

# Environmental *Burkholderia cepacia* Strain Cs5 Acting by Two Analogous Alkyl-Quinolones and a Didecyl-Phthalate Against a Broad Spectrum of Phytopathogens Fungi

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**Abstract** An environmental *Burkholderia cepacia* strain named Cs5 was isolated and identified first using API biochemical identification system and then with 16S rDNA and *recA* sequence homology search. This bacterium exhibited a broad spectrum of fungicidal activities against *Alternaria alternata*, *Aspergillus niger*, *Fusarium culmorum*, *F. graminearum*, *F. oxysporum* and *Rhizoctonia solani*. In the liquid conditions, the MIC of *A. niger* and *R. solani* were reached with, respectively, 1.25–2% of the Cs5 liquid culture supernatant. However, in the solid conditions, the same inhibition was caused in the presence of 3% of the Cs5 supernatant. The exhibition of these two fungi at low concentrations of supernatant Cs5 caused various morphological changes of their mycelia which were observed by confocal microscopy. Three antifungal compounds, named Cs5-255, Cs5-257 and Cs5-446, were purified from the Cs5 culture. The structural analysis of these molecules showed that Cs5-255 and Cs5-257 are analogous and belonged to the alkyl-quinolone family,

while Cs5-446 was a didecyl-phthalate, isolated for the first time from a bacterium.

## Introduction

Chemical treatments have been the main method for controlling plant diseases. However, public concerns about side effects of these products have triggered the search for alternative means that are less harmful to human health and to the environment as well. The last decade has known an increasing interest in biological control methods. Such methods are believed to be environmentally friendly and are widely considered in plant protection against fungal pathogens [5, 9]. Numerous micro-organisms which produce antifungal factors were identified and several modes of action have been described. Antagonists are naturally occurring organisms which are characterized by their action against pathogen proliferation by several mechanisms: (i) secretion of antibiotics and toxins, (ii) competition for the space or nutrients, (iii) parasitism of the pathogen by the biological control agent and (iv) induction of the plant self defence [16]. Only few micro-organisms have been fully commercialized for the control of plant pathogens [4].

*Burkholderia cepacia* is a ubiquitous bacterium [1]. Several strains of this species have been shown to enhance disease resistance in plants, improve nitrogen fixation and contribute to better water management and overall host adaptation to environmental stresses [2]. Indeed, several *B. cepacia* strains have been characterized for their antagonistic activities against a wide range of plant pathogens. This bacterium is known to produce a rich array of secondary metabolites such as pyrrolnitrin, phenazines, 2,4-diacéthylephloroglucinol and lipopeptides [20]. It has

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been already used as an effective biological control agent which could suppress damping-off caused by *Pythium* spp. [11] and root rot caused by *Rhizoctonia solani* [17].

In this study, a *B. cepacia* (Cs5) was isolated and identified. This strain harboured a broad range of antifungal activities against several phytopathogenic fungi at very low concentrations. Three antifungal molecules were purified and their chemical structures were determined using spectrometric techniques.

## Materials and Methods

### Fungal Strains

*Alternaria alternata*, *Aspergillus niger*, *Fusarium oxysporum* and *R. solani* were provided by the strain collection of the Centre of Biotechnology of Sfax, Tunisia. *F. culmorum* and *F. graminearum* were kindly provided by Dr. Quirico Mighelli, Sassari University, Italy. Fungal cultures were grown and maintained on Potato Dextrose Agar (PDA, Fluka). Fungal conidia were harvested by scraping the biomass, re-suspended in potato dextrose broth ( $10^7$  spores  $\text{ml}^{-1}$ ) and harvested at  $-20^\circ\text{C}$  in 15% glycerol.

### Isolation and Identification of the Antagonistic Bacterium

The strain Cs5 was isolated from the rhizosphere of almond trees (Sfax, Tunisia) using the standard serial dilution method and was characterized using physiological and biochemical tests (API 20E) and by molecular identification with 16S rDNA and *recA* sequence analyses. The Polymerase Chain Reaction (PCR) amplification of 1.5-kb fragment from 16S rDNA gene was carried out using the universal primers (*Fdl/Rdl*). For 0.9-kb fragment amplification from the *recA* gene, primers (*recAf/recAfr*) were designed on the basis of the alignment of published sequence data of recombinase A of *B. cepacia*. Details of PCR amplifications and primers sequences are given in the supplementary Table 1. Amplified PCR product was purified and sequenced with an automatic sequencer (3100-Avant Genetic Analyser).

