J Ind Microbiol Biotechnol (2008) 35:783–790
DOI 10.1007/s10295-008-0331-z

SHORT COMMUNICATION

Diversity in antifungal activity of strains of *Chromobacterium violaceum* from the Brazilian Amazon

Eriana Serpa Barreto · Adalgisa Ribeiro Torres · Marliton Rocha Barreto · Ana Tereza Ribeiro Vasconcelos · Spartaco Astolfi-Filho · Mariangela Hungria

Received: 10 November 2006 / Accepted: 13 February 2008 / Published online: 18 March 2008

Abstract *Chromobacterium violaceum* is a free-living Gram-negative bacterium found in soil and aquatic habitats; abundantly present in the Brazilian Amazon, it is an important example of exploitable microbial diversity of the tropics. In this study, 24 strains from the Brazilian Amazon and ATCC 12472^T were investigated for biocontrol potential of seven fungi pathogenic to soybean [*Glycine max* (L.) Merril] seed. Both cells and the supernatants of two Brazilian strains, 07-1 and 27-1, together with ATCC 12472^T were strongly antagonistic to six out of the seven fungi. The antifungal activity of the Brazilian strains to *Fusarium* sp., *Phomopsis* sp. and *Cercospora kikuchi* was consistently stronger than that of ATCC 12472^T. In addition, the two Brazilian strains, but not ATCC 12472^T, were effective against *Corynespora* sp., and all three strains and their supernatants were equally effective against *Aspergillus* sp. and *Colletotrichum* sp. None of the strains had antifungal activity against *Botroyodiplodia* sp. Three potential mechanisms related to the antibiosis were investigated: violacein toxicity, cyanide production and chitinolytic activity; however, it was not possible to associate any of them with the antifungal activity. The results highlight the biotechnological potential still to be explored within the poorly characterized microbial biodiversity of the tropics.

Keywords Antifungal activity · *Chromobacterium violaceum* · Cyanide · Fungicide · Violacein

E. S. Barreto · A. R. Torres · M. R. Barreto · M. Hungria (✉)
Embrapa Soja, Cx. Postal 231, Londrina,
Paraná 86001-970, Brazil
e-mail: hungria@cnpso.embrapa.br

E. S. Barreto · A. R. Torres · A. T. R. Vasconcelos · S. Astolfi-Filho · M. Hungria
CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasilia, Brazil

A. T. R. Vasconcelos
LNCC/MCT, Labinfo, Rua Getúlio Vargas 333,
Petrópolis, Rio de Janeiro 25651-071, Brazil

S. Astolfi-Filho
Laboratório de Tecnologias de DNA, UFAM,
Manaus, Amazonas 69077-000, Brazil

Introduction

Chromobacterium violaceum is a free-living Gram-negative bacterium that inhabits soil and water of tropical and subtropical regions; it is abundant in, and on the banks of the Negro river in the Brazilian Amazon region [8, 21, 29]. The most characteristic phenotypic feature of *C. violaceum* is the production of a deep violet pigment named violacein [7]—first isolated in 1944 [55]—the chemical structure of which was partially elucidated a few years later: it consists of a 5-hydroxyindole, an α-pyrrolidone and an oxindole unit, formed from the condensation of two modified tryptophan molecules [3–5, 18, 19].

The genome sequence of *C. violaceum* type strain ATCC 12472^T revealed remarkable potential for adaptability; the bacterium has several pathways for energy generation, and an enormous number of genes related to transport and motility capability [58]. These genes may explain the versatility of the organism, which is found in a variety of ecosystems. It has the capacity to adapt to stressful, normally growth-limiting conditions [30].

The pharmaceutical potential of *C. violaceum* has been a subject of much interest, especially in Brazil, with an

emphasis on the activity of violacein [21]. Violacein, isolated from *C. violaceum*, exhibits antimicrobial activity against *Mycobacterium tuberculosis* [52], *Trypanosoma cruzi* [22, 23], and *Leishmania* sp. [34], important causes of diseases that are endemic mainly in the Amazon region. Violacein has also shown bactericidal, anti-viral [8, 35, 44], and anti-tumoral [37, 57] activities. Finally, since violacein may be protective against UV radiation, it could be useful for dermatological therapy [9]. Other antibiotics are also produced by *C. violaceum* [41, 48]. However, although the antibiotic properties of *C. violaceum* have been long known [18, 24, 35], the genome sequencing of strain ATCC 12472^T raised the international attention of its pharmaceutical potential [20].

Further biotechnological potential of *C. violaceum* includes the synthesis of 3-hydroxyvalerate homopolymer (polyhydroxyvalerate) and other short-chain polyhydroxyalkanoates (PHAs), bioplastics that may replace those derived from petrochemicals, with applications in medicine and industry [26, 31, 53], particularly in the production of biodegradable plastics [27].

Many genes of *C. violaceum* may contribute to solving environmental and agricultural problems [54, 58]. Major examples comprise the operons related to environmental detoxification and biometallurgy (by bioleaching and mineral biooxidation): *ars*, encoding genes for the arsenic resistance, *cyn*, with a role in cyanate detoxification, and *hcn*, related to the biogenic production of hydrogen cyanide (HCN) [1, 6, 11]. Cyanide produced by *C. violaceum* in a mercury-free process has potential for gold recovery (biometallurgy) [10, 50], as well as in suppression of fungal diseases of roots [33].

