



Identification of Cyt2Ba from a New Strain of *Bacillus thuringiensis* and Its Toxicity in *Bradysia difformis*

Fan-Fan Wang¹ · Shao-Xuan Qu^{1,2} · Jin-Sheng Lin¹ · Hui-Ping Li¹ · Li-Juan Hou¹ · Ning Jiang¹ · Xin Luo¹ · Lin Ma¹

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Abstract

Bradysia difformis is one of the most damaging pests in mushroom production in China. In this study, eight *Bacillus thuringiensis* strains were analyzed for insecticidal activity in *B. difformis*. The strain JW-1 showed the highest insecticidal activity against *B. difformis* larvae, but did not inhibit the mycelial growth of *Pleurotus ostreatus* and *P. geesteranus*. The 16S rRNA gene (1397 bp) and *cyt2* gene (792 bp) were obtained from strain JW-1. The phylogenetic tree based on 16S rRNA gene and Cyt2 toxin showed that strain JW-1 was a member of *B. thuringiensis* and Cyt2 toxin belonged to Cyt2Ba toxin cluster. The Cyt2Ba toxin from strain JW-1 was overexpressed in *E. coli* as a fusion protein and the fusion protein (70 kDa) was purified by Ni-IDA affinity chromatography. The purified Cyt2Ba fusion protein was toxic to *B. difformis* larvae (LC₅₀ was 2.25 ng/mL). The identification of Cyt2Ba from strain JW-1 and confirmation of the insecticidal activity of Cyt2Ba in *B. difformis* provided a new means of biological control of the important pest in mushroom production.

Introduction

Bradysia difformis, belonging to the Sciaridae family, is one of the most destructive greenhouse pests, primarily affecting edible mushroom cultivation [1]. Adult female flies lay eggs on the surface of the growing mushrooms, and the larvae mostly eat hyphae and fruiting bodies, directly damaging the mushroom produce [2]. Adult flies also spread fungal spores and mites when flying over mushrooms, which indirectly leads to lower yields [3]. *Bradysia* spp. can be controlled by traditional chemical agents because of their simplicity, effectiveness, and speed [4]. However, resistance and residual insecticide pose major threats to the mushroom industry [5, 6]. The current trend is to shift to integrated and eco-friendly pest-management, involving microorganisms with insecticidal activity, such as *Bacillus thuringiensis* [7, 8].

Bacillus thuringiensis (Bt) is a gram-positive, endospore-forming, ubiquitous soil bacterium that produces parasporal crystals during the sporulation phase of its growth cycle

[9, 10]. These parasporal crystals contain different types of proteins with insecticidal activity, the insecticidal crystal proteins (ICP) [11]. Different ICPs possess highly specific insecticidal activities against numerous target insects, such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, as well as some invertebrates such as Nematelminthes and Platyhelminthes [12–14]. The insecticidal specificity of *B. thuringiensis* strains is determined by *cry* and *cyt* genes. These genes code for insecticidal crystal (Cry) protein of 65–145 kDa and cytolytic (Cyt) proteins of 25–28 kDa, which exert cytolytic activity against a broad spectrum of eukaryotic cells [15, 16]. Approximately 825 *cry* and *cyt* genes have been discovered (https://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

Compared to Cry toxins, Cyt toxins show insecticidal activity only in Diptera and may suppress the resistance to Cry toxins [17–19]. Cyt toxins are composed of a single α - β domain and are classified into Cyt1, Cyt2, and Cyt3 [20, 21]. The size of Cyt2 (about 29 kDa) is slightly larger than that of Cyt1 (about 27 kDa) and contains an additional 15-residue sequence at the C-terminus [22]. Furthermore, Cyt2 toxin is readily expressed and forms cytoplasmic inclusions in bacteria without the need for a “helper” protein-like Cyt1 [23]. Cyt2 toxins act synergistically with Cry4Aa, Cry4Ba, and Cry11Aa [24, 25]. Proteolytically activated Cyt2Ba exhibits higher toxicity against *Anopheles*, *Culex*, and *Aedes* larvae than Cyt1Ab from *B. thuringiensis* subsp. *medellin* [26].

✉ Lin Ma
malin1590@sina.com

¹ Vegetable Research Institute, Jiangsu Key Laboratory for Horticultural Crop Genetic Improvement, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China

² Institute of Life Science, Jiangsu University, Zhenjiang 210023, China

JW-1 is a Bt strain with insecticidal activity against *B. difformis* and contains a *cyt2* gene. At present, there are no sufficient studies on Cyt2Ba toxin against *B. difformis* on edible mushrooms. In this study, the *cyt2* gene from JW-1 was expressed in *Escherichia coli* and purified to determine its insecticidal activity on *B. difformis* under laboratory conditions.