### Evaluation of the Cs5 Antifungal Activities on Solid Medium

The antifungal activity of the Cs5 culture supernatant was estimated using a growth inhibition assay. The liquid culture was carried out in LB at  $27^\circ\text{C}$  and 140 rpm for 48 h. The supernatant was obtained by centrifugation at  $4000\times g$  for 10 min and sterilized by an UV treatment for 20 min. Increasing volumes of the supernatant were

incorporated into molten PDA and then homogenized (Experimental plates). In parallel, equivalent volumes of PDB were added in the control plates. The fungal inoculum (10  $\mu\text{l}$ ) was placed onto the centre of each plate and was incubated for 72 h at  $27^\circ\text{C}$ . For the non-sporulating fungus, an aliquot of the fresh culture in PDB was used as the inoculum. The test was done in triplicate. The surfaces of fungal growth were carefully measured and the inhibition ratios were calculated using the following formula:

$$\text{Inhibition ratio (\%)} = ((C - E)/C) \times 100$$

: control surface, E  
: experimental surface).

### Effect of Cs5 Antifungal Compounds on the Mycelia Growth

A serial dilution of the sterile Cs5 culture supernatant from 0.25–3% was added to 100-ml Erlenmeyer flasks containing PDB at a final volume of 20 ml. In each flask, we added either  $10^6$  spores of *A. niger* or 500  $\mu\text{l}$  of a fungal mycelia suspension of *R. solani*. The control flasks contained only PDB. The flasks were incubated at  $27^\circ\text{C}$  with shaking at 140 rpm for 2–4 days. For each fungus, the minimal inhibitory concentration (MIC) was defined as the lowest concentration of the supernatant at which no visible growth was observed. The  $\text{MIC}_{50}$  and  $\text{MIC}_{90}$  were defined as the concentrations that resulted in, respectively, 50 and 90% growth reduction, where the growth ratio is calculated by weighing the dry fungal mycelia and referred to the untreated control. The minimal fungicidal concentration (MFC) is considered to be the lowest concentration at which no visible growth was observed, for the treated fungi, on solid media. For each fungus, MFC was determined by removing 100- $\mu\text{l}$  aliquot from each Erlenmeyer flask, transferring to a PDA plate, and incubating for 72 h at  $27^\circ\text{C}$ . From each treatment, an aliquot was removed for the observation of the impact of the Cs5 supernatant on the mycelia structure by confocal microscopy (LSM 510, Zeiss).

### Purification of Cs5 Antifungal Compounds

One litre of Cs5 culture was acidified to pH 2 with HCl 1 N and treated with the equal volume of ethyl acetate for 1 h in a rotary shaker and then centrifuged for 15 min at  $3000\times g$ . The organic extract was concentrated and the residue was dissolved in 10-ml methanol, then injected into preparative RP-HPLC on a C18 column (Eurosphere 100  $\mu\text{m}$ ,  $20 \times 250$  mm) using a water/acetonitrile gradient at a flow rate of  $5 \text{ ml min}^{-1}$ . The separation was monitored by UV detection at 254 nm. Major peaks were collected,

concentrated and tested for their activities against *A. niger*. The active fractions were then purified by semi-preparative RP-HPLC (Agilent 1100 series), using a C18 column (Eurospher 100  $\mu\text{m}$ ,  $8 \times 300 \text{ mm}$ ) at a flow rate of  $1 \text{ ml min}^{-1}$ . An aliquot of  $20 \mu\text{l}$  of the active fractions was analyzed by LC-MS<sup>n</sup> (Agilent, LC/MSD Trap XCT) on an analytical C18 column (Zorbax 300  $\text{\AA}$ ,  $2.1 \times 150 \text{ mm}$ ). For the structural elucidation, 1D and 2D NMR spectra were obtained in  $\text{CDCl}_3$  at 400 and 100 MHz for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively, on a Bruker Avance 400 MHz NMR spectrometer. High resolution mass spectra (HRESIMS) were conducted on a LTQ Orbitrap mass spectrometer (Thermo Finnigan).