Brazil's economy is strongly based on agribusiness; however, the biotechnological potential of *C. violaceum* to address agricultural issues has not yet been investigated. The use of microorganisms as biological control agents may be ecologically sound and economically viable [2, 14, 17], and *C. violaceum* is a strong candidate for exploitation. The antibiotic activity of *C. violaceum* can be so strong that the Negro river is known as the "hungry river", since its biomass content is as little as a two-hundredth of that of the Amazon River [8]. Antibiosis of *C. violaceum* against bacteria [45], fungi [47], protozoans [36], and nematodes [16] has also been reported in other aquatic and terrestrial ecosystems. The powerful antibiotic activity of *C. violaceum* attributable to the violacein is regulated by an *N*-acylhomoserine lactone (AHL)-dependent quorum-sensing system; however, the genome of *C. violaceum* suggests that other genes may play major roles, e.g. those related to the synthesis of cyanide and chitinases [58], which are also AHL-regulated.

The objective of this study was to investigate the antibiotic properties of *C. violaceum* against seven seed-pathogenic fungi, all of which constitute major problems for the soybean crop in Brazil, to elucidate mechanisms related to the antibiosis.

Materials and methods

Bacteria

Chromobacterium violaceum *strains*

Chromobacterium violaceum type strain ATCC 12472^T (=CCT 3496, =NCIB 9131, =NCTC 9757, =JCM 1249, =DSM 30191, =IAM 12470, =D 252, =LMG 126) was obtained from the Fundação Tropical de Pesquisas André Tosello, Campinas, São Paulo, Brazil, the source used by the Brazilian National Genome Project Consortium [58].

Previously, Hungria et al. [29] obtained 24 strains from Negro river-water samples at three sites close to Manaus city, in the State of Amazon. In this study, based on the sequencing analysis of the 16S rRNA, strain 07-1 showed the highest similarity with ATCC 12472^T, and the other strains were positioned in two different clusters (29). The strains used in this study are listed in Table 1.

Culture media and growth conditions

Bacteria were grown in four media: Luria-Bertani (LB) (1.0% bacto-tryptone; 0.5% yeast extract; 1.0% NaCl); peptone medium (2% peptone); King's B medium (2.0% proteose peptone No. 03; 0.15% K_2HPO_4; 0.15% $MgSO_4.7H_2O$; 1% glycerol), and CV medium (0.5% peptone; 0.3% yeast extract; 0.6% NaCl; 0.25% glucose—Dr. Regina Vasconcellos Antônio, UFSC, personal communication). For solid media, 1.5% of agar was added. Bacteria were grown at 28–30 °C. Culture stocks were prepared on CV solid medium and kept at 28 °C, and also in CV liquid medium, mixed with 30% glycerol (v/v), at −70 °C, in a deep freezer, after a fast-freezing procedure placing cryotubes in liquid nitrogen.

Characterization of the bacteria

Characterization of violacein production in vitro

Production of violacein in vitro by the strains was verified based on the presence of a violet pigment, after 72 h of growth at 28 °C on CV solid medium.

Chitinolytic activity in vitro

Colloidal chitin was obtained by partial hydrolysis with concentrated HCl: crab-shell chitin (20 g) (Sigma, practical

Table 1 Origin of the isolates of *Chromobacterium violaceum*, color of the colonies in CV solid medium, production of cyanide, chitinolitic activity and antifungal activity in vitro

Strain	Origin	Color	HCN production[a]		Chitinolytic activity[b,c]	Antifungal activity[c,d]	
			24 h	48 h		*Fusarium* sp.	*Phomopsis* sp.
ATCC 12472^T	Mentekab, Malaysia	Dark violet	++	+++	1.6 e–h	11 b	14 c
CVACIM-1	Acajatuba	Violet	++	+++	1.7 c–h	7 c	5 d
CVAC3M-1	Acajatuba	Violet	++	+++	1.8 a–h	8 c	4 d
CVAC2-1	Acajatuba	Light violet	+	+++	1.3 gh	12 b	13 c
CVAC2-2	Acajatuba	Dark violet	+	+++	1.5 e–h	11 b	12 c
CVAC3-1	Acajatuba	Light violet	+	++	2.3 a–f	13 b	14 c
CVAC3-2	Acajatuba	Dark violet	+++	+++	2.4 a–f	12 b	13 c
CVAC5-1	Acajatuba	Light violet	+	++	2.3 a–f	6 c	4 d
CVAC5-2	Acajatuba	Dark violet	++	+++	2.2 a–g	7 c	4 d
CVAC6-1	Acajatuba	Violet	+++	+++	2.3 a–f	7 c	5 d
CVAC7-1	Acajatuba	Violet	++	++	1.6 d–h	11 b	14 c
CVRP4-1	Presidente figueiredo	Violet	++	++	2.5 a–d	12 b	13 c
CVTGRP5	Tarumã Grande	Violet	+++	+++	2.8 a	13 b	14 c
CVTGRP7	Tarumã Grande	Violet	+	+	0.0	0 d	0 d
CVPF4-1	Presidente Figueiredo	Light violet	+++	+++	2.4 a–e	6 c	6 d
CVPF06-1	Presidente Figueiredo	Violet	+++	+++	2.7 ab	6 c	5 d
CVIN07-1	INPA's Campus	Violet	+++	+++	1.8 a–h	22 a	29 a
CVRP27-1	Rio Preto da Eva	Violet	++	+++	1.8 b–h	23 a	20 b
CVPF29	Presidente Figueiredo	Violet	++	+++	1.2 h	7 c	4 d
CVIN46	INPA's Campus	Violet	++	+++	1.4 f–h	5 c	4 d
CVRP55-1	Rio Preto da Eva	Violet	++	++	1.8 a–h	13 b	13 c
CVRP58-1	Rio Preto da Eva	Violet	+++	+++	2.2 a–f	12 b	13 c
CVRP62-1	Rio Preto da Eva	Violet	++	++	2.6 a–c	13 b	13 c
CVRP67-1	Rio Preto da Eva	Violet	+++	+++	1.5 e–h	7 c	5 d
CVRP69-1	Rio Preto da Eva	Violet	+++	+++	1.7 d–h	6 c	5 d

