Antifungal activity and bioactive compounds produced by *Bacillus mojavensis* and *Bacillus subtilis*

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The soil bacteria, CWBI-B1567 and CWBI-B1568, isolated from arid regions and identified as *Bacillus subtilis* (accession number KC341751) and *Bacillus mojavensis* (accession number KC341749) respectively, were screened and evaluated for their antifungal activity against *Candida albicans*, one of the most important human fungal pathogens. *In vitro* assay with the antagonists and their cell-free culture supernatants on agar plates showed that the two *Bacillus* strains effectively inhibited growth of the yeast. The capacity of these *Bacillus* strains to produce the cell-wall degrading enzymes was further studied. *B. subtilis* and *B. mojavensis* are able to produce cellulase and protease, but not chitinase. Bioactive molecules were produced by *B. subtilis* CWBI-B1567 and *B. mojavensis* CWBI-B1568 in a liquid culture medium optimised for lipopeptide production. The antifungal activity was equally demonstrated by testing the resulting supernatants and lipopeptide-enriched extracts. The electro-spray mass spectrometry coupled to liquid chromatography (ESI-LC-MS) analysis showed that both the *B. subtilis* and the *B. mojavensis* produced surfactin and iturin. However the fengycin group was produced only by *B. mojavensis*. Through this study, we have demonstrated that *B. subtilis* and *B. mojavensis* have a strong antifungal activity especially against *C. albicans*. This growth inhibition is probably due to the production of cell-wall degrading enzymes and different families of lipopeptide including iturins, fengycins and surfactins.

Key words: *Bacillus subtilis* CWBI-B1567, *Bacillus mojavensis* CWBI-B1568, antifungal activity, *Candida albicans*, lipopeptides, electro-spray mass spectrometry coupled to liquid chromatography (ESI-LC-MS), arid regions.

INTRODUCTION

*Candida albicans* is a dimorphic fungus (Molero et al., 1998); it is both, a commensal on human mucosal surfaces and one of the most important human fungal pathogens which become the fourth leading cause of nosocomial infections. The diseases caused by *C. albicans* ranges from relatively benign infections of skin to oral and vaginal thrush, deep-seated mycoses and life-threatening sepsis. *Candida* infections constitute today a problem of growing clinical importance. The incidence of infections has increased dramatically over the past three decades, and this trend, will inevitably continue into the 21st century (Odds, 1988; Pfaffer et al., 1998; Edmond et al., 1999), especially, when some microorganisms continue to develop resistance against antibiotics treatment.

Bioactive natural products constitute a library of compounds with a large and privileged structural diversity,
showing a variety of biological activities. In fact, many of them have been used for pharmaceutical applications (Salas and Mendez, 2007). Inside microorganisms, several species of Bacillus genus are able to produce biological active substances that are capable of disintegrating the fungal cell walls (Tserkovniak et al., 2009). In the midst of these substances, several lipopeptide compounds, serve as a class of microbial surfactants with increasing scientific, pharmaceutical and biotechnological interest.

Lipopeptides from Bacillus are classified in three families of cyclic compounds: surfactin, iturin and fengycin. These lipopeptides, made of amino acids and a fatty acid, are amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antifungal activities. Each family contains variants with the same peptide length but with different residues at specific positions. Moreover, each variant can have several homologues of different length and isomer of the fatty acid chain, leading to a remarkable structural heterogeneity (Ongena and Jacques, 2008; Moyne et al., 2004).

In recent years, there has been a considerable interest in using Bacillus strains producing lipopeptide antibiotics like iturin A and surfactin as biocontrol agents due to their antagonistic and repressive activities against pathogens (Bais et al., 2004). These amphiphilic cyclic biosurfactants have many advantages: low toxicity, high biodegradability and environmentally friendly characteristics (Kim et al., 2004). In literature, several approaches for lipopeptide identification have been proposed but their biological activity needs to be determined after isolation procedures (Price et al., 2007).

In fact, the list of novel microorganisms, especially Bacillus, and products found in microbiologically unexplored ecosystems around the world suggest that a careful exploration of other habitats such as arid regions might continue to be useful. The Algerian arid soil presents an ecosystem with numerous opportunities as a source of novel microorganisms producing new compounds.