## Results

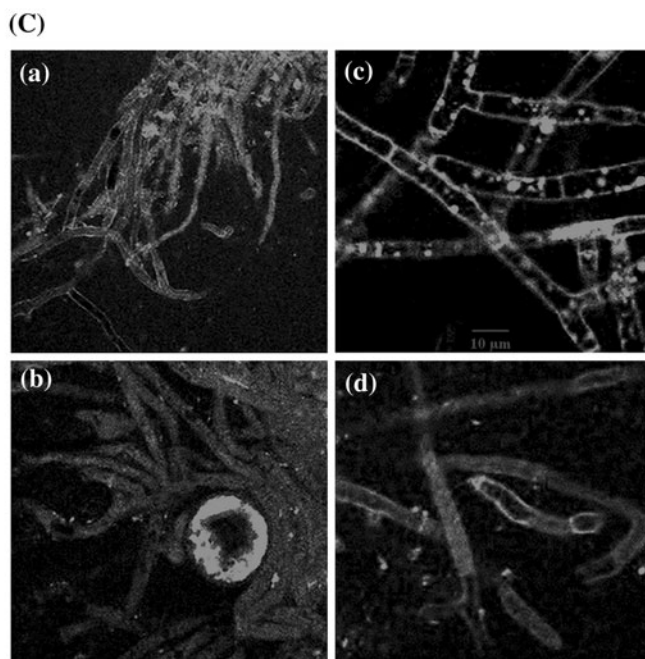
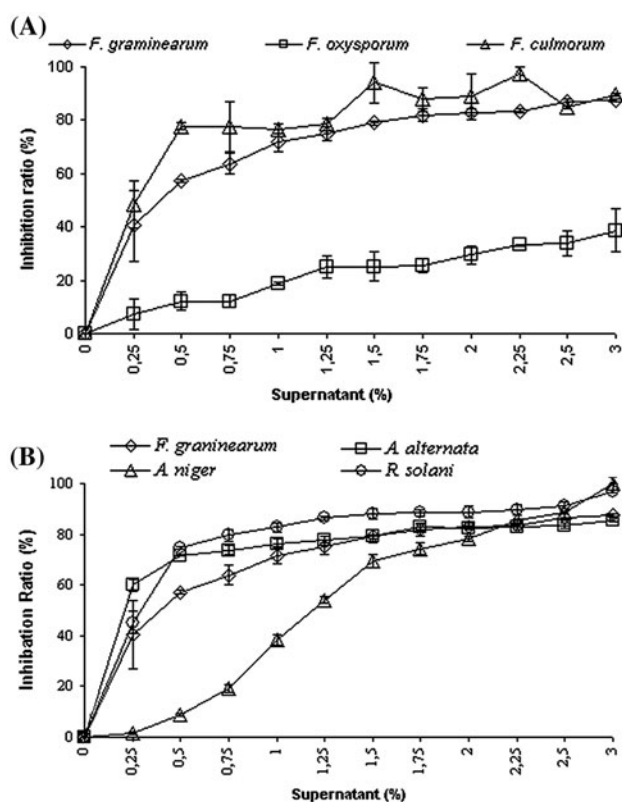
### Isolation and Identification of the Cs5 Strain

The isolated Cs5 strain, showing a good activity against *A. niger*, was identified as a member of *Burkholderia* genus using the API biochemical identification system (API 20E)

and the API-LAB software. The sequence analysis of its PCR 1.5 kb 16S rDNA showed that Cs5 isolate belongs to the *B. cepacia* complex group. To confirm this identification, *recA* gene PCR amplification and sequence analysis were performed using specific primers of genomovar I. The phylogenetic tree of the Cs5 *recA* gene and seven other *Burkholderia* species showed that the strain Cs5 was closely apparent to the strain *B. cepacia* (AF456066) as it was shown in Supplementary Fig. 1.

