**BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY**

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Antibiosis and Dark-Pigments Secretion by the Phytopathogenic and Environmental Fungal Species after Interaction *in vitro* with a *Bacillus subtilis* Isolate

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ABSTRACT

In this work, different reactions in vitro between an environmental bacterial isolate and fungal species were related. The Gram-positive bacteria had terminal and subterminal endospores, presented metabolic characteristics of mesophilic and acidophilic growth, halotolerance, positive to nitrate reduction and enzyme production, as caseinase and catalase. The analysis of partial sequences containing 400 to 700 bases of the 16S ribosomal RNA gene showed identity with the genus Bacillus. However, its identity as B. subtilis was confirmed after analyses of the rpoB, gyrA, and 16S rRNA near-full-length sequences. Strong inhibitory activity of environmental microorganisms, such as Penicillium sp, Aspergillus flavus, A. niger, and phytopathogens, such as Colletotrichum sp, Alternaria alternata, Fusarium solani and F. oxysporum f.sp vasinfectum, was shown on co-cultures with B. subtilis strain, particularly on Sabouraud dextrose agar (SDA) and DNase media. Red and red-ochre color pigments, probably phaeomelanins, were secreted by A. alternata and A. niger respectively after seven days of co-culture.

Key-words: *Bacillus subtilis*, phytopathogenic fungi, environmental fungi, microbial interaction

INTRODUCTION

The genus *Bacillus* is widely diversified and spread throughout the world from many sources such as air, soil, water, and gastrointestinal tracts of animals and humans (Guo et al., 2006, Earl et al., 2007, Rintala et al., 2008). Many species are extremophyles, or tolerant to more strict conditions such as high alkalinity, acidity, salinity or temperature (Niehaus et al., 1999, Horikoshi, 2008). Endospore production by such bacilli may be resistant to desiccation, heat, UV light and organic solvents (Nicholson et al., 2000). In general, these microorganisms are saprobes and rarely pathogenic.

Bacillus spp have powerful biotechnological applications such as natural biocontrol of pests in agriculture, production of insecticides, antibiotics and enzymes and probiotics for human and animal microbiota regulation (Niehaus et al., 1999, Luna et al., 2005, Monteiro et al., 2005, Souza et al., 2005, Guo et al., 2006, Whipps, 2001).

Antibiosis against the phytopathogenic microorganisms has been frequently associated with secondary metabolites (Romero et al., 2007, Pryor et al., 2007, Keel and Défago, 1997).

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Co-cultures of filamentous fungi and *Bacillus* spp can lead to increased bacteriocins production (Abee et al., 1995, Cornea et al., 2003). The synthesis of these inhibitory substances have been described by different species, such as *Bacillus subtilis, B. cereus, B. polymixa, B. amyloliquefaciens, B. stearothermophilus, B. brevis, B. thuringiensis, B. megaterium, B. licheniformis* and *B. circulans* (Abee et al., 1995, Whipps, 2001, Stein, 2005).

Many misclassifications have been found in the phylum Firmicutes (Wang et al., 2007). De Clerck *et al.* (2004) reported that the analysis of only the 16S rRNA gene was unsuitable for the species or sub-species identification of the genus *Bacillus*, particularly of the cereus and subtilis groups. Sequencing of other genes, such as gyrA and rpoB, was suggested to aid in molecular identification of the subtilis group because of the great homology among 16S rRNA sequences (Chun and Bae, 2000, Earl et al., 2007).

The aim of this work was to study different interactions *in vitro* of environmental and phytopathogenic fungi with a Gram-positive bacillus isolated from soil. This bacterial species was identified through their phenotypical profiles and molecular sequencing analyses.

MATERIAL AND METHODS

Microorganisms

Approximately 10 g of soil were randomly collected in the University Federal of Mato Grosso Campus, Cuiabá (Mato Grosso, Brazil). The samples were homogenized separately with 100 mL phosphate buffered saline (PBS, pH 7.2) in 200 mL glass beakers, filtered in a common paper filter, and decanted for two hours. Supernatants were diluted to concentrations of 1/10 and 1/100, plated on trypticase™ soy agar (TSA, Difco/BBL, pH 7.3), and incubated at 30° C for one week. Several fungi and bacterial colonies were observed in these cultures. However, only the bacteria that exhibited antifungical activity were selected for later studies against environmental fungi (*Penicillum* sp, *Aspergillus flavus, A. niger*) and phytopathogens (*Alternaria alternata, Fusarium solani, F. oxysporum f.sp vasinfectum, Colletotrichum* sp). All fungal species used in this study were previously cultivated on Sabouraud

dextrose agar (SDA, Difco/BBL), supplemented with 80 mg/L gentamycin.

