Arch Biol Sci. 2016;68(2):319-324 DOI:10.2298/ABS150325022J

A NOVEL ANTIFUNGAL PEPTIDE PURIFIED FROM *BACILLUS SUBTILIS* STRAIN EDR4

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Received: March 25, 2015; **Revised**: December 1, 2015; **Accepted**: December 4, 2015; **Published online**: March 25, 2016

Abstract: *Bacillus subtilis* strain EDR4 is a potential biocontrol agent against *Valsa mali* (*Vm*), the fungus causing apple canker disease. In this study, we identified and characterized the antifungal peptide produced by *B. subtilis* EDR4. Peptides were isolated by 30% ammonium sulfate precipitation and purified by column chromatography. A 4-kDa peptide exhibiting antifungal activity was obtained and designated as P6. The amino acid sequence of P6 was determined by liquid chromatography combined with tandem mass spectrometry. P6 showed inhibitory effects against eight different pathogenic plant fungi, and was stable (i.e., retained its biological activity) at temperatures as high as 121°C for 20 min and at pH values ranging from 3 to 11. Microscopic examination of *Vm* hyphae treated with P6 showed protoplasm release, and *in vitro* spore germination was also inhibited. These results suggest that P6 is the active substance responsible for the antifungal activity of *B. subtilis* EDR4 against *Vm* and that P6 may be effective in the biocontrol of *Vm* as well as other plant pathogenic fungi.

Key words: *Bacillus subtilis*; purification; antifungal peptide; thermostability; antifungal activity

INTRODUCTION

Apple Valsa canker, caused by the fungus *Valsa mali* (*Vm*) [1], is a disease affecting apples, causing considerable economic losses in East Asia [2]. Current strategies for controlling *Vm* rely on chemical pesticides such as tebuconazole and diniconazole [3]. However, these pesticides have limited efficacy and cause environmental pollution; therefore, it is necessary to develop safer and more effective fungicides based on natural products.

Biocontrol is a promising alternative approach for the control of plant diseases [4,5]. Endophytes, which grow within plants without causing any detrimental symptoms of infection or disease [6,7], are effective biocontrol agents against plant pathogens. They compete with pathogens for nutrition and space, while secreting metabolites that protect the host from fungi, insects and mammals, and inducing plant resistance [8-10]. Thus, screening endophytes for their ability to produce antifungal substances can potentially yield novel biocontrol agents.

One of the most prominent groups of endophytes is the genus *Bacillus*, comprising Gram-positive bacteria. *Bacillus* spp. are widely distributed in plants and soil, and several produce diverse antimicrobial and antifungal proteins [11], including glucanase [12] and chitinase [13], as well as antifungal peptides [14] and lipopeptides [15].

A previous study found that the endophytic bacterial strain EDR4 of *Bacillus subtilis* [16] strongly inhibits a variety of fungal plant pathogens, including the wheat take-all fungal pathogen (*Gaeumannomyces graminis* var. *tritici, Ggt*). The fungicidal activity against *Ggt* was attributed to an antifungal protein E2, which was isolated by precipitation with ammonium sulfate at a relative saturation between 30% and 70%

[17]. Therefore, in this study, we sought to purify and characterize an EDR4 metabolite with antifungal activity against *Vm* and to determine its potential as a biocontrol agent against this fungus.

MATERIALS AND METHODS

Microorganisms and culture media

B. subtilis EDR4 was previously isolated from wheat roots and stored at -80°C in 25% glycerol-containing nutrient broth/yeast extract [18]. Cells were activated by culturing on nutrient agar consisting of 1% peptone, 0.5% NaCl, 0.3% beef extract and 1.4% agar for 48 h.

The plant pathogens (*Vm*, *Botryosphaeria dothidea*, *Botryosphaeria berengeriana* pv. *piricola*, *Curvularia lunata*, *Fusarium graminearum*, *Monilinia fructigena*, *Sclerotinia cepivorum* and *Ggt*) obtained from the Department of Plant Pathology of Northwest A&F University, Shaanxi, China, were grown on potato dextrose agar (PDA) consisting of 200 g potato, 20 g dextrose, 15 g agar and 1000 mL distilled water at 25°C for 7 days, and then stored at 4°C.