### Evaluation of Cs5 Antifungal Activities

The antifungal activity of Cs5 against a selection of phytopathogenic fungi was quantified by the fungicides incorporation in PDA. This test showed that all of them were very sensitive to Cs5 fungicides. However, it was observed that the sensitivity could be different between fungi of a same genus as it was shown for *F. oxysporum*, *F. culmorum* and *F. graminearum* (Fig. 1A). Actually, for *R. solani* and *F. graminearum*, 50% growth inhibition was observed using 0.25% of the Cs5 supernatant, whereas for *A. niger*, similar inhibition ratio was reached with 1.1% of



**Fig. 1** Quantification and observation of fungicidal activities of Cs5 culture supernatant: **A** Evaluation of fungal growth inhibition of *F. culmorum*, *F. graminearum* and *F. oxysporum* on solid medium amended with Cs5 culture supernatant. **B** *F. graminearum*, *A. alternata*, *A. niger* and *R. solani*. **C** Confocal microscopic

observations of the Cs5 culture supernatant effect on fungal growth: *a* and *c* untreated mycelium of *A. niger* and *R. solani*, respectively; *b* *A. niger* mycelium treated with 0.4% Cs5 culture supernatant; *d* *R. solani* mycelium treated with 1.5% Cs5 culture supernatant

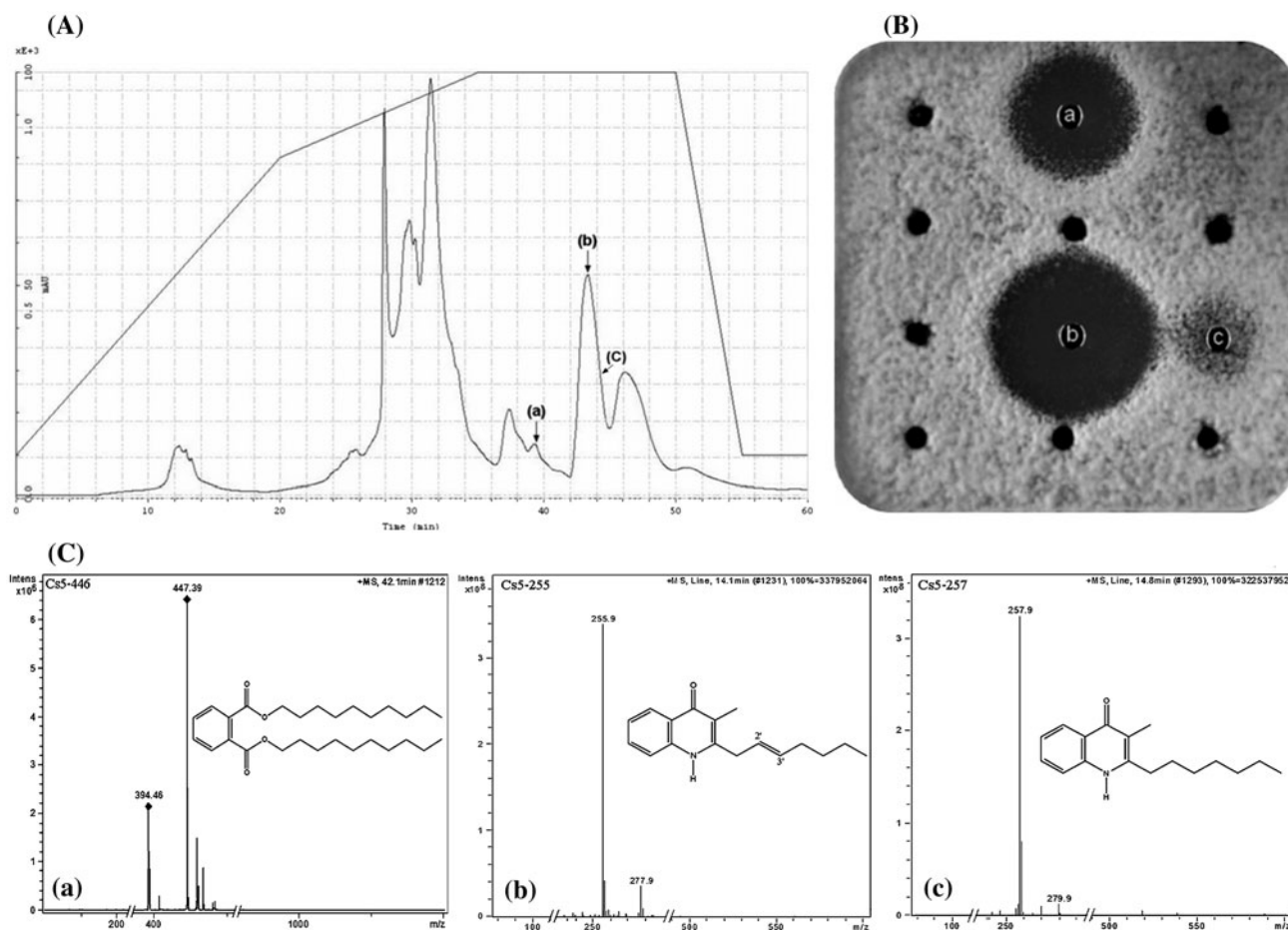
supernatant. Furthermore, *A. alternata* showed a high sensitivity to Cs5 fungicides since the same inhibition was reached with 0.05% of supernatant (Fig. 1B).