Classical characterization

The preliminary identification of the bacteria was performed by macroscopic analysis of colonies and microscopic morphology, including endospore position and Gram stain. Biochemical tests were performed for citrate utilization; nitrate reduction; growth on mannitol and growth in 6.5 and 10% NaCl; production of H_2S, indole, methyl red, Voges-Proskauer (VP); and hydrolysis of esculin (Foldes et al., 2000, Reva et al., 1995, Winn et al., 2006). Enzymatic tests were performed to detect the production of amylase, ornithine-decarboxylase, oxidase, catalase, urease, lecithinase, DNase, lipase, caseinase and haemolysins (Winn et al., 2006, Sanchez-Porro et al., 2003). The growth of the bacterial strain on TSA and tryptic soy broth (TSB, Difco), pH 7.3, and SDA, pH 5.6, was observed at different temperatures (4, 20, 25, 37, 42, and 55° C) over intervals of 24 and 48 h.

Co-cultures

Bacterial and fungal inocula were cultivated at a distance of 3 to 5 cm from each other on Petri plates containing TSA or SDA. Co-cultures were maintained at 28°C and were observed daily for one week. Antagonistic activity was verified by inhibition halo growth of fungal species (Cornea et al., 2003, Lisboa et al., 2006). Co-cultures of the bacterial and fungal species were also performed in DNase agar with 0.1 g/L toluidine blue (Difco/BBL, pH 7.3), using *Staphylococcus aureus* ATCC 25923 as the positive control and *Staphylococcus epidermidis* ATCC 12228 as the negative control. DNase agar was used specifically for co-cultivation because previous observations showed that the zones with enzymatic activity in this medium coincided with a green fluorescent light ring around the bacterial colonies. Green fluorescent light was correlated with the zones of fungal inhibition *in vitro* in the co-cultures. Furthermore, the relationship between nucleic acid hydrolysis and microbial antagonism was also investigated. To verify whether the substances produced by the *Bacillus* sp reacted with dye used in the DNase medium, co-cultures were performed on SDA Petri-plates supplemented with 0.1 g/L toluidine blue.

Extraction and purification of genomic DNA

After 24 h growth at 30° C on TSA, the bacterial colonies were suspended in 400 µL of lysis buffer containing 50 µL of lysozyme and 25 µL of RNase, yielding final concentrations of approximately 1 mg/Eppendorf tube and 10 µg/mL, respectively. Eppendorf tubes were incubated at 37° C for 15 min with careful shaking. Then, 50 µL 20% lauroylsarcosine (Sigma Aldrich) was added to the lysates, which were kept on ice for 5 min to obtain a translucent material. Approximately 500 µL of phenol:chloroform (Sigma Aldrich) was added to the tubes. Genetic material was precipitated in 1 mL ethanol with 0.3 M NaOAc for 5 min at room temperature. The pellet was centrifuged and quickly washed with 70% ethanol and resuspended in 100 µL TE buffer. Genomic preparations were quantified using a spectrophotometer at 260nm and samples were kept at -20° C until use.

Amplification of 16S rRNA, rpoB and gyrA genes

The PCR-reactions were performed with the following universal primers: 16S rRNA (8bF, 5'-ACTGGGATCCACAGGAGTGCAAACGACG A-3'; 518f, 5'- CCAGCAGCCGCGGTAAT-3'; 1406r, 5'-ACGGGCGGTGTGTRC-3'; and 1512r, 5'- CTGAAGCTTACGGYTAGCTTGTTACGAC TT-3'), gyrA (gyrA-f, 5'-CAGTCAGGAAATGC GTACGTCCTT-3' and gyrA-r, 5'-CAAGGTAA TGCTCCAGGCATTGCT-3'), and rpoB (*rpoB*-f, 5'-AGGTCAACTAGTTCAGTATGGAC-3' and *rpoB*-r, 5'-AAGAACCGTAACCGGCAACTT-3') (De Clerck et al., 2004). Each 50 µL PCR reaction mix contained 5 µL Taq buffer 10x, 3 µL MgCl$_2$ 25 mM, 0.4 µL dNTPs 25 mM, 0.5 µL Taq-polymerase 5 U/mL (all reagents from Fermentas), 1.25 µL primer 20 mM, 50 ng genomic DNA and 36.1 µL H$_2$O milli-Q. A positive control reaction was directly performed from bacterial cells and PCR mix. The PCR reactions were performed in a BioRad thermal cycler (PTC 1148 MJ mini thermal cycler) using 35 cycles (1 min denaturing at 94° C, 1 min annealing at 48° C, 2 min extension at 72° C, and finally a 9 min extension at 72° C. The resulting amplicons were subjected to ultrapure 1% agarose gel electrophoresis (Invitrogen) using GeneRuler 1 kb DNA ladder (Life Sciences) as a molecular weight marker.

