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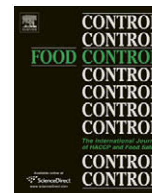

Iturin produced by *Bacillus pumilus* HY1 from Korean soybean sauce (*kanjang*) inhibits growth of aflatoxin producing fungi

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ABSTRACT

A new strain of *Bacillus pumilus*, designated HY1, was isolated from Korean soybean sauce (*kanjang*). This classification was based on morphological, physiological, and chemotaxonomic features of the organism that identified it as a Gram-positive bacillus, and confirmed by 16S rDNA based phylogenetic analysis. Strain HY1 showed strong antifungal activity against the aflatoxin-producing fungi *Aspergillus flavus* and *Aspergillus parasiticus*, two common contaminants of fermented soybean foods. MALDI-TOF mass analysis revealed that the antifungal compound was similar to the known lipopeptide iturin. Iturin purified from strain HY1 had three isoforms with protonated masses of *m/z* 1,043.4, 1,057.4, and 1,071.4, and different structures in combination with Na^+ ion using MALDI-TOF MS. Purified iturin from HY1 also exhibited antifungal activity against *A. flavus* and *A. parasiticus*.

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1. Introduction

Aflatoxins are fungal secondary metabolites known to be highly toxic, mutagenic, and carcinogenic to animals and humans (Ali et al., 2005). For example, aflatoxins can cause liver cancer in animals and humans when ingested as contaminants in feeds or food (Moyne, Shelby, Cleveland, & Tuzun, 2001). Aflatoxins are produced by certain strains of *Aspergillus* including *Aspergillus flavus, Aspergillus parasiticus*, *Aspergillus nomius*, and *Aspergillus tamarii*, and are known contaminants during fermentation of the soybean products *meju*, *doenjang*, and *kanjang* (Bae, Kwak, Park, Kim, & Shon, 2003; Kim & Roh, 1985; Kim et al., 2001; Ok et al., 2007; Park, Kang, Oh, & Chung, 2001). Previous studies reported that isolation of *Bacillus* sp. with antifungal activity against aflatoxin-producing fungi such as *A. flavus* and *A. parasiticus* (Bottone & Peluso, 2003; Kang, Park, & Cho, 2000; Kim & Roh, 1998; Kimura & Hirano, 1988; Klich, Lax, Bland, & Scharfenstein, 1993; Moyne et al., 2001).

Several *Bacillus* sp. secrete antibiotics, some of which are antifungal peptides (Bottone & Peluso, 2003; Cho et al., 2002; Kimura & Hirano, 1988; Klich et al., 1993; Moyne et al., 2001). Most of these antifungal peptides have a molecular weight of less than 2000 Da and are synthesized non-ribosomally via a multi-enzyme-catalyzed process (Moyne et al., 2001). The antibiotics synthesized non-ribosomally include the lipopeptides iturin (Maget-Dana & Peypoux, 1994), surfactin (Cho et al., 2003), fengycin (Vanittanakom, Loeffler, Koch, & Jung, 1986), plipastatin (Tsuge, Ano, & Shoda, 1996), and di- and tripeptides such as bacilysin (Walker & Abraham, 1970). Iturins are a family of cyclic lipopeptides that have a peptide ring of seven amino acid residues with the chiral sequence LDDLLDL closed by a β-amino acid bearing a hydrophobic tail of 11–12 carbon atoms. Iturin has a broad antifungal spectrum, but its antibacterial activity is limited to only a few species (Grau, Ortiz, de Godos, & Gómez-Fernández, 2000).

Fermented soybean foods such as *doenjang* (soybean paste), *kanjang* (soybean sauce), and *cheonggukjang* (soybean cook) are often served as side dishes in Korea and have been a major source of protein in the Korean diet for thousands of years. The production of Korean traditional soybean sauce and soybean paste from the fermentation of soybean depends predominantly on *Bacillus* sp., notably *Bacillus subtilis, Bacillus licheniformis* and *Bacillus pumilus* (Cho & Seo, 2007). In this study, bacterial strain HY1 isolated from Korean traditional soybean sauce was identified as *B. pumilus* by 16S rDNA analysis and by biochemical and biophysical characterization. In addition, an iturin with inhibitory activity against aflatoxin-producing fungi was purified from HY1 using thin layer chromatography (TLC) and reverse phase high pressure liquid chromatography (HPLC) and structurally identified by MALDI-TOF.

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2. Materials and methods

2.1. Isolation of an antibiotic producing Bacillus sp. and culture conditions

The aflatoxin-producing fungi *A. flavus* KCTC 6905 and *A. parasiticus* KCTC 6598 were kindly provided by Korean Collected Type Culture (KCTC), Daejeon, Korea. The aflatoxin-producing fungi were maintained on potato dextrose agar (PDA, Difco), and were grown at 28 °C. Strain HY1 producing antibiotic was isolated from Korean soybean sauce (*kanjang*). The sample of Korean soybean sauce was suspended in sterile distilled water with 0.85% NaCl and spread on 5% sheep blood agar plates. The plates were incubated at 30 °C for 24 h and one colony showed a clear zone and was screened for lipopeptide production. The isolate was cultivated on tryptic soy agar (TSA, Difco) plate and was grown at 30 °C. Strain HY1 was cultured on PDA and its antifungal activities against the aflatoxin-producing fungi was detected by bioassay.