The Cs5 activity was also quantified in a liquid medium against the sporulate fungus, *A. niger* and the non-sporulate one, *R. solani*. As shown in supplementary Table 2, Cs5 exhibited a powerful suppression of fungi growth with low percentages of crude supernatant with a MIC<sub>50</sub> of 0.45% for *A. niger* and 1% for *R. solani*. The effect of Cs5 fungicides on growth and morphological changes of these fungi was examined by confocal microscopy in the presence of different percentages supernatant (Fig. 1C). Observations showed that for *A. niger*, spores germination was considerably delayed and the germ tube changed its aspect starting from 0.25% of supernatant. After 24 h of incubation at 0.4%, the formation of swollen bulbous with condensed granulation on the border was observed. This structure could not show up at 0.6% of supernatant. However, mycelium fragments were observed with spread

fluorescence (Fig. 1Ca, Cb). *R. solani* was less sensitive to Cs5 fungicides. Indeed, 1.5% was needed to break most of mycelium and to lead to fluorescence scattering (Fig. 1Cc, Cd).

#### Purification of Antifungal Compounds of Cs5

The chromatographic analysis of the chemical composition of the Cs5 organic extract was realized using RP-HPLC. The bioguided antifungal evaluation of the HPLC fractions against *A. niger* yielded three active fractions (a), (b) and (c), all eluted with 100% acetonitrile (Fig. 2A). After a subsequent step of purification by semi-preparative RP-HPLC, the main chemical constituent of each fraction was purified: Cs5-446 from fraction (a), Cs5-255 from fraction (b) and Cs5-257 from fraction (c). To confirm the existence of these three active fractions, the culture was reproduced several times and the compounds were separated by two different HPLC and to avoid contamination with plastic,



**Fig. 2** A Separation of compounds contained in the organic extract of Cs5 culture supernatant by preparative RP-HPLC using C18 column and water/acetonitrile gradient. B Antifungal activity of HPLC fractions against *A. niger* using the technique of well test:

*a* 2.5  $\mu$ g of purified Cs5-446, *b* and *c* 10  $\mu$ g of purified Cs5-255 and Cs5-257. C Chemical structures and LCMS profiles of antifungal molecules secreted by the strain Cs5: *a* Cs5-446, *b* Cs5-255 and *c* Cs5-257

only glass material were used during the purification. The antifungal activities of the three purified molecules were estimated against *A. niger*. With 10  $\mu\text{g}$  of the pure Cs5-255, a clear zone of 45 mm was observed and with the same quantity, Cs5-257 showed a reversible inhibition zone of 21 mm. However, the compound Cs5-446 was tested at 2.5  $\mu\text{g}$  and showed an inhibition zone of 38 mm (Fig. 2B).