Sequencing of 16S rRNA, rpoB, and gyrA genes and sequence analyses.

Five PCR products from each amplified gene were extracted from agarose gel with QIAEX II Gel Extraction Kit (QIAGEN). Each 15 µL PCR reaction contained 100 ng purified DNA, 3 µL sequencing buffer 5X, 2 µL Big Dye, 10 pmol primers and H$_2$O. Gene sequences were determined on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). The Bioedit program and ClustalW2 (www.ebi.ac.uk/Tools /clustalw2/index.html) were used to sequence the alignments. Partial sequences were blasted in the bank databases, such as GenBank (www.ncbi.nlm. nih.gov), EMBL (www-db.embl.de), RDP II (rdp.cme.msu.edu), DDBJ (http://blast.ddbj.nig. ac.jp/top-e.html).

Nucleotide sequencing accession numbers

Partial sequences of environmental *Bacillus subtilis* strain were deposited in GenBank under accession numbers FJ025759 (rpoB), FJ025758 (gyrA), FJ025757 (16S rRNA).

RESULTS

Classical and molecular classification

The Gram-positive bacteria presented terminal and subterminal endospores, growth at 4 to 40° C, halotolerance and good growth on acid media (SDA, pH 5.6). The reactions were positive for methyl red, Voges-Proskauer, motility, mannitol growth, nitrate utilization, as well as production of catalase, oxidase, caseinase, ornithine decarboxylase. The reactions were negative for citrate utilization, esculin growth, as well as the production of amylase, urease, indole, lecithinase, lipase, hemolysins and gas. The 16S rRNA gene was amplified directly from the bacterial cells or genomic DNA using primers 8bf and 1512r. Fragments of approximately 1.5kb were sequenced, and partial 5' and 3' sequences containing 400 to 700 bases were aligned and analyzed. These sequences demonstrated 100% identity to the *Bacillus* genus in all the bank databases. However, a crossed identity of ribosomal genes over 95% was detected among the species of *B. amyloliquefaciens, B. velezensis* and *B. subtilis*. Subsequently, the primer 518f was used to amplify the central region of 16S gene.

A near-full-length cDNA sequence of 16 S rRNA gene with approximately 1.437 nucleotides was produced after manual alignment of 3' and 5' sequences from the PCR-products generated with primers 8bf, 518f and 1512r. Other genes, such as rpoB and gyrA, were analyzed to confirm *B. subtilis*.

In vitro interactions

Antibioses of *Bacillus* sp was observed in co-cultures against all the fungal species, including environmental fungi such as *Penicillum* sp, *A. flavus*, *A. niger* (Fig. 1A), and phytopathogens such as *A. alternata, F. solani, F. oxysporum f.sp vasinfectum* and *Colletotrichum* sp. Antifungal activity was more pronounced on SDA medium,

suggesting increased synthesis of inhibitory substances in acidic pH. In many instances, a line of precipitation was observed in co-cultures plates near the bacterial colony (Fig. 1-B).

Green fluorescence was observed after exposure to ultraviolet light (UV), particularly in the inhibition zone. Inhibitory zones on DNase medium (Fig. 2) containing toluidine-blue dye were initially light pink in color and then became yellow, whereas inhibitory zones on SDA medium with the same dye did not exhibit this pigmentation.

The diffusion of reddish-brown and red pigments, which were most likely phaeomelanin, on agar plates by fungal colonies of *A. niger* (Fig. 3-A) and *A. alternata* (Fig. 3-B) were observed after one week of co-culture with *B. subtilis*.
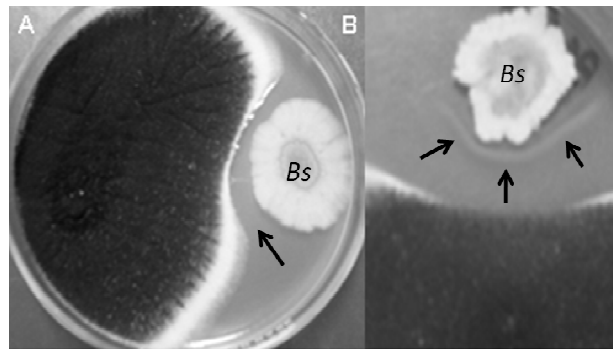