[a] Intensity of HCN reaction with picrate indicator: none, −; weak, +; moderate, ++; strong, +++

[b] Chitinolytic activity index was given by the diameter of chitin degradation clear zone/diameter of bacterial colony

[c] Tests performed in triplicate for each isolate and repeated three times; means followed by the same letter are not statistically different (Tukey, $P \leq 0.05$)

[d] Data represent the inhibition halo (mm) after 7 days, evaluated by the pairing method

grade) was dissolved in 200 mL of HCl and maintained under agitation for 3 min at 40 °C. The chitin was precipitated by the addition of water at 5 °C, resulting in a colloidal suspension. The suspension was filtered (filter paper) and the colloid remaining in the filter was successively washed with water until the suspension reached pH 4.0. The colloidal chitin was maintained at 4 °C.

Chitinolytic activity of *C. violaceum* strains was verified using the method proposed by Chernin et al. [12, 13], but optimized for *C. violaceum*. First, strains were streaked on the semi-minimum (SM) medium [59] [containing, per liter: 15 g glucose; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.6 g K_2HPO_4; 0.15 g KCl; 1 g de NH_4NO_3; 1 mL of micronutrient solution (containing, per liter, 0.005 g $FeSO_4 \cdot 7H_2O$; 0.006 g $MnSO_4$; 0.004 g $ZnSO_4 \cdot H_2O$; 0.002 g CoCl)] and 10% (v/v) of CV medium (Sect. "Culture media and growth conditions"). The mixture was supplemented with colloidal chitin (0.2%) and, for solid medium, with 1.5% agar. Bacteria streaked on SM–CV-chitin solid medium were incubated at 28 °C for 96 h, when the presence or the absence of a halo—due to the chitinolytic activity—was determined. The chitinolytic activity index was given by the ratio of the diameter of chitin degradation clear zone in relation to that of the bacterial colony, according to Teather and Wood [56].

Cyanide production in vitro

Qualitative production of cyanide by *C. violaceum* strains was evaluated as described by Kremer and Souissi [32]. Bacteria were streaked on one-fourth-strength solid (1.5% agar) King's B medium. In the lid of each Petri dish a filter-paper disk was fixed, saturated with two solutions—picric

acid at 1.4% and Na_2CO_3 at 10%; the plates were sealed with parafilm and bacteria were grown at 28 °C. Cyanide production was detected by visually comparing inoculated and non-inoculated plates at 24 and 48 h after sealing.

Crystallized violacein used in the assays

Crystallized violacein from *C. violaceum* type strain ATCC 12472^T was kindly supplied by Dr. Regina Vasconcellos Antônio (UFSC), and obtained using the procedure described by Rettori and Durán [44]. Violacein solutions used in the assays (Sect. "Inhibition by violacein") were prepared as described by Shirata et al. [47]. Separately, 1 g of dried extract of the violacein was dissolved in 50 mL of acetone to prepare a stock solution. The stock solution was mixed with an equal volume of distilled water to prepare a diluted half-strength solution, which was then diluted successively, to half strength each time, using 50% acetone; four concentrations were prepared: 0.01, 0.05, 0.1 and 0.5%.

Fungi

Seven seed-pathogenic fungi were kindly supplied by Dr. Ademir A. Henning of the Plant Pathology Laboratory (Embrapa Soja), where they were classified as *Cercospora kikuchi*, *Colletotrichum* sp., *Fusarium* sp., *Phomopsis* sp., *Corynespora* sp., *Aspergillus* sp. and *Botroyodiplodia* sp. The fungi were grown on potato-dextrose agar (PDA) medium [containing, per liter: 200 g of sliced potatoes boiled in water for 5–10 min, with the broth decanted and filtered; 10 g of dextrose (glucose); 1.5% agar], the purity of the cultures was confirmed and the stocks were maintained in PDA medium at 4 °C.

