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Comparative proteomic analysis reveals intracellular targets for bacillomycin L to induce *Rhizoctonia solani* Kühn hyphal cell death

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ABSTRACT

Bacillomycin L, a natural iturinic lipopeptide produced by *Bacillus amyloliquefaciens*, is characterized by strong antifungal activity against a variety of agronomically important filamentous fungi including *Rhizoctonia solani* Kühn. To further understand its antifungal actions, proteomes were comparatively studied within *R. solani* hyphal cells treated with or without bacillomycin L. The results show that 39 proteins were alternatively expressed within cells in response to this lipopeptide, which are involved in stress response, carbohydrate, amino acid and nucleotide metabolism, cellular component organization, calcium homeostasis, protein degradation, RNA processing, gene transcription, and others, suggesting that, in addition to inducing cell membrane permeabilization, iturin exhibits antibiotic activities by targeting intracellular molecules. Based on these results, a model of action of bacillomycin L against *R. solani* hyphal cells was proposed. Our study provides new insight into the antibiotic mechanisms of iturins.

1. Introduction

A lipopeptide is a molecule consisting of a lipid connected to a peptide. *Bacillus* sp. produces three types of lipopeptides: surfactins, fengycins, and iturins. Iturins are a family of cyclic lipopeptides. Members of this family, including iturin A and C, bacillomycin D, F, L, and LC, and mycosubtilin, share structural and functional similarities [1]. These amphiphilic compounds are featured by a heptapeptide, with the constant LDDLLDL α-amino acid configuration including invariable D-Tyr 2 and D-Asn 3, cyclized in a ring with a β-amino fatty acid chain in a length of 14 to 17 carbons. Iturins exhibit strong antifungal activities against a wide variety of yeasts and plant pathogenic fungi but limited antibacterial activities [2]. The structural features of iturin lipopeptide are important for their biological activity and may play a critical role in their mechanism of action against microbial cells [3,4].

In the past several decades, a large body of literature about the mechanisms of iturinic antibiotics has proposed that the antimicrobial activity of iturins predominantly depends on their capacity to increase membrane permeability, which is attributed to aggregates formed by iturin molecules, iturin-phospholipid complex or iturin-phospholipid-sterol complex. However, most of these studies are carried out on model membranes rather than real cells by using various experimental approaches [5–8]. It is still open to discuss whether membrane permeabilization is the only mechanism by which iturin lipopeptides kill microbial cells, or peptides have various intracellular targets [9,10]. In another word, we know little so far about the intracellular response or molecular events underlying iturin-induced cell inhibition or death. For this reason, studies using live microbial cells, especially fungal hyphal cells, are necessary for our better understanding of the antibiotic actions of iturins.

Previously, we originally isolated bacillomycin L, an iturin lipidpeptide, from the culture broth of *Bacillus amyloliquefaciens* K103. This peptide displays a strong in vitro antifungal activity against a wide variety of plant fungal pathogens, including *Rhizoctonia solani* Kühn, a pathogen of considerable economic importance particularly at the seedling stage of most agronomic plant [11,12]. In addition, antibiotic study against *R. solani* hyphae indicates that induction of membrane permeabilization is not the only way that bacillomycin L kills cells [12]. Bacillomycin L may target intracullar molecules to induce cell death, but no literature has proved it.

Proteomic analysis is a powerful tool to profile protein expression in response to various stresses in bacteria, yeasts, and fungi [13–17]. In this study, protein expression within cells was comparatively studied by 2-D PAGE proteomic analysis after *R. solani* was treated with or without bacillomycin L. The data obtained show that 39 proteins were altered in expression after cells were treated with the lipidpeptide. Several signaling pathways are proposed to be involved

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in the bacillomycin L-induced cell death. Our study provides new insight into the antibiotic actions of iturins.

2. Materials and methods

2.1. Peptide preparation

Bacillomycin L was isolated and purified from the culture broth of *B. amyloliquefaciens* K103 by a combination of Sephadex LH-20 gel permeation chromatography and reversed-phase high-performance liquid chromatography using a Shim-pack PREP-ODS C18 column (20 mm × 250 mm, 10 μm, Shimadzu) with gradient elution of 20% to 80% acetonitrile with 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 mL/min. The HPLC-purified peptide was identified by its amino acid composition and quadrupole-time of flight mass spectrometry (Q-TOF-MS) [11]. Bacillomycin L was prepared in PBS before use.

2.2. Fungal culture and treatments

The plant fungal pathogen *R. solani* Kühn (No. 3.2871), purchased from the China General Microbiological Culture Collection Center, was cultured in potato dextrose broth (PDB) at 28 °C with shaking at 140 rpm. After growth, hyphae at logarithmic phase were treated with 100 μg/mL bacillomycin L for 24 h as in literature [12]. Same volume of vehicle was used for control. The hyphal cells were harvested, washed, lyophilized and stored at −20 °C until analysis.

2.3. Protein extraction

Total proteins were extracted using TCA-acetone precipitation procedures as previously described with some modification [18,19]. Briefly, 1.0 g of lyophilized mycelium was powdered in liquid nitrogen with a mortar and pestle and suspended in 15 mL of 10% (*w*/*v*) TCA in acetone containing 0.07% (*v*/*v*) DTT. The suspension was vortexed and incubated at −20 °C for 24 h with intermittent stirring. Total protein was recovered by centrifugation at 12,000 rpm for 20 min at 4 °C. The pellet was washed with acetone containing 0.07% (*v*/*v*) DTT, vacuum dried for 30 min and resuspended in 1 mL of lysis buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT and 0.5% biolytes (Bio-Rad, Hercules, CA,USA) by sonication on ice. Cell debris was removed by centrifugation at 12,000 rpm for 10 min at 4 °C. The protein concentration was determined by a modified Bradford protein assay (Bio-Rad) with BSA as the standard. The protein samples were snap-frozen in liquid nitrogen and stored at −80 °C for later analysis.