Materials and Methods

Bradysia difformis

Bradysia difformis eggs were initially collected from a mushroom greenhouse in Jiangsu Province, China, in 2014. The larvae were fed mushroom mycelia and placed in plastic boxes in the laboratory. Adult flies oviposited on the surface of mycelium. The third instar larvae were used in insecticidal activity assays. All experiments were conducted at 25 ± 1 °C and relative humidity at $65 \pm 5\%$.

Bacterial Strains, Plasmid, and Culture Conditions

Eight Bt strains were isolated from the soil collected from the Purple Mountain of Nanjing City, Jiangsu Province, China (GPS locality: N118° 86' 16.94"; E32° 07' 44.11"). The strain *B. thuringiensis* var. *israelensis* (Bti) was isolated from the Bti wettable powder from Wuhan Kernel Bio-tech Co., Ltd. (Wuhan, China). For assessment of insecticidal activity, Bt strains were grown in beef extract-peptone-glucose broth (BPG medium; beef extract: 3 g/L, peptone: 5 g/L, and glucose: 10 g/L) for 72 h at 30 °C, 220 rpm. *E. coli* were cultured in Luria–Bertani (LB) broth at 37 °C, 220 rpm. The plasmids and bacterial strains used in this study are listed in Table 1.

Insecticidal Activity Assay

Insecticidal toxicity of the eight Bt strains and their Cyt2 toxins were analyzed in third instar larvae of *B. difformis* by larval feeding assay [27]. Single colonies of the eight Bt strains were individually inoculated into 5 mL of BPG broth overnight at 30 °C, 200 rpm, and 0.5 mL of this culture solution was then transferred to 50 mL of BPG broth and inoculated again for 72 h using the same culture conditions. The liquid cultures of the strains were normalized to 0.7 at OD₆₀₀ using the BPG broth. Three fresh pieces (diameter of 1 cm) of fruiting *A. polytricha*, soaked in Bt strain normalized broth (2 mL) or Cyt2 toxin diluent (2 mL) were placed on a 9-cm plate. One piece of the filter paper (Sangon Biotech, Shanghai, China), wetted by 0.8 mL of same liquid as used for *A. polytricha*, was laid on the bottom of the plate. Thirty larvae were transferred into every plate and kept at 25 ± 1 °C and $65 \pm 5\%$ relative humidity for 72 h. All assays were performed in triplicates. BPG broth was used as a control, and the Bti strain was used as a positive control. Mortality and correct mortality were calculated [27], and statistical analysis was performed [28].

Antifungal Activity Assay

The effects of Bt strains that showed the highest insecticidal activity against *B. difformis* on the hyphal growth of *Pleurotus ostreatus* and *P. geesteranus* were determined by the confrontation culture method [29]. A PDA disc (5 mm in diameter) containing the mycelia above was placed onto the center of each 9-cm plate. A 5- μ L aliquot of Bt strain fermentation broth was immediately spotted onto the same plate equidistant from the edge of the fungal disc. Bti fermentation broth was used as a control. Three replicates of plate bioassays

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
Bt strains		
JW-1, 51-4, 68-6, 69-1, 69-3, 88-1, 99-3, 214-4	Wild-type strains with insecticidal activity	This study
Bti	Purified from <i>Bacillus thuringiensis</i> subsp. <i>israelensis</i> wettable powder	Wuhan Kernel
Edible fungus strains	<i>Pleurotus ostreatus</i> , <i>Pleurotus geesteranus</i> , <i>Auricularia polytricha</i>	This study
<i>Escherichia coli</i>		
TOP10	F- <i>mcrA</i> Δ (<i>mrr</i> - <i>hsd</i> RMS- <i>mcr</i> BC) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX</i> 74 <i>recA1</i> <i>ara</i> Δ 139(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> rps (Strr) endA1 <i>nupG</i>	Zoonbio
Arctic	F- <i>ompT</i> <i>hsdS</i> (rB- mB-) <i>dcm</i> + Tetr <i>gal</i> λ (DE3) end A Hte [cpn10 cpn60 Gentr] [argU proL Strr]	
Plasmids		
PMAL-c5x	Expression vector for <i>cyt2</i> , MBP, Ap ^r	Zoonbio
pCYT-2	pMAL-c5x carrying 792-bp PCR product of the <i>cyt2</i> , Ap ^r	This study

were performed independently. The inoculated plates were incubated in the dark for one week at 25 ± 1 °C.