Inhibition of pathogenic fungi in vitro

Fungal disks placed at equidistant points in Petri dishes

Antifungal activity of each bacterium and its supernatant was tested using the pairing method on PDA medium, as described by Araújo et al. [2]. Pure cultures of each fungus were initially grown for 7 days at 25 °C on PDA medium supplemented with streptomycin at 200 μg/mL to reduce bacterial contamination; all *C. violaceum* strains were resistant to this concentration of antibiotic. After that, 1-cm disks were cut from the edges of active colonies of each fungus with a cork borer. Four disks were transferred to equidistant sites of new 9-cm Petri dishes containing PDA medium, 24 h prior to inoculation of *C. violaceum*.

To obtain cells and supernatants of *C. violaceum*, each bacterium was grown in CV liquid medium at 28 °C for 72 h under constant agitation (100 rev./min). Cells were removed by centrifugation (10,000 *g* for 10 min) and the supernatant was sterilized by passage through a 0.45-μm Millipore filter. Cells were washed three times with 0.2 M sterile phosphate buffer pH 6.5 and resuspended to produce concentrations of about 10^{12} cells/mL, in phosphate buffer. Each *C. violaceum* strain or its supernatant (80 μL of each) was applied onto the central area of each plate. Plates were incubated at 28 °C for 7 days, when the inhibition halos were measured.

Fungal disks placed singly in the center of the Petri dishes

The essential difference between this method and that described in Sect. "Fungal disks placed at equidistant points in Petri dishes" was that the 1-cm disks containing fungus were placed singly in the central part of the Petri dishes containing PDA supplemented with streptomycin at 200 μg/mL, also 24 h prior to the inoculation of *C. violaceum* cells or supernatants. *C. violaceum* cells and supernatants were produced as described in Sect. "Fungal disks placed at equidistant points in Petri dishes" and were applied in a circle around the fungus disk.

Inhibition by violacein

One-centimeter pieces of colonies of each fungus were placed in Petri dishes containing PDA supplemented with streptomycin at 200 μg/mL. Twenty-microliter of diluted violacein solution (at concentrations of 0.01, 0.05, 0.1 and 0.5%, as described in Sect. "Crystallized violacein used in the assays") was dropped onto each colony. The diameters of the fungal colonies were measured after 7 days of growth at 28 °C and the inhibition activity against each fungus was estimated by comparison with the diameter of the fungal colony in the control treatment.

Inhibition by chitinases

Pure cultures of each fungus were grown at 25 °C for 7 days, as described in Sect. "Fungal disks placed at equidistant points in Petri dishes". After this period, 1-cm disks were cut from the edges of active colonies of each fungus with a cork borer. Four disks were then transferred to equidistant sites on new 9-cm Petri dishes containing PDA medium, 24 h prior to inoculation with *C. violaceum*.

C. violaceum strains were grown in SM + 10% CV liquid medium (v/v) with 0.2% of colloidal chitin (Sect. "Chitinolytic activity in vitro"), at 28 °C, for 72 h, with constant agitation (100 rev./min). Cultures were then centrifuged at 10,000*g* for 10 min and the supernatant was filtered (0.45 μm, Millipore). An 80-μL aliquot of filtered supernatant was placed in a central hole in each Petri dish containing a fungal culture. Fungi were allowed to grow at 25 °C for 7 days, when the halos were measured for each strain in comparison to the pathogenic fungi.

Inhibition of fungi by *C. violaceum* chitinases was also evaluated using a second method: 80 μL of filtered supernatant was inoculated in an external circle on the Petri dishes containing the 1-cm fungal disks in the center.

Statistical analysis

All tests (Sects. "Chitinolytic activity in vitro" and "Cyanide production in vitro") and experiments (Sects. "Fungal disks placed at equidistant points in Petri dishes", "Fungal disks placed singly in the center of the Petri dishes", "Inhibition by violacein" and "Inhibition by chitinases") were performed in triplicate for each isolate, and each test/experiment was repeated three times. The tests/experiments were performed with a completely randomized statistical design, and means were compared using the Tukey's test.

Results

Bacterial growth and characterization

Similar growth was observed in all four media from 24 h after inoculation, with incubation at 28–30 °C; CV medium was selected for all assays due its low cost. Maintenance of bacteria required stocking on solid CV medium at 28 °C, or in CV liquid medium mixed with glycerol (30%), with a fast-freezing in liquid nitrogen and storage in deep-freezer at −70 °C. In general, bacteria from the Amazon region did not survive at temperatures below 15 °C for 2 weeks.

Characterization of potential antibiotic compounds in *C. violaceum*: violacein, chitinases and cyanide

Although bacterial growth was observed from 24 h, violet pigmentation was not usually observed until after 72 h. All 25 strains produced purple pigment in vitro, with variability in color intensity. They fit into three-color categories: dark violet, violet and light violet (Table 1). Four strains, including ATCC 12472^T, produced a dark violet pigment, whereas the great majority of the strains from the Amazon (17) were characterized by a violet color, and four were of light violet (Table 1) color.