Bacillus strains have been investigated previously for agricultural applications and have proven to be an effective biocontrol agent against important phytopathogens (Monteiro et al., 2005). In this paper, the antifungal activity of Bacillus subtilis CWBI-B1567 and Bacillus mojavensis CWBI-B1568 against Candida albicans was investigated. These two strains were isolated from soil of different palm trees situated in South-East of Algeria.

MATERIALS AND METHODS

Isolation of bacterial strains

Soil samples were collected from soil of different palm trees of arid regions, situated in South-East of Algeria. 1 g of each sample was suspended in 9 ml sterile physiological water and shaken vigorously for 2 min. The soil suspension was serially diluted in sterile physiological salt (0.85% NaCl), (from 10−1 to 10−5); 0.1 ml of all dilutions were plated on YPD medium (Glucose, 20 g; Peptone, 10 g; Yeast extract, 10 g; Agar. 15 g; distilled water, 1 L) supplemented with a commercial antifungal to inhibit fungi growth. Petri dishes were incubated at 30°C (Gerhardt, 1994). The bacterial strains were maintained on YPD medium at 4°C before experimental use, and stored at -80°C in cryotubes according to the manufacturer recommendations (Microbank, Prolab Diagnostic, Richmond Hill, Canada) for long-term storage.

Screening of antagonists strains by in vitro antagonism experiments

The antifungal activity of the isolates against C. albicans was determined according to the plate diffusion method (Barakate et al., 2002; Errakhi et al., 2007). The yeast strain was isolated in Laboratory of Parasitology and Mycology (CHU Constantine, Algeria) from a patient’s mouth suffering from candidiasis. It was maintained on Yeast Malt Agar (YMA: Glucose, 20 g; Peptone, 10 g; Yeast extract 10 g; Agar, 15 g; distilled water 1L) containing gentamicin and chloramphenicol.

Isolates were grown on YPD medium for 24 h, discs (6 mm in diameter) were cut and placed on YMA which was seeded by spreading of the yeast suspension. Plates were first kept in a refrigerator (4°C) for at least 2 h to allow the diffusion of any antibiotics produced, then incubated at 30°C. Inhibition zones were determined after 24 and 48 h of incubation. The same method was used to test the antagonistic effect of cell-free culture supernatant. In this case, 80 µl aliquots of filter (0.2 µm) sterilized supernatant samples were dispensed in wells (performed with a sterile cork borer, of 6mm diameter) made in the gelified medium previously spread by the yeast suspension (Touré et al., 2004).

Among the strains which developed antifungal activity, two potential bacterial strains (coded as CWBI-B1567 and CWBI-1568) were selected for further study and screened for lipopeptide and cell-wall degrading enzymes production.

Characterisation of the selected bacteria

To identify the strains CWBI-B1567 and CWBI-B1568, Gram staining, catalase test, morphology and standard biochemical tests, as listed in Bergey’s Manual of Determinative Bacteriology, and their ability to utilise various carbon sources (elements) in the API 50CH (Biomerieux) were assayed (Logan and Berkeley, 1984). The two selected antagonistic bacterial strains were also identified by sequencing of their 16S rDNA.

Sporulation test

Microscopy

The two strains were grown in a liquid culture medium (YPD: Glucose, 20g; Peptone, 10g; Yeast extract, 10g; distilled water, 1 L) at 30°C for 3 days in agitated flasks at 200 rpm. The cells were examined under optic Zeiss Axioskop 2 MOT microscope.

Thermal treatment

The strains CWBI-B1567 and CWBI-B1568 were inoculated into
nutrient broth and incubated with shaking for 24 h at 30°C. The cultured samples were diluted into a minimal medium salt base, and the cell suspensions were incubated for 12 min at 80°C. Samples of treated and untreated cultures were diluted and plated on Nutrient Agar for CFU counting.

Identification by sequencing 16S rDNA gene

The 16S rDNA gene sequence was determined by direct sequencing. Total DNA was isolated by using the Wizard® genomic DNA purification kit (Promega, Madison, USA). Primers used for PCR and DNA sequencing were presented in Table 1. PCR amplification was performed with the primer pair SPO/SP6 targeted against regions of 16S rDNA (Ventura et al., 2001).