Preparation of crude protein extract

Activated *B. subtilis* EDR4 was cultured in a 250-ml Erlenmeyer flask in 100 ml Luria Bertani broth (5 g yeast extract, 10 g tryptone and 10 g/L solution of NaCl in water) at 28°C and 150 rpm for 48 h [17]. The culture supernatant was collected by centrifugation at 8000 rpm for 20 min at 4°C and was used for peptide isolation. The supernatant was precipitated with solid ammonium sulfate at 30% saturation, stored overnight at 4°C, and then collected by centrifugation for 20 min at 12000 rpm at 4°C. The pellet was redissolved in 10 ml of 25 mM phosphate buffer (PB; pH 7.0), dialyzed for 48 h against distilled water to remove $(NH_4)_2SO_4$, and the solution was freeze-dried to a powder.

Purification of antifungal peptide

The lyophilized crude protein extract was resuspended in 20 mM PB (pH 7.0) and passed through a Superdex peptide 10/300 GL column on an AKTA Prime system (Amersham Biosciences, Uppsala, Sweden), pre-equilibrated with 20 mM phosphate-buffered saline (PBS; pH 7.0) at a flow rate of 0.5 mL/min. The antifungal activity of the protein mixture against *Vm* was determined for each 2-mL fraction collected. The fractions exhibiting antifungal activity were pooled and applied to a Resource RPC 15 (Amersham Biosciences) column, pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) (Merck, Darmstadt, Germany) and eluted with a gradient of 0-80% (v/v) acetonitrile (Merck) containing 0.09% (v/v) trifluoroacetic acid (TFA) at a flow rate of 1.0 mL/min over 60 min. The active fractions were collected, freeze-dried and used for analysis. All purification steps were performed at room temperature, and the column effluent was monitored by measuring the absorbance at 215 nm.

Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Tricine-SDS-PAGE was carried out according to a previously published method [19]. Acrylamide and bisacrylamide compositions for each type of gel were as follows: stacking, 4% and 0.5%; spacer, 10% and 0.5%; and separating, 16.5% and 0.5%, respectively. A 15-μL protein aliquot was mixed with 5 μL of sample buffer concentrated 4-fold and heated for 5 min at 95°C. Molecular mass markers (3300-20100 Da) were from Solarbio (Beijing, China). Electrophoresis was carried out at 30 V for the stacking and 100 V for the spacer and separating gels. The gel was stained with Coomassie brilliant blue G250.

Peptide sequencing

Following tricine-SDS-PAGE, the band corresponding to the antifungal peptide was excised and sequenced by the Beijing Genomics Institute (Beijing, China).

In vitro antifungal activity assay

The inhibitory effect of strain EDR4 on *Vm* hyphal growth was examined *in vitro* with the Oxford cup

test. Briefly, 20 μg of the antifungal peptide suspension was transferred to a sterile Oxford cup placed at the center of a PDA plate. Two 40-mm diameter agar disks excised from the edge of actively growing colonies of each species of fungi were placed symmetrically at a distance of 25 mm from the plate center. The same PBS buffer without peptide was used as control. Plates were incubated at 25°C for 72 h until mycelial growth in control plates had enveloped the disks. This experiment consisted of three replicates in a completely random design. The diameter (in mm) of inhibition zones around the Oxford cups containing the samples was measured on the third day. The experiment was performed three times. *Vm* was simultaneously cultured on apple leaves after their immersion in protein solution for 5 min or PBS (pH 7.0) for control leaves. The plates were incubated at 25°C for 3 days before lesion expansion was measured. Experiments were performed in triplicate with each trial repeated three times.

Determination of the inhibition spectrum of the peptide

Antifungal activity of the peptide against *Vm*, *Botryosphaeria dothidea*, *Botryosphaeria berengeriana* pv. *Piricola*, *Curvularia lunata*, *Ggt*, *Curvularia lunata*, *Monilinia fructigena*, and *Sclerotinia cepivorum* was assessed using the Oxford cup test described above.