All strains, except RP7, hydrolyzed colloidal chitin after 72–96 h of growth on semi-minimal-CV medium supplemented with colloidal chitin as the sole source of carbon. Variability in the chitinolytic activity among the strains was suggested by the different sizes of the clearing zones around the bacteria after 96 h of growth (Table 1). Bacteria could be classified into three classes of chitinolytic activity, according to halo diameter: low (1.2–1.8 mm), medium (1.9–2.3 mm) and high (>2.3 mm) activities, and 13, 5 and 6 strains fit these categories, respectively. Type strain ATCC 12472^T was of low chitinolytic activity, and the strains showing high activity were obtained in four Amazonian sites: Açajatuba, Presidente Figueiredo, Tarumã Grande and Rio Preto da Eva. There was no apparent association between chitinolytic activity and violacein color in vitro (Table 1).

A qualitative evaluation of cyanide production, based on color intensity developed in paper impregnated with picrate/Na_2CO_3, confirmed production by all strains in this study (Table 1). However, variability among the strains was verified, as final paper color ranged from yellow to light brown, brown, or reddish brown, indicating weak, moderate, or strong cyanogenic potential, respectively; these categories included one, six and eighteen strains, respectively, with ATCC 12472^T being a strong producer after 48 h (Table 1). Strain RP7 from Tarumã Grande produced violet pigment in vitro and was the only one showing weak production of cyanide and absence of chitinases. No apparent association was found between cyanogenic activity and either violacein color or chitinolytic activity (Table 1).

Antifungal activity

An initial screening was performed in which the antibiotic activity of all 25 strains was verified against two seed-pathogenic fungi, *Fusarium* sp. and *Phomopsis* sp. Ten Brazilian strains were as effective as type strain ATCC 12472^T against the fungi and two others, strains 07-1 and 27-1, showed higher antifungal activity (Table 1).

Accordingly, strains 07-1 and 27-1 together with ATCC 12472^T were used in further studies against seven seed-pathogenic fungi. Using the paring method, *C. violaceum* cells and their metabolites (supernatants) showed antibiosis against six major seed-pathogenic fungi (Table 2). The Brazilian strains and their supernatants had stronger activity than the type strain ATCC 12472^T against *Fusarium* sp., *Phomopsis* sp., and *Cercospora kikuchi*. In addition, the two Brazilian strains, but not AT CC 12472^T, were effective against *Corynespora* sp., and all three strains and their supernatants were equally effective against *Aspergillus* sp. and *Colletotrichum* sp. None of the strains had antifungal activity against *Botroyodiplodia* sp. (Table 2). Antifungal activity was confirmed both; when the fungal disks were placed at equidistant points of the Petri dishes and when the disks were placed in the center.

Discussion

It has been suggested that Brazil represents the world's most important reservoir of biodiversity, particularly the

Table 2 Growth inhibition against seven fungi pathogenic on soybean seed, by *Chromobacterium violaceum* cells and their sterile supernatants

Strain		*Fusarium* sp.	*Phomopsis* sp.	*Corynespora* sp.	*Cercospora kikuchi*	*Aspergillus* sp.	*Colletotrichum* sp.
ATCC 12472^T	Cells	11 c	14 c	0 c	5 b	14 a	12 a
	Supernatant	12 c	18 c	0 c	4 b	13 a	12 a
07-1	Cells	22 b	29 a	17 a	12 a	14 a	10 a
	Supernatant	29 a	32 a	19 a	13 a	14 a	10 a
27-1	Cells	23 b	20 b	10 b	12 a	14 a	11 a
	Supernatant	20 b	24 ab	15 ab	12 a	14 a	9 a

No antifungal activity was verified against *Botroyodiplodia* sp.

Data represent the inhibition halo (mm) after 7 days, evaluated by the pairing method: tests performed in triplicate for each isolate and repeated three times; means followed by the same letter within each column are not statistically different (Tukey, $P \leq 0.05$)

Amazon basin. We chose *C. violaceum* as a model representing Amazonian biodiversity as it occurs abundantly in that region. However, diversity even within this species is still poorly understood, despite evidence of its biotechnological potential. *C. violaceum* typically has a purple color related to the production of violacein, and studies performed in Brazil have shown that the pigment has antibiotic properties toward important human pathogens such as *Mycobacterium tuberculosis*, *Trypanosoma cruzi* and *Leishmania* sp. However, those studies were not performed with Brazilian strains [9, 22–24, 34, 52]. The biotechnological potential of Brazilian strains of *C. violaceum* thus deserved further investigation, and as the country's economy is strongly based on agriculture, antibiotic properties of the bacterium merited investigation.

Typical properties of the Brazilian *C. violaceum* were highlighted in this study, starting with optimum temperature conditions for growth. In the species definition, Sneath [51] reports that optimum temperatures for *C. violaceum* growth range from 30 to 37 °C, with minima of 1 to 15 °C and a maximum of 40 °C. Survival in a hot tropical environment probably relates to the reports of *C. violaceum* strains from the Amazon—eight in this study—being capable of growing at 44 °C and adversely affected by temperatures below 15 °C [29]. Efthimion and Corpe [25] also reported sensitivity of *C. violaceum* to low temperatures, with severe loss of cell viability after only 2 h of exposure to 0–2 °C; losses were observed even at 12 °C. These properties are consistent with loss of viability of the strains from the Amazon region—temperatures often reach 40 °C—when exposed to temperatures below 15 °C for 2 weeks [29].