2.4. Two-dimensional electrophoresis and image analysis

Protein expression was comparatively analyzed by two-dimensional electrophoresis as in literature with some modification [19]. The immobilized pH-gradient (IPG) strips (17 cm, nonlinear pH 3–10; Bio-Rad) were rehydrated with 450 μg protein in 300 μL rehydration buffer containing 0.2% (*v*/*v*) ampholytes (Bio-Lyte, Bio-Rad) and 0.002% (*w*/*v*) bromophenol blue for 14 h at 20 °C. Proteins on IPG strips were focused (PROTEAN IEF Cell system, Bio-Rad) at a constant current of 50 μA per strip until 60,000 Vh was reached. Strips were then equilibrated in 5 mL equilibration buffer [6 M urea, 50 mM Tris/HCl, pH 8.8, 30% (*v*/*v*) glycerol, 2% (*w*/*v*) SDS, 0.002% bromophenol blue and 2% (*w*/*v*) DTT] with gentle shaking for 15 min followed by a second equilibration in the same buffer containing 2.5% (*w*/*v*) iodoacetamide instead of DTT. Equilibrated IPG strips were placed on the top of 12.5% polyacrylamide gels. Gels were run in buffer containing 25 mM Tris, 192 mM glycine and 0.1% (*w*/*v*) SDS at 16 mA per gel for 30 min, and then 30 mA per gel until the bromophenol blue reached the gel bottom. Gels were stained with Coomassie Blue G-250 dubbed blue silver (Amresco, Solon, OH, USA) as previously described [20]. Gels were imaged with a densitometer (GS-800, Bio-Rad) and analyzed with PDQuest software (Bio-Rad) using a guided protein spot detection method. A protein was considered to be differentially expressed when it was significantly different ($P < 0.05$) and at least a 2-fold change was determined in abundance between the control and iturin treated cells. The molecular mass (Mr) of each protein was estimated by comparison with the standard marker, and the isoelectric point (pI) was determined by the spot position along the IPG strip. Three biological replicates were performed for each condition.

2.5. Protein identification

The selected protein spots were excised and destined with 25 mM ammonium bicarbonate and 50% (*w*/*v*) acetonitrile (ACN) for 15 min with agitation. Gel specimen was dried by centrifugal lyophilization (Savant AES1010 SpeedVac, Savant, Holbrook, NY, USA). In-gel digestion was performed with 0.01 μg/μL trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate for 15 h at 37 °C. The supernatants were then collected, and the tryptic peptides were extracted from the gel sequentially with 5% (*w*/*v*) trifluoroacetic acid (TFA) at 40 °C for 1 h, and with 2.5% (*w*/*v*) TFA and 50% (*w*/*v*) ACN at 30 °C for 1 h. The extracts were pooled and dried by centrifugal lyophilization. For protein identification, peptide extracts were dissolved in 0.5% (*w*/*v*) TFA. 1 μL of peptide solution was mixed with 1 μL of matrix (4-hydroxy-α-

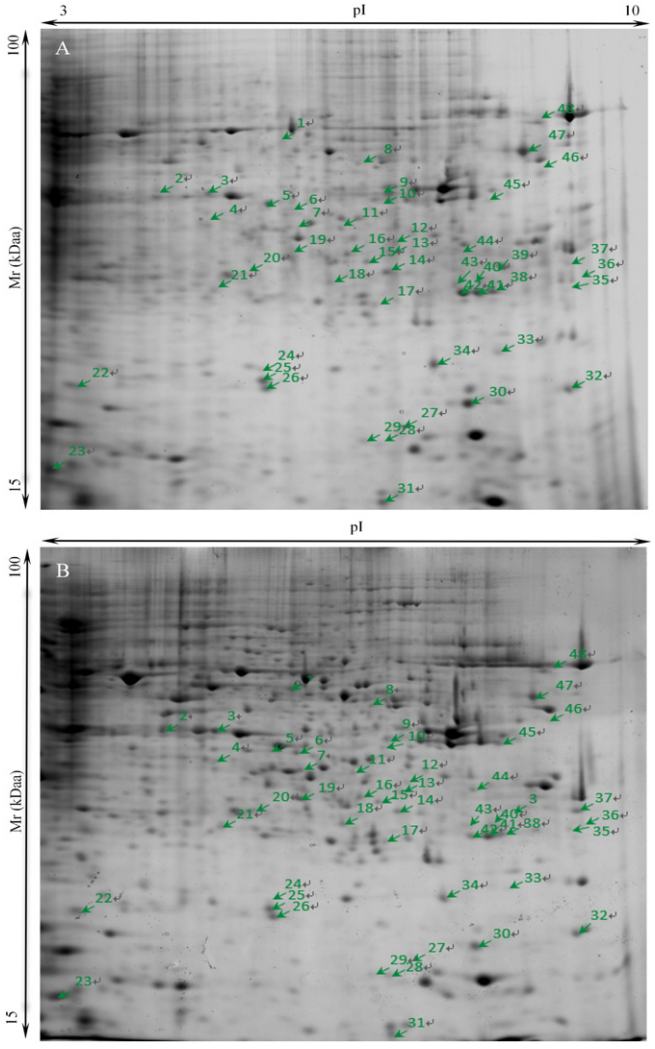

Fig. 1. 2-D PAGE analysis of proteins extracted from *R. solani* hyphal cells treated without (A) and with 100 μg/mL bacillomycin L (B). Differentially expressed proteins were subjected to MALDI-TOF mass spectrometry and tandem TOF/TOF mass spectrometry, which resulted in the identification of 48 proteins (marked with arrows).