Measurement of the effects of temperature and pH on peptide stability and activity

Thermal stability was evaluated by incubating the supernatant in 20 mM PB at 20°C, 40°C, 60°C, 80°C, 100°C and 121°C for 20 min before analyzing residual inhibitory activity. Similarly, pH stability was determined in the range of pH 3.0-12.0, using 1 M NaOH or HCl. Antifungal activity after incubation at different temperature and pH values was measured with the Oxford cup method. Samples treated with PBS (pH 7.0) were used as controls. Three replicates of each treatment were carried out, and each trial was repeated three times.

Testing the effect of antifungal peptide on Vm hyphal morphology

Vm mycelia grown on 50-mm diameter agar disks were placed at the center of PDA culture plates. EDR4 cells were transferred to the same plates at a distance of 25 mm from the center, and the plates were incubated at 25°C for 3 days. Each treatment was repeated three times. For scanning electron microscopy (SEM), 1-cm^2 samples of mycelia at the periphery close to the Oxford cups were removed and processed as previously described [20].

RESULTS

Purification and identification of antifungal peptides

The fermentation supernatant of *B. subtilis* strain EDR4 was precipitated with ammonium sulfate at a relative saturation of 30%. Chromatographic analysis of the crude protein revealed two adsorbed fractions (Fig. 1A-A); of these, peak A1 showed antifungal activity. Chromatographic separation of the peptide corresponding to peak A1 yielded six fractions, P1-P6 (Fig. 1A-B), of which one (P6) had anti-fungal activity (Fig. 1B). Tricine-SDS-PAGE analysis of P6 revealed a single band with a relative molecular mass of about 4 kDa (Fig. 1C), and a BLAST search of the sequence yielded seven potential matches: ABC1-like protein, iturin A synthetase B, ATP synthase subunit alpha, DNA primase, protein YvaM and two hypothetical proteins (Table 1), one of which had a mass of 4.6 kDa.

Antifungal activity of the isolated peptide

Purified P6 suppressed the growth of *Vm* on apple leaves and significantly reduced lesion diameter compared to control leaves treated with PBS (Fig. 1D). The peptide had broad-spectrum antifungal activity against eight pathogenic plant fungi (Table 2), with inhibition zone diameters ranging from 13.42 to 25.74 mm.