2.2. Morphological and physiological characteristics

Cell morphology was examined by light microscopy after Gram staining. Flagellum type was determined by transmission electron microscopy (TEM 1010, Jeol) using preparations negatively stained with 1% phosphotungstic acid. Phenotypic characterization was carried out by standard methods, using API50CHB kits (Bio-Mérieux). The methods described by Cowan and Steel (1965) were used for the following physiological test: catalase, indole production, oxidase, Voges–Proskauer reaction and hydrolysis of gelatin, casein and starch. Tests for utilization of substrates as a sole carbon source were performed with BIOLOG GP2 MicroPlates containing 95 different carbon compounds and the results were checked over 24 h. Growth in the presence of NaCl concentration and the temperature range were determined using TSA as basal medium.

2.3. Chemotaxonomy

The biomass for cellular fatty acid analysis was prepared from 1 day culture grown on a TSA plate at 28 °C. Fatty acid methyl esters were prepared using the method described in the MIDI Microbial Identification System (Hewlett Packard) manual. The resultant esters were separated using a gas chromatograph (model 5890, Hewlett Packard) fitted with a phenylmethyl silicone fused silica capillary column (25 m $\times$ 0.2 mm, Hewlett Packard).

2.4. Sequencing of 16S rDNA and phylogenetic analysis

16S rDNA of strain HY1 was amplified by PCR. The PCR primers used to amplify 16S rRNA gene fragments were the *Bacillus* specific primers (478F, 5′-TTCTACGGAGAGTTGATCC-3′; 479R, 5′-CACCTTCCGGTACGGCTACC-3′) (Cho et al., 2007). 16S rRNA genes were amplified by PCR using the extracted DNA, Super-Therm DNA polymerase (JMR, Side Cup, Kent, UK), 1.5 mM $MgCl_2$, 2 mM dNTP, and primers in a final volume of 50 μl 30 cycles (Denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min 30 s) were followed by a final incubation at 72 °C for 10 min. The anticipated product of approximately 1300 bp was isolated after agarose gel electrophoresis of the amplified mixture using a gel extraction kit (iNtRON Biotechnology, Suwon, Korea). PCR products were directly cloned into the pGEM-T Easy vector (Promega, WI, USA) and recombinant colonies were randomly picked. Samples for nucleotide sequencing were prepared by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin–Elmer Corp., Norwalk, CN, USA). The samples were analyzed with an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Assembly of the nucleotide sequences was performed with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). All reference sequences were obtained from the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) databases. The 16S rRNA similarity sequences searches were performed using the BLASTN as well as PSI-BLAST tools in the NCBI website (McGinnis & Madden, 2004). Sequences were aligned using the multiple sequence alignment program, CLUSTAL W (Tompson, Higgins, & Gibson, 1994). Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Phylogenetic analysis was performed using neighbor-joining methods (Saito & Nei, 1987). Bootstrap analysis was performed using data re-sampled 1000 times using the DNAMAN analysis system.

2.5. Isolation of an iturin fraction

Isolation of iturin produced by strain HY1 was performed as described by Cho et al. (2003). Cells were grown in number 3 medium (No. 3: 10 g polypeptone, 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ per liter, pH 6.8) at 30 °C. After 3 days of cultivation, the supernatant was collected by centrifugation and adjusted to pH 2.0 using concentrated HCl. The precipitate was collected by centrifugation and extracted three times with methanol. The methanolic extracts were concentrated and then dissolved in methanol.

2.6. TLC and RP-HPLC

The culture supernatant of *B. pumilus* HY1 was precipitated with concentrated HCl and then extracted with methanol. The methanolic extracts were examined using thin layer chromatography on silica gel DC 60 plates (Merck, Darmstadt, FRG) using chloroform/methanol/water 65:25:5 (v/v/v) as the mobile phase. The antifungal iturin was detected by charring with concentrated 10% sulfuric acid and showed a broad spot with R_f of 0.36. For preparative isolation of the iturin fraction, the corresponding spots were scratched out from the thin layer chromatography (TLC) and the silica gel material was extracted with methanol. For further purification, the extract (1 mg) was subjected to reversed phase high performance liquid chromatography (RP-HPLC; LC-908, JAIGEL-1H column, Japan Analytical Industry, Japan) and eluted with the following conditions: flow of 2.5 ml/min, acetonitrile/water (1:1) as mobile phase. Iturin standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and composed of C14 and C15 β-amino acids.

2.7. MALDI-TOF analysis of an iturin fraction

The iturin fraction was analyzed by using fast atom bombardment (FAB) mass spectrometry (data not shown) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF, Vg-Instruments, Manchester, UK) mass spectrometry. Five micrograms of sample was dissolved in dimethyl sulfoxideglycerol and introduced on copper probe tip using a mixture of glycerol and triglycerol as matrix. A saturated solution of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile/0.1% trifluoroacetic acid (v/v) was mixed with an equal volume sample for the MALDI-TOF mass analysis. One microliter of the sample (2–3 ρmol) was deposited on a sample plate and air-dried. Ions were accelerated with a voltage of 20 kV. The positive-ion and reflector mode was applied.

2.8. Antifungal assay of an iturin fraction

The ability of *B. pumilus* strain HY1 to inhibit growth of aflatoxin-producing fungi *in vitro* was determined in a solid medium growth assay. Agar plugs of growing aflatoxin-producing fungi

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