16S rRNA Gene and *cyt2* Gene Analysis

The single colony of Bt strain JW-1 was incubated in 5 mL of LB broth overnight at 30 °C, 200 rpm. The bacterial cells were resuspended in 600 µL sorbitol buffer (1.2 M with 0.1 M phosphate-buffered saline (PBS) and 5 µL lysozyme (0.05 g/mL) and kept at 30 °C overnight in the water bath. Total DNA of JW-1 was extracted using the Bacterial Genome Extraction Kit (TsingKe, Nanjing, China).

The 16S rRNA gene of the strain JW-1 was amplified using the universal primers 27F and 1492R [30]. According to the complete *cyt2Ba* gene of strain HD-567 (GenBank accession no. GQ919039.1), primers JW-1-*cyt2F/R* (JW-1-*cyt2F*: 5'-CCATATGGATGCACCTTAATAATTTGAA-3'; JW-1-*cyt2R*: 5'-CCCGCTTGTTACGATTTTATTGGA TTAACAT-3') were designed using the Primer Premier 5 software [31] and were used to amplify the full *cyt2* gene of JW-1. Amplification was carried out with I-5™ Hi-Fi DNA Polymerase (TsingKe Company). The cycling program for PCR of the 16S rRNA gene included 35 cycles of 98 °C for 10 s, 50 °C for 10 s, and 72 °C for 15 s and the cycling program for PCR of the *cyt2* gene included 35 cycles of 98 °C for 10 s, 50 °C for 10 s, and 72 °C for 10 s. Sequencing reads of 16S rRNA and *cyt2* were assembled by DNASTAR Lasergene package version 7.1.0, and the DNA nucleotide sequences of 16S rRNA and *cyt2* were deposited in the GenBank database under the Accession Numbers (MN539648.1 and MN539642.1). Phylogenetic trees were constructed based on 16S rRNA gene and Cyt2 toxin sequences of JW-1 using MEGA 6.0 software [32]. The evolutionary history was inferred by using the Maximum Likelihood method and a bootstrap analysis of 1000 replications was performed [33].

Expression Vector Construction

To investigate the insecticidal activity of the Cyt2 toxin from JW-1, the Cyt2 protein was expressed in *E. coli*, by inserting the blunt-end PCR product (complete sequence of the 792 bp *cyt2* gene) into the multiple cloning site of pMAL-c5X, following manufacturer's instructions, to produce the plasmid pCYT-2. pCYT-2 was then transformed into *E. coli* TOP10 competent cells, and the transformants were identified on LB agar containing ampicillin (100 µg/mL). The *cyt2* gene was amplified from the *E. coli* colonies with the primers JW-1-*cyt2F/R* to confirm the presence of the plasmid.

The expression of Cyt2 in *E. coli* with pCYT-2 was induced by addition of 0.5 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to the culture and incubation at 20 °C overnight, as described previously [34]. Following this, 1 mL of the induced bacterial solution was

centrifuged at 10,000 rpm for 2 min, and the cell pellets were suspended in 100 µL of × 1 loading buffer (SDS: 20 mg/mL, bromophenol blue: 0.1 mg/mL, 1 M Tris-HCl, pH6.8: 25 µL/mL, glycerinum: 0.1 mL/mL, and adding 5 µL/mL of β-mercaptoethanol before using). The solution was centrifuged at 4000 rpm for 10 min, and the cell pellets were resuspended in PBS. After disrupting the suspension by ultrasonication, the proteins in the supernatant and the precipitate were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [35].

Purification and Western Blot Analysis of Cyt2 Fusion Protein

The supernatant from the Cyt2 preparation from above was loaded onto a Ni-IDA Sepharose CL-6B affinity chromatography column pre-equilibrated with Ni-IDA binding buffer at 0.5 mL/min. The column was washed by Ni-IDA binding buffer at 0.5 mL/min and Ni-IDA washing buffer (20 mM Tris-HCl, pH 8.0, containing 20 mM imidazole and 150 mM NaCl) at 1 mL/min until the OD₂₈₀ value of the effluent reached the baseline. The column was then eluted with Ni-IDA elution buffer (20 mM Tris-HCl, pH 8.0, containing 250 mM imidazole and 150 mM NaCl) at 1 mL/min to obtain the target protein. The collected protein solution was dialyzed overnight against tris-buffered saline (TBS) (20 mM Tris-HCl, 150 mM NaCl, pH 8.0). The dialysate was analyzed by 12% SDS-PAGE.

