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Isolation and identification of antifungal peptides from *Bacillus* BH072, a novel bacterium isolated from honey

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ABSTRACT

A bacterial strain BH072 isolated from a honey sample showed antifungal activity against mold. Based on morphological, biochemical, physiological tests, and analysis of 16S rDNA sequence, the strain was identified to be a new subspecies of *Bacillus* sp. It had a broad spectrum of antifungal activity against various mold, such as *Aspergillus niger*, *Pythium*, and *Botrytis cinerea*. Six pairs of antifungal genes primers were designed and synthesized, and *ituA*, *hag*, *tasA* genes were detected by PCR analysis. The remarkable antifungal activity could be associated with the co-production of these three peptides. One of them was purified by 30–40% ammonium sulfate precipitation, Sephadex G-75 gel filtration and anion exchange chromatography on D201 resin. The purified peptide was estimated to be 35.615 kDa and identified to be flagellin by micrOTOF-Q II. By using methanol extraction, another substance was isolated from fermentation liquor, and determined to be iturin with liquid chromatography–mass spectrometry (LC–MS) method. The third possible peptide encoded by *tasA* was not isolated in this study. The culture liquor displayed antifungal activity in a wide pH range (5.0–9.0) and at 40–100 °C. The result of the present work suggested that *Bacillus* BH072 might be a bio-control bacterium of research value.

1. Introduction

Fungi are widespread in nature. It is the important reason for contamination of food crops, food raw materials and processed products. Fungal contamination causes not only huge economic losses, but also food safety issues. Fungi also lead to industrial contamination and some result in human and animal diseases (Hawksworth 2001). Prevention and control of fungal contamination is an important issue in the field of industry, agriculture and medicine. To food industry, chemical preservatives are generally used to control the growth and reproduction of fungi. However, due to abuse of chemical preservatives, its residues and food safety issues become more and more serious. Some microbes can produce antimicrobial metabolites which can inhibit or kill other microorganisms. Antifungal peptides are one of the most important natural defences which were against the invading of most fungal pathogens. Some has been developed to be the food preservatives and bio-pesticides, which has provided a new choice to prevent and control the fungal contamination of agricultural products.

Through the research of bio-control against fungi, antifungal peptides play an important role. According to the Antimicrobial Peptide Database (APD), 756 different antifungal peptides were isolated from different organisms, such as humans, animals, reptiles, birds, insects and microbes (http://aps.unmc.edu/AP/main.php). By blocking, destroying the synthesis of fungal cell wall or forming holes in lipid membranes leading to the important contents of the fungi that causes death. Some bacteria reacted to fungal mitochondria and nucleic acid within cells causes death, too. According to reports, *Pseudomonas*, *Bacillus*, *Aspergillus*, *Streptomyces*, edible fungi and other microorganisms can produce different antifungal substances, such as antifungal polypeptide. Among them, the *Bacillus* species are widely used. Therefore, the study of the unknown bacteria and their antibiotic substances is of great significance.

Most of the genus *Bacillus* are Gram-positive, aerobic endospore-forming and rod-shaped bacteria, which are found in diverse environments such as soil and clays, rocks, dust, aquatic environments, vegetation, food and the gastrointestinal tracts of various insects and animals (Nicholson 2002). Commercial products including enzymes, antibiotics, amino acids and insecticides are produced by *Bacillus* sp. The potential of *Bacillus* species to secrete various peptides which have shown distinct capacities to inhibit plant pathogens, such as fungi and bacteria with high concentrations, have been known for more than 50 years. To date, a lot of antimicrobial peptides produced by *Bacillus* sp. were reported. A *Bacillus amyloliquefaciens* CCMI 1051 strain was isolated which had strong inhibition on both *Rhizopus* sp. L-122 and *Trichoderma harzianum* CCMI 783 (Caldeira et al. 2007, 2008). Arrebola et al.

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(2010) reported that *B. amyloliquefaciens* PPCB004 could inhibit *Penicillium crustosum* hyphal extension. Lee et al. (2008) discovered *Botrytis cinerea* was inhibited by *Bacillus* WJ5 (*Paenibacillus lentimorbus*) and its antifungal substances were extracted. Quan et al. (2006) and Zhang et al. (2010) isolated *B. amyloliquefaciens* Q-12 and NK10.B and displayed a strong inhibitory effect on *Fusarium oxysporum*, *Fusarium solani* and other fungi. Many researches indicated that the *Bacillus* sp. strains themselves and their antimicrobial substances had huge application potential in bio-control of plant diseases. Some antibiotics have been a certain degree of practical application. The United States has 4 bio-control *Bacillus subtilis* strains including GBO3, MBI600, QST713 and *B. subtilis* var. *amyloliquefaciens* FZB24, obtained the Environmental Protection Agency (EPA) approval, which can be applied to commercial production (http://www.epa.gov/pesticides/biopesticides).

Antifungal materials produced by *Bacillus* strains have the following two kinds. Ribosome synthesis antimicrobial proteins contain bacteriocins (Oscáriz and Pisabarro 2000; Zheng and Slavik 1999), cell wall degrading enzymes (such as proteases, chitinase, β-1,3-glucanase) as well as some unidentified inhibitory proteins (Manjula et al. 2004; Simi 1994). The non-ribosomal synthesis antibiotics primarily include lipopeptide antibiotics, such as Surfactin, Iturin and Fengyein (Huszcza and Burczyk 2006; Peypoux et al. 1999; Mukherjee and Das 2005; Bonmatin et al. 2003; Koumoutsi et al. 2004; Tsuge et al. 1999; Kim et al. 2004; Jacques et al. 1999). Lipopeptide antibiotics with antimicrobial activity against filamentous fungi and yeasts are mostly D-type and L-type amino acid cyclic peptides, with a fatty acid chain.