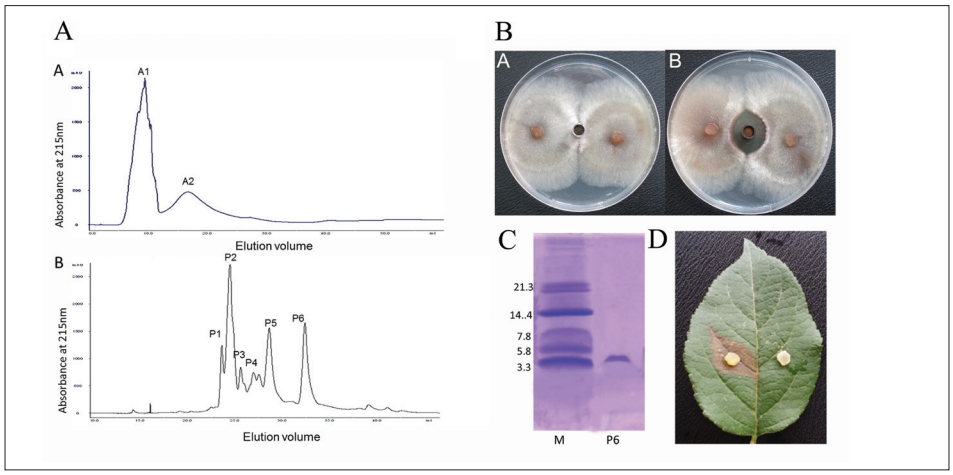

Fig. 1. Purification and identification of antifungal peptide from *B. subtilis* EDR4 by gel filtration (A); antifungal activity test (B); tricine-SDS-PAGE (C); antifungal activity on leaves (D). A – purification of antifungal protein from EDR4 by chromatography; gel permeation elution profile from a Superdex peptide column. B – reverse chromatography of fraction A1 on a Resource RPC 15 column; the antifungal activity test. A: CK, B: peak P6. C – tricine-SDS-PAGE analysis of antifungal peptide P6 isolated from *B. subtilis* EDR4; left lane: ultra-low molecular weight protein marker; right lane: 4-kDa band corresponding to peptide P6. D – lesions on apple leaves caused by *Vm* with or without the addition of P6 peptide isolated from *B. subtilis* EDR4; left: Lesions on PBS-treated control leaves 3 days after inoculation; right: lesions on leaves treated with P6 at the time of inoculation were smaller than those of control leaves.

Effect of temperature and pH on antifungal activity

The peptide P6 retained high antifungal activity after incubation in 20°C, 40°C, 60°C, 80°C, 100°C and 121°C for 30 min. The antifungal activity still had 97.8% antifungal activity after 20-min incubation at temperatures up to 121°C (Fig. 2A). This result showed that the antifungal peptide P6 from strain EDR4 possessed good thermal stability. The antifungal activity of the peptide was highest at pH 7.0 and decreased at lower and higher pH values (Fig. 2B); for instance, a 24.41% decline was observed at pH 3.0. This result indicates that the peptide could tolerate a wide pH range.

Table 1. Results of BLAST search of P6 peptide sequence.

Protein ID†	Molecular function	Protein mass (kDa)	Coverage
gi|407961210	ABC1-like	77.20	1.03
gi|321315478	Hypothetical protein	4.60	19.05
gi|16040971	Iturin A synthetase B	610.70	0.13
gi|16080736	ATP synthase subunit alpha	54.60	1.39
gi|407961821	DNA primase	73.0	1.10
gi|350267581	Protein YvaM	29.40	3.12
gi|407958092	Hypothetical protein	210.50	0.67

†All proteins were from *B. subtilis*.

Table 2. Test of antifungal activity of peptide P6 isolated from Bacillus subtilis EDR4

Plant pathogens	Inhibition zone (mm)†
Valsa mali	12.88 ± 0.49
Botryosphaeria dothidea	11.02 ± 0.86
Botryosphaeria berengeriana pv. *piricola*	10.57 ± 0.99
*Curvularia luna*ta	10.38 ± 0.39
Gaeumannomyces graminis f. sp. *tritici*	9.71 ± 0.57
Fusarium graminearum	9.50 ± 0.42
Monilinia fructigena	8.72 ± 0.32
Sclerotinia cepivorum	6.68 ± 0.33

†Inhibition zone diameter was measured on inoculated plates incubated in the dark at 25°C for 3 days; 20 μg of peptide was used in each Oxford cup.

Effect of antifungal peptide on *Vm* hyphal morphology

SEM studies revealed severe morphological alterations in *Vm* hyphae treated with peptide P6 relative to untreated hyphae (Fig. 3A). The morphological alterations included abnormal hyphal growth and swollen, shriveled tips (Fig. 3B), cytoplasmic leakage (Fig. 3C) and rupture, which caused them to appear as beads on a string (Fig. 3D).