Western blot analysis was performed as previously described [36]. The purified recombinant Cyt2 toxin was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane, and then the PVDF membrane was washed four times by PBS-Tween 20 (PBST, 0.1% Tween 20) buffer and incubated with His-tag primary antibody (Zoonbio Biotechnology, Nanjing, China) diluted by 5% skim milk blocking buffer (1:1000) at 4 °C overnight. After washing three times with PBST buffer, the membrane was incubated with goat anti-mouse IgG-conjugated alkaline phosphatase (Zoonbio Biotechnology, Nanjing, China) prepared in 5% skim milk blocking buffer (1:5000) at 37 °C for 1 h. The membrane was then washed four times with PBST buffer. Images were developed by enhanced chemiluminescence (ECL). Protein concentrations were determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) on samples solubilized as previously described.

Results

Determination of Toxicity and Inhibitory Activity

Eight laboratory Bt strains that produced parasporal crystals were used to determine the virulence on the larvae

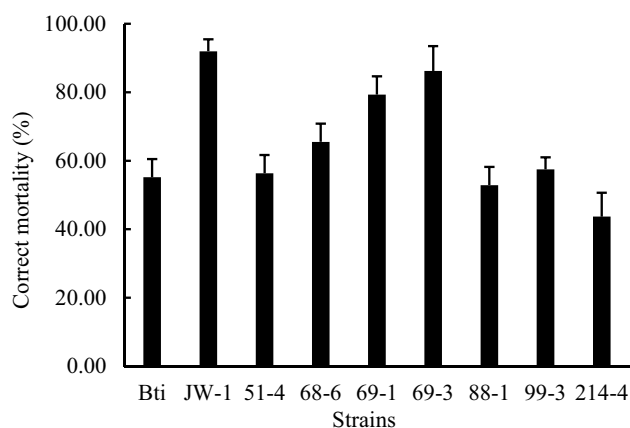


Fig. 1 The toxicity of Bt strains in *B. difformis* larvae. Insecticidal toxicity of the eight Bt strains and their Cyt2 toxins were analyzed in third instar larvae of *B. difformis* by larval feeding assay. Bti strain was used as a positive control

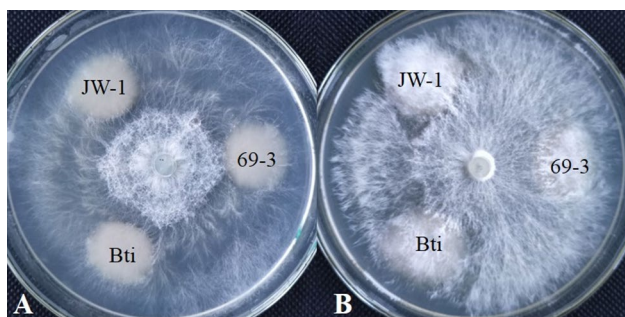


Fig. 2 Antifungal activities of Bt strains against hyphae of *P. ostreatus* (a) and *P. geesteranus* (b). The three kinds of Bt strains (5 μ l aliquot) was inoculated onto the PDA plate simultaneously with *P. ostreatus* (a) and *P. geesteranus* (b) (5 mm PDA discs with mycelia), then incubated in the dark for one week at $25 \pm 1^\circ\text{C}$

(Fig. 1). After 72 h, the corrected mortality of *B. difformis* larvae treated with all bacterial strains was above 50% except for strain 214-4. Strains JW-1 and 69-3 showed the highest insecticidal activity, and the corrected mortality was $91.96 \pm 3.49\%$ and $86.21 \pm 7.26\%$, respectively. After treatment with Bt for 72 h, most larvae became darkened or started to disintegrate.

Strains JW-1 and 69-3 were tested for their antifungal activity against hyphae of *P. ostreatus* and *P. geesteranus*. The hyphae of the two fungi grew normally on the plates without showing inhibition by the bacteria. Therefore, we identified that the three Bt strains (JW-1, 69-3, Bti) did not inhibit mycelial growth (Fig. 2). Based on the results from the insecticidal and antifungal activity assays, the strain JW-1 was selected as the candidate strain for further research.

Amplification of 16s rRNA and cyt2 genes

The nearly full-length 16S rRNA gene sequence (1397 bp) of strain JW-1 shared highest homologous to *Bacillus* species archived in GenBank. The phylogenetic tree based on 16S rRNA gene showed that strain JW-1 was clustered together with *B. thuringiensis* (Fig. 3).