Soybean is a major crop in Brazil, occupying 46% of the cropped land. Seed-transmitted fungi represent an important limitation to productivity, requiring chemical treatments to seeds by more than 90% of farmers (Henning 28]. The majority of the Brazilian strains inhibited two important fungal pathogens of soybean seed—*Fusarium* sp., and *Phomopsis* sp.—and two of those strains (07-1 and 27-1) were strong inhibitors of four other fungi: *Cercospora kikuchi*, *Colletotrichum* sp., *Corynespora* sp. and *Aspergillus* sp. In general inhibition of fungi by the Amazon strains was stronger than by ATCC 12472^T strain.

In a second stage of this study, the strong antibiosis of *C. violaceum* against important seed-pathogenic fungi led us to investigate some mechanisms that could be related to the fungicidal activity. The first candidate was violacein, as Shirata et al. [47] detected antibiotic activity of the pigment produced by *Janthinobacterium lividum* against the plant pathogenic fungi *Colletotrichum dematium* and *Rosellinia necatrix*, which cause anthracnose and white root-rot diseases of mulberry, respectively. All 24 strains from the Amazon region showed the typical purple pigment in vitro after 72 h of growth; however, color nuances differed among strains, ranging from dark to light violet. Most Brazilian isolates (71%) were violet in color, whereas type strain ATCC 12472^T produced a dark violet pigment. It is noteworthy that despite the fact that *C. violaceum*'s characteristic feature is the production of violacein [51], non-pigmented variants [39, 49, 51], and variability in color of strains isolated from nature [15] have been reported, indicating that pigmentation should not be held as an essential feature of the genus. In this study, contrary to what was observed by Shirata et al. [47], fungicidal activity was not related to pure violacein, evaluated at four concentrations (0.01, 0.05, 0.1 and 0.5%). Furthermore, although experiments testing higher concentrations of violacein are in progress, the application of violacein at high concentrations may not be economically or ecologically viable.

Another major characteristic of *C. violaceum* is the production of the secondary metabolite hydrogen cyanide [38, 51], confirmed in all strains in this study. Cyanide synthesis is controlled by the *hcnABC* gene set [1], and in *C. violaceum* [58] the operon shows strong similarity to that of *Pseudomonas fluorescens* [33]. Cyanide may play a role in the control of plant diseases, similar to *P. fluorescens* controlling *Thielaviopsis basicola* in tobacco (*Nicotiana tabacum*) [33]. Both *C. violaceum* strains

showing stronger fungicidal activity were characterized by high cyanogenic activity; however, other strains showing low biocidal activity were also strong cyanide producers (Table 1), therefore these characteristics may not be directly related.

Chitin and chitinolytic enzymes are gaining biotechnological importance, and might be particularly useful to control plant pathogens [42]. Chitin, an insoluble linear polymer, is a major structural component of cell walls of most fungi and arthropods, and many species of bacteria [43, 46], including *C. violaceum* [13], produce chitinolytic enzymes. Importantly, Park et al. [40] reported fungicidal activity against *Rhizoctonia* related to chitinases produced by *C. violaceum*; in contrast, in this study the chitinases produced by the Brazilian and the type strains apparently had no adverse effects on fungi pathogenic to soybean seed.

The continuous use of chemical inputs in agriculture is one of the main causes of imbalances in soil microbial communities, which can cause outbreaks of diseases of crop plants, highlighting the need for methods of biological control [2]. In this study, two strains of *C. violaceum* isolated from the Amazon region showed strong antibiotic activity against six major fungi pathogenic to soybean seed. The anti-fungal activity was verified in assays using both cells and supernatants of *C. violaceum*; however, our preliminary survey failed to show correlations of the antibiotic activity to production of violacein, cyanide, or chitinases, therefore our search for other metabolites related to the antibiosis will continue. Finally, the antagonistic potential of the strains from the Amazon region emphasizes the biotechnological potential still to be explored within the vast biodiversity that exists in the tropics.

Acknowledgments To Ministério de Ciência e Tecnologia (MCT)/ Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support (6800220/00-3) and fellowships to the authors. Rodrigo Masel Capeletti Cioato and Adrian Augusto Sosa Gómez Rolim received under-graduated fellowships from the CNPq and helped in the experiments. We thank Dr. Allan R. J. Eaglesham for suggestions on the manuscript.