According to the literature, most strains of *Bacillus* sp. produced only one or two antimicrobial substances. Mora et al. (2011) cloned six antimicrobial peptide genes from *Bacillus* strains of environment origin, and determined that they encoded different lipopeptides. Large molecular antimicrobial proteins have rarely been reported. Hammami et al. (2009, 2012) pointed out that *B. subtilis* 14B produced a novel bacteriocin (Bac 14B) weighted 31 kDa that was highly effective as a bio-control agent against crown gall disease. Most of the references reported that TasA and flagellin were obtained through genetic engineering method. Yang et al. (2010) cloned and expressed the *tasA* gene, and the expressed product showed antifungal activity against cucumber gray mold. Asano et al. (2001) cloned the *hag* gene, the antifungal activity of its expressed product flagellin explained the inhibitory appearance of *Bacillus* strains B-3, NK-330 and NK-C-3. By now, TasA and flagellin have not been purified directly by normal protein extract methods from *Bacillus* strains fermentation liquor. The novel bacterium BH072 isolated from honey showed strong antifungal activity against a lot of mold. In order to explain the antifungal activity, the peptides of fermentation broth were isolated and identified by a combination of several purification methods, and their genes were retrieved and analyzed. We try to elucidate the nature of antifungal substances and explore the possibility of their further application.

2. Materials and methods

2.1. Microbial strains and culture medium

The microorganism BH072 used in this study was isolated from a honey sample. Three *Bacillus* strains *B. subtilis* BH121, *B. licheniformis* BH122 and *B. subtilis Natto* BH123 and the indicator strains of *Escherichia coli*, *Staphylococcus aureus*, *Pythium*, *Penicillium*, *Colletotrichum orbiculare* and *Saccharomyces cerevisiae* were preserved at Food Biotechnology Laboratory of Tianjin University. The indicator *A. niger* CGMCC3.03928, *Botrytis cinerea* CGMCC3.4584 and *Fusarium oxysorum* CGMCC3.2830 were purchased from China General Microbiological Culture Collection Center. All the medium components used in this study were reagent pure grade purchased from Jiangtian Chemical Technology Co., Ltd. (Tianjin, China). Solid media used in the antifungal study consisted of potato dextrose agar (PDA) for fungi (potato, 200 g; glucose, 20 g; agar, 18 g; and distilled water, 1 L) and Luria–Bertani agar (LBA) for bacteria (peptone, 10 g; yeast extracts, 5 g; NaCl, 10 g; agar, 18 g; and distilled water, 1 L). Liquid media, the same agar-lacking LBA, were used for fermentation test. The strain BH072 was activated by transferring single colonies of the strain from plates to 10 mL activation LB medium extract in 50-mL flasks. The flasks were shaken at 37 °C, 150 rpm for 16 h.

2.2. Microscopic examination and Physiological–biochemical identification

According to Deng et al. (2012), sample was prepared and applied to scanning electron microscope (XL-30 TMP; Philips, the Netherlands). Physiological–biochemical tests includes sugar fermentation experiments, acid yield experiment, optimum growth temperature and pH measurement, salt tolerance test, resistance to organic acid experiment, gelatin puncture, casein and starch hydrolysis.

2.3. 16S ribosomal DNA sequence analysis

The genomic DNA of strain BH072 was used as a template. The 16S ribosomal DNA sequence was amplified and sequenced using universal primers 8F: 5′-AGAGTTTGATCATGGCTCAG-3′ and 1492R: 5′-ACGGTTACCTTGTTACGACTT-3′ (Maiwald et al. 1994). The amplification was conducted by polymerase chain reaction in a PCR thermal cycler (MyCycler; Bio-Rad Laboratories Inc., USA). The PCR amplification system was 25 μL, including 0.2 μL Taq enzyme (0.5 U/mL), 2.5 μL 10× buffer, 1.8 μL Mg^{2+}, 1 μL dNTPs mixture, 1 μL template DNA, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM) and 17.5 μL ddH_2O. Amplification factor: 95 °C, 3 min; 95 °C, 30 s, 55 °C, 60 s, 72 °C, 90 s, 30 cycle; 72 °C, 5 min; 4 °C termination reaction. The amplified products were purified and sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

2.4. Phylogenic tree construction

According to 16S rDNA sequencing results, combined with GenBank in the genus *Bacillus* in 16S rDNA sequences, using MEGA 4.0 (Tamura et al. 2007) software by the neighbor-joining analysis (Saitou and Nei 1987), phylogenetic tree was constructed.

2.5. Cloning of antifungal genes by PCR analysis

DNA was extracted respectively from overnight culture of *Bacillus* BH072, *B. subtilis* BH121, *B. licheniformis* BH122 and *B. subtilis Natto* BH123. Specific primers for the functional genes of the peptides (Abriouel et al. 2011): *ituA* (iturin A), *hag* (flagellin), *tasA* (TasA), *srf* (surfactin), *spaS* (subtilin) and *mrsA* (mersacidin) genes are listed in Table 1. The amplification system was the same as 16S rDNA sequences amplification system. Amplification factor: 95 °C, 3 min; 95 °C, 30 s, 51 °C, 60 s, 72 °C, 90 s, 30 cycle; 72 °C, 5 min; 4 °C termination reaction. The PCR product was gel-purified and ligated to pUCm-T vector (Sangon Biotech Co., Ltd., Shanghai, China) to facilitate DNA sequencing. After transformation into competent cells of *E. coli* DH5α, the recombinants were selected in LB agar plates supplemented with ampicillin. By a colonial PCR reaction using the corresponding primers, further identification was conducted. And the aimed plasmid was sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). Sequencing data were applied to retrieve for homologous sequences with the BLAST algorithm