#### Structural Elucidation of Antifungal Molecules

The molecular weights of the three purified antifungal compounds were determined by LC-MS<sup>n</sup> as 255, 257 and 446 Da for Cs5-255, Cs5-257 and Cs5-446, respectively. Concerning the last compound Cs5-446, a molecular formula of C<sub>28</sub>H<sub>46</sub>O<sub>4</sub> was assigned on the basis of HR-MS data and a careful analysis of its full NMR dataset conducted to its characterization as didecyl-phthalate (DDP) (Fig. 2Ca). The LC-MS<sup>n</sup> analyses of Cs5-255 and Cs5-257 showed similar fragmentation patterns. It was suggested that these two molecules were structurally related. On the basis of the comparison of their NMR data, these compounds have been unambiguously identified as 4-oxo-3-methyl-2-alkylquinolone derivatives (Fig. 2Cb, Cc). The only difference between these two analogue molecules was found on the alkyl side-chain, which was saturated for Cs5-257 and  $\Delta^{2',3'}$ -monounsaturated in the case of Cs5-255.

#### Discussion

In order to develop an environmentally friendly method for the control of plant pathogenic fungi, several antagonistic bacteria were isolated from the region of Sfax (Tunisia). The isolate named Cs5 was retained for this study for its interesting activity against *A. niger*. The molecular identification showed that this isolate belongs to *B. cepacia* genomovar I. The investigation of Cs5 antifungal activities against *A. alternata*, *A. niger*, *F. culmorum*, *F. graminearum* and *R. solani* showed a total fungal growth inhibition at very low percentage of the sterile Cs5 supernatant. *Fusarium oxysporum* has a relative resistance to Cs5 fungicides with an inhibition ratio of 40%. Nevertheless, this inhibition ratio was still considered high since it was reported that 20% suppression is an acceptable antifungal activity [7]. To better understand the Cs5 fungicidal activities, confocal microscopy was used to observe the impact of Cs5 culture supernatant on the germination and growth as well as on the aspect of fungi mycelium in a liquid culture. *Aspergillus niger* mycelium was irreversibly damaged and spore germination was delayed. Moreover, formations of bulbs, condensation of protoplast and to a

total fragmentation of mycelium were observed. Concerning *R. solani*, fluorescence dissemination inside and outside the mycelium was noticed. This fact was evidenced by a deterioration of the fungus cell wall and an alteration of the permeability. These observations prove a specific action mode of the antifungal compounds and probably each effect is related to a specific molecule [13].

The antimicrobial activities of *B. cepacia* are known to be related to the production of several molecules. Most of them have antifungal activities, such as pyrrolnitrin or cepaciamide A. The last one is a fungitoxic compound with a narrow activity spectrum but it was unstable at UV irradiation. Pyrrolnitrin was thoroughly studied due to its high antibiotic activities against fungi and Gram positive bacteria [20]. The fungicides of Cs5 exhibited a broad spectrum of antifungal activities against plant pathogenic fungi but a very low antibacterial activity has been detected against *Bacillus cereus*. The HPLC separation and LC-MS<sup>n</sup> analysis revealed three active molecules. Cs5-255 was found to be abundant in the supernatant of Cs5, while Cs5-446, the most active molecule, was produced at a low level. The mass spectrometric analysis showed that Cs5-255 and Cs5-257 were structurally analogous. The structural difference between these two molecules is the origin of the difference in activity which proves the involvement of the carbon chain in their action mechanism. Despite their close molecular masses with pyrrolnitrin and its derivatives they do not have any similarity [18]. The NMR characterization of these two molecules revealed that they belong to the alkyl-quinolone family with a methylation in position 3, characteristic of the genus *Burkholderia*, which play an important role in their biological activities [21]. These compounds present a growing interest for their involvement in the cell-cell communication and their diverse biological activities [3, 21]. The antifungal activity of quinolones was rarely described and it may result from another action mode than that described on prokaryotes [14, 15]. Cs5-446 was identified as didecyl-phthalate. This compound is well known as an industrial plasticiser. Recently, many natural phthalates were isolated from microorganisms, algae and plants [8, 12, 22] and more recently, three phthalates were isolated from *B. cepacia* strain K87 [19]. To the knowledge, this is the first time that a didecyl-phthalate was purified from a bacterium [6, 10].

In conclusion, *B. cepacia* Cs5 is an environmental strain with an important antagonistic effect against various phytopathogenic fungi. This strain is an interesting biofungicide producer due to the secretion of several active factors produced in a significant quantities and active at low concentrations. Cs5 produce two known quinolones but not for their fungicidal activities and didecyl-phthalate isolated for the first time from this bacterium.

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