References

1. Anderson PM, Sung YC, Fuchs JA (1990) The cyanase operon and cyanate metabolism. FEMS Microbiol Rev 7:247–252
2. Araújo FF, Henning AA, Hungria M (2005) Phytohormones and antibiotics produced by *Bacillus subtilis* and their efects on seed pathogenic fungi and on soybean root development. World J Microbiol Biotech 21:1639–1645
3. August PR, Grossman TH, Minor C, Draper MP, MacNeil IA, Pemberton JM, Call KM, Holt D, Osburne MS (2000) Sequence analysis and functional characterization of the violacein biosynthetic pathway from *Chromobacterium violaceum*. J Mol Microbiol Biotechnol 2:513–519
4. Ballantine JA, Beer RJS, Crutchley DJ, Dodd GM, Palmer DR (1958). The synthesis of violacein and related compounds. Proc Chem Soc 1:232–234
5. Ballantine JA, Beer RJS, Crutchley DJ, Dodd GM, Palmer DR (1960) The chemistry of bacteria. Part VIII. The synthesis of violacein and related compounds. J Chem Soc 2292–2299
6. Blumer C, Haas D (2000) Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch Microbiol 173:170–177
7. Boisbaudran L (1882) Matière colorante se formant dans la colle de farine. Comp Rend Acad Sci 94:562–563
8. Caldas LR (1990) Um pigmento nas águas negras. Cienc. Hoje 11:55–57
9. Caldas LR, Leitão AAC, Santos SM, Tyrrell RM (1978) Preliminary experiments on the photobiological properties of violacein. International Symposium on Current Topics Radiobiology and Photobiology, Rio de Janeiro, Brasil, pp 121–132
10. Campbell SC, Olson GJ, Clark TR, McFeters G (2001) Biogenic production of cyanide and its application to gold recovery. J Ind Microbiol Biotechnol 26:134–139
11. Carepo MSP, Azevedo JSN, Porto JIR, Bentes-Souza AR, Batista JS, Silva ALC, Schneider MPC (2003) Identification of *Chromobacterium violaceum* genes with potential biotechnological application in environmental detoxification. Genet Mol Res 3:181–194
12. Chernin LS, Ismailov Z, Haran S, Chet I (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Appl Environ Microbiol 61:1720–1726
13. Chernin LS, Winson MK, Thompson JM, Haran S, Bycroft BW, Chet I, Williams P, Stewart GS (1998) Chitinolytic acitivity in *Chromobacterium violaceum*: a substrate analysis and regulation by quorum sensing. J Bacteriol 180:4435–4441
14. Cook RJ (1993) Making greater use of introduced microorganisms for biocontrol of plant pathogens. Ann Rev Phytopathol 31:53–80
15. Corpe WA (1953) Variation in pigmentation and morphology of colonies of gelatinous strains of *Chromobacterium* species from soils. J Bacteriol 66:470–477
16. Cronin D, Moenne-Loccoz Y, Dunne C, O'Gara F (1997) Inhibition of egg hatch of the potato cyst nematode *Globodera rostochiensis* by chitinase-producing bacteria. Eur J Plant Pathol 103:443–440
17. Cubeta MA, Hartman GL, Sinclair JB (1985) Interaction between *Bacillus subtilis* and fungi associated with soybean seeds. Plant Dis 69:506–509
18. DeMoss RD (1967) Violacein. Antibiotics 2:77–81
19. DeMoss RD, Evans NR (1959) Physiological aspects of violacein biosynthesis in nonproliferating cells. J Bacteriol 78:583–588
20. Dessaux Y, Elmerich C, Faure D (2004) La violacéine : une molécule d'intérêt biologique, issue de la bactérie tellurique *Chromobacterium violaceum*. Rev Med Interne 25:659–662
21. Durán N, Menck CFM (2001) *Chromobacterium violaceum*: a review of pharmacological and industrial perspectives. Critl Rev Microbiol 27:201–222
22. Durán N, Antonio RV, Haun M, Pilli RA (1994) Biosynthesis of a trypanocide by *Chromobacterium violaceum*. World J Microbiol Biotechnol 10:686–690
23. Durán N, Campos V, Riveros R, Joyas A, Pereira MF, Haun M (1989) Bacterial chemistry: III. Preliminary studies on the trypanosomal activities of *Chromobacterium violaceum* products. An Acad Bras Cienc 61:31–36
24. Durán N, Erazo S, Campos V (1983) Bacterial chemistry-II: antimicrobial photoproduct from pigment of *Chromobacterium violaceum*. An Acad Bras Ci 55:231–234
25. Efthimion MH, Corpe WA (1968) Effect of cold temperatures on the viability of *Chromobacterium violaceum*. Appl Microbiol 17:169–175