Amplification of DNA was performed in a Mastercycler thermal cycler (Eppendorf). PCR conditions included a hot start at 96°C (5 min), 25 cycles consisting of hybridisation at 55°C (30 s), elongation at 72°C (2 min), denaturation at 95°C (30 s) and a final extension at 72°C (10 min). PCR products were resolved by electrophoresis in 1% (w/v) agarose gel and visualised by ethidium bromide (1 μl/10 ml). 16S rDNA PCR amplicons were purified following the microcon YM-100 kit (Bedford, MA, USA) and sequenced using the Big Dye Terminator V3.0 kit as specified by the supplier with the primers described in Table 1. Phylogenetic analysis was realised by the alignment of sequences of the 16S rDNA with genes collected in an international database (GenBank). The results were then expressed as a percentage of homology between the submitted sequences and the sequences contained in the database.

Determination of the enzymatic activities

Enzymatic activities (cellulase, protease and chitinase) were assessed in a qualitative way through halo formation on solid media. To obtain active cell populations, the strains were streaked on solid YPD medium 24 h before transfer to enzymatic test medium.

Cellulase activity

Bacterial strains were first grown on carboxymethyl cellulose liquid medium (CMC: Peptone, 1.5 g; Yeast extract, 1.5 g, CMC, 35 g; Distilled water, 1 L) at 30°C for 48 h, and cultures were centrifuged at 10,000 g for 20 min. Bacterial cultures and their supernatants were tested against a commercial cellulase on CMC agar containing (g/l): KH₂PO₄, 1.0; MgSO₄.7H₂O, 0.5; NaCl, 0.5; FeSO₄.7H₂O, 0.01; MnSO₄.H₂O, 0.01, NH₄NO₃, 0.3; CMC, 10.0; agar, 12.0. The pH was adjusted to 7.0 with 1 M NaOH. The CMC agar plates were incubated at 37°C for 5 days to allow for the secretion of cellulase. At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 min. The formation of a clear zone of hydrolysis indicated cellulose degradation (Ariffin et al., 2006).

Protease activity

For protease activity, the fresh colonies were used to inoculate liquid Luria cultures (Peptone, 10 g; Yeast extract, 5 g; NaCl, 5 g; glucose, 1 g; distilled water, 1 L). After overnight incubation at 30°C, these cultures were filtered (0.2 μm acetyl cellulose membrane) in order to obtain cell free supernatants which were spotted (15 μl on a circle of Whatmann paper, 5 mm diameter) on protease test medium plates. Plates were observed after 24 h of incubations at 30°C. The detection of enzymatic activity was based on the formation of clear haloes. Papain 1 mg/ml (powder at 35 000 USP) was used as a positive control.

Chitinase activity

The bacterial strains were grown on solid medium, containing colloidal chitin, at 30°C for 2-7 days. The formation of a clear zone of hydrolysis indicated positive chitinase activity.

Production and purification of lipopeptides

The strains CWBI-B1567 and CWBI-B1568 were grown at 30°C for 72 h in agitated flasks at 200 rpm in 350 ml of a liquid culture medium optimised for lipopeptide production (named Opt medium) as described by Jacques et al. (1999). Cultures were centrifuged at 17,400 g for 20 min. The supernatant was filtered through a 0.2 μm sterile filter membrane for testing by the plate diffusion method. For safety, these tests were performed against Yarrowia lipolytica.

In order to obtain lipopeptide-enriched extracts, purification of the resulting supernatants was realised by solid-phase extraction on a 300mg C18 column (Touré et al., 2004); this is sufficient to fraction 10-20 ml of classic supernatant (agitated culture of 3 days in rich medium).

Table 1. Primers used for PCR and sequencing of 16S rDNA of bacterial strain.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Primer</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>16SPO</td>
<td>5'-AAGAGTTTGATCCTGGCTCAG-3'</td>
<td>Ventura et al., 2001</td>
</tr>
<tr>
<td></td>
<td>16SP6</td>
<td>5'-CTACGGCTACCTTGTACGA-3'</td>
<td>Ventura et al., 2001</td>
</tr>
<tr>
<td>Sequencing*</td>
<td>F1</td>
<td>5'-CTTGCTCGAGAYGAACG-3'</td>
<td>(Sigma-Proligo)</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>5'-GAGGCGACAGTRGGGAAT-3'</td>
<td>(Sigma-Proligo)</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>5'-ACACCGTGCGAAGGC-3'</td>
<td>(Sigma-Proligo)</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>5'-GCACAAGCGGYGGACAT-3'</td>
<td>(Sigma-Proligo)</td>
</tr>
</tbody>
</table>

*Synthetic primers used for sequencing were deduced from the alignment of 16S rDNA genes collected from EMBL (European Molecular Biology Laboratory) databases and were supplied by Sigma-Proligo.
Table 2. Concentrations of the solvents used and their times.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (H₂O 0.1 FA) %</th>
<th>Solvent B (ACN 0.1 FA) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>18</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>19</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>24</td>
<td>55</td>
<td>45</td>
</tr>
</tbody>
</table>

ACN, Acetonitrile; FA, formic acid.