Figure 1 - Co-cultures between environmental strains of *A. niger* and *B. subtilis* (*Bs*). A) Inhibition of the fungal colony on SDA medium. B) Precipitation line formed near the bacterial colony.
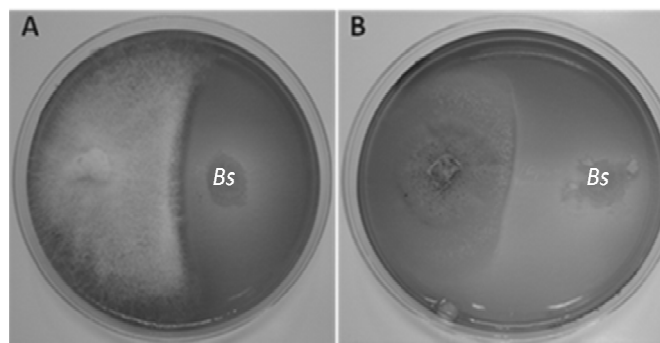

Figure 2 - Inhibitory growth of phytopathogens on DNase medium and zone linked to the antifungical activity of *B. subtilis* (*Bs*). A) *F. oxysporum* B) *Colletotrichum* sp.
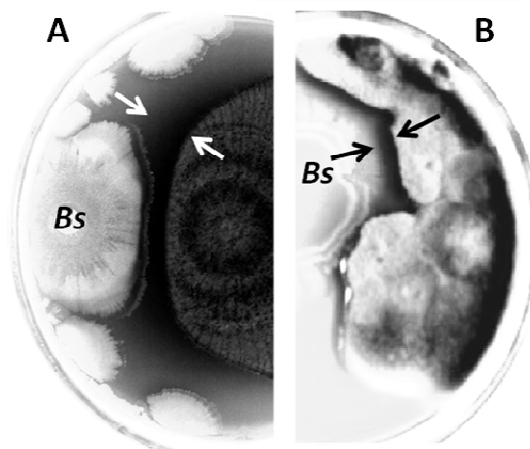
Figure 3 - Reddish-brown and red pigments secreted by *Asp. niger* (A) and *A. alternata* (B) after one week of co-culture with *B. subtilis* (*Bs*).

DISCUSSION

The identification of the *Bacillus* species is very difficult due to the high level of phenotypic variability, environmental diversity, and absence of suitable qualitative methods. For this reason, molecular analyses have been advantageous tools in characterization of the *Bacillus* spp. The phylum Firmicutes presents high homology in gene targets for phylogenetic characterization, such as 16S and 23S rRNA (Earl et al., 2007, Wang et al., 2007, Bavykin et al., 2004). Several conserved proteins related to cellular transcription and replication, such as RNA polymerases and DNA gyrases, respectively, can also be used in gene sequencing and phylogeny (Earl et al., 2007, Chun and Bae, 2000). Although the genetic heterogeneity of these proteins could be interesting for molecular characterization, a high intra-specific homology on subunits α or β may be found across *Bacillus* groups making this a poor marker for differentiation of species and subspecies. The recovery of partial (over 400 bp) and near-full-length sequences have resulted in more accurate molecular identification than short sequences (near 200 bp) (Wang et al., 2007). Similarity among the aligned sequences should be ≥ 99% and the ideal is above 99.5%. In this investigation, species-specific characterization after sequencing the 16S rRNA gene was not successful. The identity of 100% with sequences of *B. subtilis* available in the database banks was observed only after crossing the near-full-sequence of 16S rRNA gene and gene sequences of gyrA and rpoB.

Gram-positive bacilli are found in soil, acting as bioregulators, biocontrollers, and decompositors (Whipps, 2001, Araujo et al., 2005, Keel and Défago, 1997). Microbial interaction studies are very important in the discovery of antimicrobials, insecticides, and pro- and pre-biotics (Whipps, 2001, Guo et al., 2006, Stein, 2005). A variety of interactions, such as competition for nutrients or space, may be the cause of antagonistic reactions (Whipps, 1997, Keel and Défago, 1997). Antibiosis normally occurs via the release of substances that inhibit microbial growth (Whipps, 2001). The production of secondary metabolites by *Bacillus* spp, including antibiotics of low-molecular-weight and natural cyclic-peptides, has been related to the inhibition of many organisms, including fungal species (Stein, 2005, Romero et al., 2007). The bacterium studied has shown good antifungal activity to environmental fungi and phytopathogens, particularly by antibiosis. As expected, *B. subtilis* that presented biocidal effectors against fungi has been utilized as a biocontroller of phytopathogenic fungi (Broggini et al., 2005). Precipitation lines between *Bacillus* and fungi colonies have been reported (Cornea et al., 2003). These are most likely due to compounds produced by the fungus whose spreading on agar may be associated with inhibitory substances produced by the *Bacillus* sp.