26. Forsyth WGC, Hayward AC, Roberts JB (1958) Occurrence of poly-β-hydroxybutyric acid in aerobic gram-negative bacteria. Nature 182:800–801
27. Gourson C, Benhaddou R, Granet R, Krausz P, Verneuil B, Branland P, Chauvelon G, Tribault JF, Saulnier L (1999) Valorization of maize bran to obtain biodegradable plastic films. J Appl Pollut Sci 74:3040–3045
28. Henning, AA (2004) Patologia e tratamento de sementes: noções gerais. Londrina: Embrapa Soja, p 51 (Embrapa Soja. Documentos, 235)
29. Hungria M, Astolfi-Filho S, Chueire LMO, Nicola's MF, Santos EBP, Bulbol MR, Souza-Filho A, Nogueira Assunção E, Germano MG, Vasconcelos ATR (2005) Genetic characterization of *Chromobacterium* isolates from black water environments in the Brazilian Amazon. Lett Appl Microbiol 41:17–23
30. Hungria M., Nicolás MF, Guimarães CT, Vasconcelos ATR (2004) Tolerance to stresses and environmental adaptability of *Chromobacterium violaceum*. Gen Mol Res 3:102–116
31. Kolibachuk D, Miller A, Dennis D (1999) Cloning, molecular analysis, and expression of the polyhydroxyalkanoic acid synthase (*phaC*) gene from *Chromobacterium violaceum*. Appl Environ Microbiol 65:3561–3565
32. Kremer RJ, Souissi T (2001) Cyanide production by rhizobacteria and potencial for supression of weed seedling growth. Curr Microbiol 43:182–186
33. Laville J, Blumer C, von Schroetter C, Gaia V, Défago G, Keel C, Haas D (1998) Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. J Bacteriol 180:3187–3196
34. Leon LL, Miranda CC, Souza AO de, Durán N (2001) Antileishmanial activity of the violacein extracted from *Chromobacterium violaceum*. J Antimicrob Chemother 48:449–450
35. Lischstein HC, van de Sand VF (1945) Violacein, an antibiotic pigment produced by *Chromobacterium violaceum*. J Infect Dis 76:47–51
36. Matz C, Deines P, Boenigk J, Arndt H, Eberl L, Kjelleberg S, Jurgens K (2004) Impact of violacein-producing bacteria on survival and feeding of bacterivorous nanoflagellates. Appl Environ Microbiol 70:1593–1599
37. Melo PS, Maria SS, Vidal BC, Haun M, Durán N (2000) Violacein cytotoxicity and induction of apoptosis in V79 cells. In Vitro Cell Dev Biol Anim 36:539–543
38. Michaels R, Corpe WA (1965) Cyanide formation by *Chromobacterium violaceum*. J Bacteriol 89:106–112
39. Miller DP, Blevins WT, Steele DB, Stowers MD (1988) A comparative study of virulent and avirulent strains of *Chromobacterium violaceum*. Can J Microbiol 34:249–255
40. Park SK, Lee HY, Kim KC (1995) Role of chitinase produced by *Chromobacterium violaceum* in the suppression of *Rhizoctonia* damping-off. Korean J Plant Pathol 11:304–311
41. Parker WL, Rathnum ML, Johnson JH (1988) Aerocyanidin, a new antibiotic produced by *Chromobacterium violaceum*. J Antibiot 41:454–460
42. Patil RS, Ghormade V, Despande MV (2000) Chitinolytic enzymes: an exploration. Enzyme Microb Technol 26:473–483
43. Perrakis A, Wilson KS, Chet I, Oppenheim AB, Vorgias CE (1993) Phylogenetic relationships of chitinases. In: Muzzarelli RAA (ed) Chitin enzymology. European Chitin Society, Ancona, pp 217–232
44. Rettori D, Durán N (1998) Production, extraction and purification of violacein: an antibiotic pigment produced by *Chromobacterium violaceum*. World J Microbiol Biotechnol 14:665–668
45. Richard C (1993) *Chromobacterium violaceum*, opportunist pathogenic bacteria in tropical and subtropical regions. Bull Soc Pathol Exot 86:169–173
46. Sahai AS, Manocha MS (1993) Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiol Rev 11:317–338
47. Shirata A, Tsukamoto T, Yasui H, Hata T, Hayasaka S, Kojima A, Kato H (2000) Isolation of bacteria producing bluish-purple pigment and use for dyeing. Jpn Agric Res Q 34:131–140
48. Singh PD, Liu WC, Gougoutas JZ, Malley MF, Porubcan MA, Trejo WH, Wells JS, Sykes RB (1988) Aerocavin, a new antibiotic produced by *Chromobacterium violaceum*. J Antibiot 41:446–453
49. Sivendra R, Lo HS (1975) Identification of *Chromobacterium violaceum*: pigmented and non-pigmented strains. J Gen Microbiol 90:21–31
50. Smith AD, Hunt RJ (1985) Solubilization of gold by *Chromobacterium violaceum*. J Chem Technol Biotechnol 35:110–116
51. Sneath PHA (1984) Genus *Chromobacterium* Bergonzini 1881, 153^{AL}. In: Krieg NH (ed), Holt JG (ed-in-chief). Bergey's manual of systematic bacteriology, vol 1. Williams & Wilkins, Baltimore, pp 580–582
52. Souza AO de, Aily DCG, Sato DN, Durán N (1999). Atividade da violaceína in vitro sobre o *Mycobacterium turbeculosis* H37RA. Rev Inst Adolfo Lutz 58:59–62
53. Steinbüchel A, Debzi EM, Marchessault RH, Timm A (1993) Synthesis and production of poly(3-hydroxyvaleric acid) homopolyester by *Chromobacterium violaceum*. Appl Microbiol Biotechnol 39:443–449
54. Stephens C (2004) Microbial genomics: tropical treasure? Curr Biol 14:65–66
55. Strong FM (1944) Isolation of violacein. Science 100:287
56. Teather RM, Wood PJ (1982) Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from bovine rumen. Appl Environ Microbiol 43:777–780
57. Ueda H, Nakajima H, Hori Y, Goto T, Okuhara M (1994). Action of FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* no. 968, on Ha-ras transformed NIH3T3 cells. Biosci Biotechnol Biochem 58:1579–1583
58. Vasconcelos ATR, Almeida DF, Hungria M, 106 other authors (2003) The complete genome sequence of *Chromobacterium violaceum* reveals remarkable and exploitable bacterial adaptability. Proc Natl Acad Sci USA 100:11660–11665
59. Yedidia I, Benhamou N, Chet I (1999) Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Appl Environ Microbiol 65:1061–1070