RESULTS

Isolation and screening of antagonistic strains by in vitro antagonism experiments

Over 42 bacterial isolates were obtained from soil of arid regions situated in South-East of Algeria. In order to assess whether the bacterial strains isolated may be useful sources for natural bioactive compounds, the antifungal activity of the isolates and the supernatants was determined. Two strains showed a strong antagonistic effect when faced with C. albicans by developing clear zones of lyses of 23 and 19 mm by strain CWBI-B1567 and CWBI-B1568 respectively (Figure 1).

Identification of bacterial strains

Upon microscopic examination, catalase activity and the Gram stain showed that the two strains were catalase and Gram positive. The two sporulation tests and identification made by API 50CH revealed that these strain belongs to the genus: Bacillus.

However, nucleotide sequences of 16S rDNA of the two Bacillus sp. were determined to confirm biochemical identification. The obtained 16S rDNA sequences were compared with available 16S rDNA sequences in the GenBank database. Obtained sequence identifications were very reliable (>99% similarity with the database). This comparison showed that the strain CWBI-B1567 was B. subtilis (accession number KC341751) while; the strain CWBI-B1568 was B. mojavensis (accession number KC341749).

Determination of the enzymatic activities developed by the antagonistic Bacillus strains

The capacity of the Bacillus strains to produce cell-wall degrading enzymes was qualitatively studied. The results show that both B. subtilis and B. mojavensis produced protease and cellulase (Figures 2 and 3) but not chitinase (Table 3).
Purification and identification of lipopeptides produced by the selected Bacillus strains

Implication of lipopeptides in the observed antagonism was at first studied by testing filter-sterilised crude supernatants obtained from culture in the Opt medium. A total of 80 µl of this solution was sufficient to clearly inhibit the development of the test yeast suggesting the involvement of secreted antifungal metabolites. A similar level of antagonistic activity was observed by testing lipopeptide-enriched extracts.

Members of the different families were detected in the methanolic extract obtained from the culture supernatant. They were identified by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) on the basis of their retention times (Figure 4) compared to purified molecules used as standards and on the basis of their fragmentation profiles (Figure 5). A wide variety of homologous compounds were detected within each group; both the B. subtilis and the B. mojavensis produced surfactin and iturin. However, the fengycin group was produced only by B. mojavensis (Table 4).

DISCUSSION

Bacillus species have the interesting property of producing secondary metabolites with a wide spectrum of antibiotic activity and very diverse structures. Therefore, they have been very valuable for medical applications. Bacillus strains produce gene-encoded antibiotics as well as a variety of small antibiotic peptides (<2000 Da) synthesized non-ribosomally (Moyné et al., 2004). They include lipopeptide antibiotics such as iturin, surfactin, and fengycin.

In this article, we have described the use of Bacillus strains CWBI-B1567 and CWBI-B1568 isolated from arid soil as well as their secreted secondary metabolites to act as antifungal products against C. albicans. These strains showed a powerful antifungal activity; both the antagostic strains and their cell-free culture supernatants significantly inhibited growth of the yeast (inhibition zone ≥ 19 mm). This strong activity expressed by a large zone of inhibition on agar plates indicated, as mentioned by Barakate et al. (2002), that these two isolates produce water soluble antimicrobial metabolites. It is well known that several species of Bacillus are capable of producing biologically active molecules, including antifungal substances (Yun-Feng et al., 2012; Caldeira et al., 2011; Zhenzhen et al., 2010).

Based on the physiological, biochemical characteristics and 16S rDNA sequence analysis, strains CWBI-B1567 and CWBI-1568 were identified as B. subtilis and B. mojavensis respectively. B. mojavensis Roberts, Nakamura, and Cohan was distinguished from the B. subtilis complex on the basis of whole-cell fatty acid composition, divergence in DNA sequence, and resistance to genetic transformation (Roberts et al., 1994). The type of this bacterium is an isolate from the Mojave Desert, California. This group of species in addition to the desert origins has additional physiological traits that serve as characteristics of the species. All strains of this species are antagonistic to fungi and are all endophytic. These traits suggest that this species has outstanding traits as an endophytic biocontrol agent.