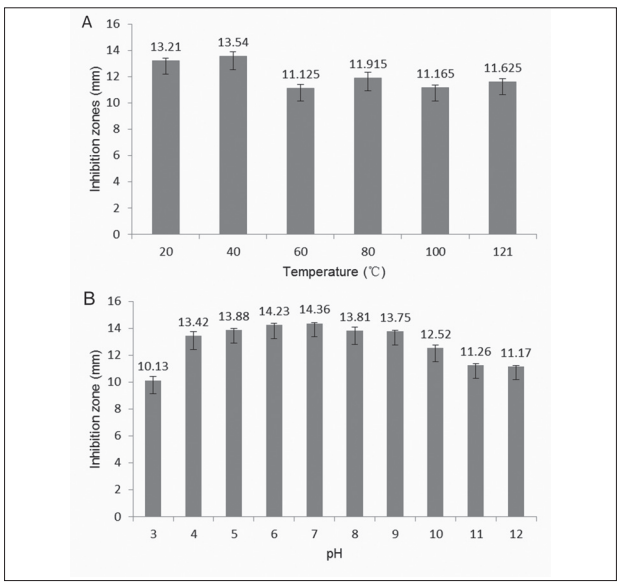

Fig. 2. Effect of temperature and pH on antifungal activity of peptide P6. Zones of *Vm* mycelium growth inhibition by P6 treatment were measured at the indicated temperatures (A) and pH (B).

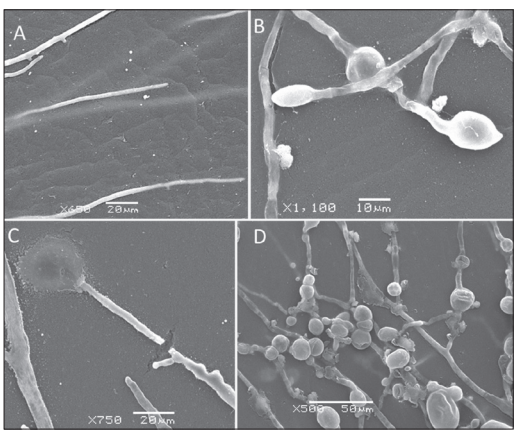

Fig. 3. Effects of *B. subtilis* EDR4 peptide P6 on *Vm* hyphae morphology as observed by scanning electron microscopy. A – untreated hyphae; B-D – hyphae treated with P6 had swollen or shriveled tips (B), displayed cytoplasmic leakage (C), or were ruptured with a beads-on-a-string structure surrounded by exudates (D).

DISCUSSION

Bacillus spp. act as biocontrol agents against plant pathogens by producing metabolites with antimicrobial activity, including peptides with antifungal properties [21,22]. In this study, we purified and characterized an EDR4 metabolite from *B. subtilis* and found that EDR4 potently inhibited *Vm* growth. This is consistent with the findings of previous studies that found that *B. subtilis* EDR4 inhibits a variety of fungal plant pathogens, including *Sclerotinia sclerotiorum* on greenhouse-cultivated rapeseed [23] and *Ggt*, both *in vitro* and *in vivo* [17].

The antifungal effect was attributed to a novel peptide P6 that shared similarities with other promising biocontrol peptides. For instance, P6 markedly decreased conidial germination, an effect similar to that exerted by the *B. subtilis* strain 29 protein B29I [24]. Furthermore, P6 remained stable at 121°C and was active in the pH range 3-12, consistent with the characteristics of other antifungal peptides such as *B. subtilis* peptide BCP61[25], a novel thermolabile heterodimeric ribonuclease [26], subpeptin JM4-A and subpeptin JM4-B. Together, these results suggest that P6, along with other similar peptides, can be useful biocontrol agents because these peptides would be effective against a broad spectrum of fungal species owing to their collective stability across a range of temperatures and pH values.

P6 showed the highest sequence identity with a hypothetical peptide (Protein ID: gi|321315478), a 4-kDa peptide for which antifungal activity has not yet been reported in *Bacillus* species. Thus, P6 is likely a novel antifungal peptide. Future studies should focus on cloning the gene and a detailed sequence analysis should be conducted to elucidate its function and potential uses.

Acknowledgments: This research was supported by the National Natural Science Foundation of China (NO. 31171796), Science and Technology Innovation Research of Shaanxi (No. 2011KTZB02-02-02), Special Fund for Agro-scientific Research in the Public Interest of China (201203034) and the 111 Project (B07049).

Authors' contributions: Lili HUANG and Zhensheng KANG conceived and designed the study. Jingyuan JI performed the experiments and wrote the paper. Jie YANG performed some of the experiments. Lili HUANG reviewed and edited the manuscript. All authors read and approved the manuscript.

Conflict of interest disclosure: There is no conflict of interest.

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