Antagonism *in vitro* varies according to culture media, bacterial antagonist and fungal species (Walker et al., 1998). The pH might be crucial for inducing the enzyme production by *Bacillus* spp from an intestinal source. In present work, a more pronounced inhibition of fungi occurred in the acid medium (SDA, pH 5.6) compared to TSA (pH 7.3). There are evidences that fungal inhibitory effects may be caused by enzymatic compounds (Cornea et al., 2003, Bizani and Brandelli, 2002, van der Wal et al., 1995). Active enzymes from *Bacillus* spp can damage the fungal cellular walls (Barbosa-Corona et al., 1999, Mavingui and Heulin, 1994). Bacterial exo-chitinases and glucanases may have an important antagonist role against fungi (Dijksterhuis et al., 1999, Keel and Défago, 1997). Many Gram-positive bacteria are good producers of DNase exoenzymes (Sanchez-Porro et al., 2003). In this study, a relationship between the antifungical activity and DNA-hydrolysis zones was observed, suggesting that the secreted inhibitory factors might be involved with DNases. However, future studies are needed to investigate this hypothesis.

In many instances, microbial resistance to hostile environment is directly associated with dark pigmented polymers (Henson et al., 1999, Jacobson, 2000). Melanized cells are more resistant to hydrolytic enzymes, oxidative radicals, high temperatures, antifungals, and host defenses (Nosanchuck and Casadevall, 2003, Henson et al., 1999). In this work, a striking secretion of melanin by melanogenic fungi after co-culture with *B. subtilis* was observed in agar plates, showing that fungi under stressful growth conditions could produce pigments that might give more protection against the bacterial substances or could result the melanin pathway stimulation by secondary metabolites.

Biocontrollers have been proposed as safe methods due to their natural effects against the phytopathogens, low operational cost, ease of growing, high resistance to environmental conditions, stability during the industrial process, and non-toxicity to life (Elad, 2003, Pryor et al., 2007, Whipps, 1997). The interactions of *Bacillus* spp with the plants and soils have been linked to increases in vegetable biomass, elimination of pathogenic flora and more efficient fixation of nitrogen compounds (Whipps, 2001, Keel and Défago, 1997). These microorganisms may, thus, act as "phyto-probiotics". Regional utilization of biocontrols may be more attractive and ecologically suitable than other available alternatives largely used in the local agriculture pest control, such as chemical pesticides.

In this work, a natural antimicrobial action against environmental and phytopathogenic fungi was detected from an environmental bacterium isolate, indicating that this microorganism had the potential for biotechnological application as a biocontrol agent. However, more experimental assays would be necessary because the fungal induction of melanogenesis process could be a factor related to increased resistance and virulence of many species.

ACKNOWLEDMENTS

ABBREVIATIONS

SDA = Sabouraud dextrose agar, SDB = Sabouraud dextrose broth, PBS = phosphate buffered saline, PCR = Polymerase Chain Reaction, rpoB = RNA polymerase subunit B, gyrA = DNA gyrase subunit A, rRNA = ribosomal RNA.

RESUMO

Na presente investigação, nosso objetivo principal foi relatar diferentes interações *in vitro* de um isolado bacteriano ambiental com espécies fúngicas. Através da identificação clássica, nós verificamos que o bacilo ambiental apresentava endósporos terminais e subterminais, características metabólicas de mesofilia, acidofilia, halotolerância, redução de nitrato e produção de enzimas, como caseinase e catalase. Análise de seqüências parciais do gene 16S RNAr contendo de 400 a 700 bases revelou identidade com gênero *Bacillus*. No entanto, a espécie *Bacillus subtilis* foi confirmada somente depois da análise de seqüências dos genes rpoB, gyrA, and 16S RNAr. Intensa atividade inibitória aos fungos ambientais, como *Penicillium* sp, *Aspergillus flavus*, *A. niger*,

e fitopatogênicos, como *Colletotrichum* sp, *Alternaria alternata, Fusarium solani* e *F. oxysporum f.sp vasinfectum*, foi observada em cocculturas com a cepa bacteriana (*B. subtilis*), particularmente em ágar Sabouraud dextrose e ágar DNase. Pigmentos de cor avermelhada e vermelho-amarronzado, provavelmente feomelaninas, foram secretados respectivamente por colônias de *A. alternata* e *A. niger* depois de sete dias de co-cultivo.

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