Antifungal power of B. subtilis and B. mojavensis was further exemplified by testing filter-sterilised crude supernatant obtained from their culture in the Opt medium. The electro-spray mass spectrometry coupled to liquid chro-matography (ESI-LC-MS) analysis showed that B. mojavensis CWBI-B1568 produced the three lipopeptides families. However, B. subtilis CWBI-B1567 produce only two types of lipopeptides (surfactin and iturin). These antifungal substances have been widely reported in Bacillus cultures such as B. amyloliquefaciens B94 (Yu et al., 2002) and B. subtilis JA (Chen et al., 2008;
Fernandes et al., 2007). The two tested *Bacillus* strains produced cellulase and protease but the chitinase activity was not observed in either *Bacillus* strain.

The antifungal activity detected in this work can be explained by the capacity of the *Bacillus* isolates to produce the cyclic lipopeptides and the cell-wall degrading enzymes (Glick et al., 1999; Dunne et al., 1993). Lipopeptides, including surfactin, iturin and fengycin, were described previously as a major class of *Bacillus* peptide antibiotics, which have a great potential

### Table 3. The cell-wall degrading enzymes produced by *B. subtilis* and *B. mojavensis* on enzymatic test mediums.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protease</th>
<th>Cellulase</th>
<th>Chitinase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>+++ (34 mm)</td>
<td>+++ (30 mm)</td>
<td>-</td>
</tr>
<tr>
<td><em>B. mojavensis</em></td>
<td>++ (20 mm)</td>
<td>+++ (30 mm)</td>
<td>-</td>
</tr>
</tbody>
</table>

### Figure 4. Chromatograms corresponding to lipopeptides analyzed by ESI/LC/MS technique. **A**, lipopeptides families produced by *B. mojavensis* CWBI-B1568; **B**, lipopeptides produced by *B. subtilis* CWBI-B1567.
for biotechnological and biopharmaceutical applications (Peypoux et al., 1999). Iturins and fengycins display a strong antifungal activity, however, surfactins are not fungitoxic by themselves but retain some synergistic effect on the antifungal activity of Iturin A (Maget-Dana et al., 1992). Results found by Ongenae et al. (2005), strongly suggest that lipopeptides are responsible for most of the antifungal activity developed in vitro by \textit{B. subtilis} toward multiple plant pathogens.

Through this study, we have demonstrated that \textit{B. subtilis} and \textit{B. mojavensis} have a strong antifungal activity especially against \textit{C. albicans}. Both the \textit{B. subtilis} and the \textit{B. mojavensis} have a potential of lipopeptides and cell-wall degrading enzymes production. Thus, these bioactive components might provide an alternative resource for the pharmaceutical control of \textit{C. albicans}. Further studies are needed to determine the ability of these \textit{Bacillus} strains to control diseases caused by this...
Table 4. Lipopeptide homologues produced by *B. subtilis* and *B. mojavensis* after growth for 72h in Opt medium.

<table>
<thead>
<tr>
<th>Bacillus isolate</th>
<th>Lipopptides</th>
<th>Retention times (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fengycins</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>Iturins</td>
<td>/</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CWBI-B1567</td>
<td>C15</td>
<td>6.22</td>
</tr>
<tr>
<td></td>
<td>C18</td>
<td>6.753</td>
</tr>
<tr>
<td></td>
<td>Surfactins</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus mojavensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CWBI-B1568</td>
<td>C12</td>
<td>12,121</td>
</tr>
<tr>
<td></td>
<td>C13</td>
<td>12,645</td>
</tr>
<tr>
<td></td>
<td>C14</td>
<td>13,291</td>
</tr>
<tr>
<td></td>
<td>C15</td>
<td>14,373</td>
</tr>
<tr>
<td></td>
<td>C16+C15</td>
<td>14,609</td>
</tr>
<tr>
<td></td>
<td>C15+C16</td>
<td>14,906</td>
</tr>
<tr>
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<td>C16</td>
<td>15,098</td>
</tr>
<tr>
<td></td>
<td>Fengycins</td>
<td>6.22</td>
</tr>
<tr>
<td></td>
<td>Iturins</td>
<td>3.602</td>
</tr>
</tbody>
</table>

human pathogenic yeast.

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