; ii) the use of xylose or trehalose, as sole carbon sources, did not increase the level of antagonism exhibited by strain PVFi5A; and iii) antagonism against fungi was higher on PDA than on other growth media (NSA, KB, WMP). Growth of PVFi5A was fairly good on PDA, NSA, WM, WMP and WMP-N, but very poor on WMP-TT and WMP-TX.

All these results once again attest to the high genetic variability of *'B. cepacia'* strains, though this does not, of course, exclude the possibility that the antagonism of strain PVFi5A is influenced by physiological and environmental conditions other than those tested in this study.

In our study, the exact nature of the biocontrol activity exhibited by strain PVFi5A against fungi and bacteria, Gram-positive as well as Gram-negative, remained unknown, but most likely it depended upon the production of antibiotic compounds. We have demonstrated that Woolley's culture filtrates and a crude ethyl acetate extract from a 5-day-old culture of strain PVFi5A grown on NSA or PDA dishes showed inhibitory activity against a range of fungal and bacterial pathogens that were also inhibited by strain PVFi5A. This indicates that the antagonistic properties of strain PVFi5A are due to compounds extracted from the growth media with an organic solvent.

It has been ascertained that strains of *'B. cepacia'* produce several antibiotic substances including altericidins [27], cepacin A, cepacin B [41] and pyrrolnitrin [2, 5]. We do not yet know which of these substances are produced specifically by strain PVFi5A, but it is likely that this strain produces more than one antimicrobial substance: at least three partially purified fractions of the ethyl acetate extract, chromatographically different one from another, showed antibiotic activity. Moreover, the pattern of active substances produced by PVFi5A was quite different from that of the phytopathogenic strain ICMP5796. It will be of interest to test whether some of these substances produced by PVFI5A are also involved in phytopathogenicity.

In this study, the antagonistic activity of strain PVFi5A has been demonstrated against several plant pathogens. Moreover, preliminary experiments in which strain PVFi5A was applied to tomato seeds naturally or artificially infected with bacterial pathogens (*P. syringae* pv. *tomato*; *C. michiganensis* subsp. *michiganensis*; *E. carotovora* pv. *carotovora*) have shown that this strain protects seeds against bacterial pathogens in the early stages of disease development [54, 55]. Therefore this *'B. cepacia'* strain is potentially useful as a seed inoculant to suppress soil-borne diseases of tomato. Clearly, the release into the environment of strain PVFi5A as well as of any other *'B. cepacia'* strain must be considered with great attention because of the implication of many strains in nosocomial infection.

Résumé — Isolement, de la rhizosphère de plants de tomate sains, et caractérisation d'une nouvelle souche de *Burkholderia* antagoniste. Nous avons partiellement caractérisé une nouvelle souche de *Burkholderia* (PVFi5A) qui se montre antagoniste pour de nombreux agents bactériens et fongiques pathogènes. Cette souche a été isolée de la rhizosphère de plants de tomate, et a été apparentée au

complexe *Burkholderia cepacia* sur la base des caractéristiques de sa culture, de sa morphologie et de sa biochimie, y compris sur la base de la détermination de la séquence de l'ADNr 16S et du profil des acides gras. La souche PVFi5A est une bactérie aérobie gram-négative, oxidase- et catalase-positive, mobile grâce à une touffe de flagelles polaires, capable de croître sur divers milieux sans produire de pigments diffusibles; elle est non virulente pour l'oignon, capable de croître à 41 °C et résistante à plusieurs substances antibiotiques. Son profil en acides gras comprend les hydroxy-acides 18:1 2OH, 14:0 3OH et 16:0 3OH mais non les acides 16:0 2OH et 16:1 2OH. L'activité antagoniste de cette souche est due à divers composés antimicrobiens non encore identifiés, un ou plusieurs d'entre eux pouvant différer de ceux déjà connus chez certaines souches de *'B. cepacia'*. La capacité de la souche PVFi5A d'empêcher la croissance d'importants agents phytopathogènes, bactériens ou fongiques, fait de cette souche un agent potentiel pour la lutte biologique. © Elsevier, Paris

complexe *Burkholderia cepacia* / tomate / contrôle biologique / rhizosphère

Acknowledgments

This work was supported by a grant from the Italian Ministry of Universities and Scientific and Technological Research.

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