A 792 bp fragment was obtained from PCR amplification of the *cyt2* gene from the total DNA of Bt strain JW-1 (Fig. 4a). The Cyt2 toxin sequence (GenBank accession no. QGM12370.1) exhibited 99% identity to the reported Cyt2Ba toxin, confirming that this protein is a member of Cyt2Ba in the Cyt2 family (Fig. 5).

Construction of Expression Vector

The entire *cyt2Ba* (792 bp) gene was inserted into pMAL-c5x by seamless cloning. *Pvu*I was used for double enzyme digestion, confirming the full-length of pCYT-2 construct. The result of digestion was 2.4 kb (0.8 kb *cyt2Ba* and 1.6 kb located at vector) and 4.0 kb vector (Fig. 4b).

Induced Expression

Escherichia coli Arctic culture (pCYT-2) was incubated with IPTG in LB medium. The toxin Cyt2Ba (30 kDa) was expressed as a fusion protein with a 40 kDa maltose-binding protein (MBP) tag. The 70 kDa recombinant product was visualized in SDS-PAGE, which validated the expression of Cyt2Ba toxin (Fig. 6a). Due to the protein structure, the protein appeared to be smaller than the calculated molecular weight. The results of SDS-PAGE indicated that Cyt2 was mainly present in the supernatant and expressed in soluble form, which could be directly purified by affinity chromatography.

Purification of the Fusion Protein and Western Blot Verification

After the fusion protein was purified by Ni column affinity chromatography, the initial bacterial homogenate, effluent, and eluate were separately subjected to 12% SDS-PAGE analysis. The result revealed that a relatively pure target protein fraction was obtained in the eluted fractions (Fig. 6b). After the purified protein was subjected to Western blotting (Fig. 7), the fusion protein was solubilized on the Western blot.

Insecticidal Activity Assay of Cyt2Ba Toxin

Bioassays were performed against the third instar larvae of *B. difformis*. After 72 h of treatment, the mortality caused by different doses of Cyt2Ba toxin showed a linear

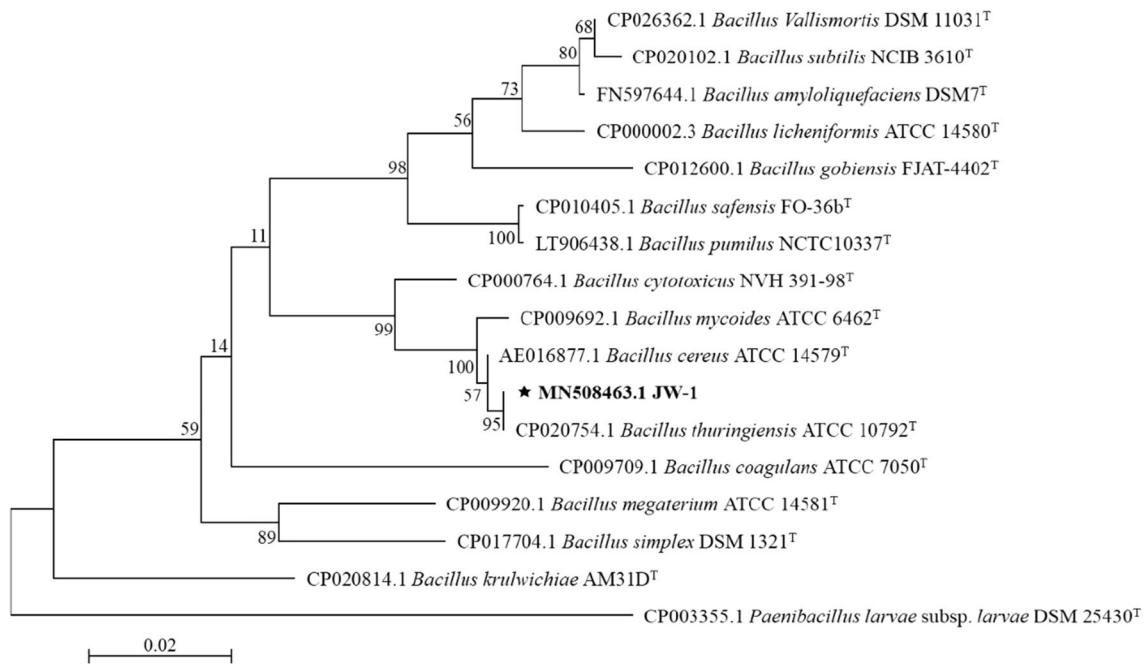


Fig. 3 Phylogenetic tree based on 16S rRNA gene sequences. The dendrograms were generated using the maximum likelihood method and gene distances were calculated using the Jukes–Cantor model

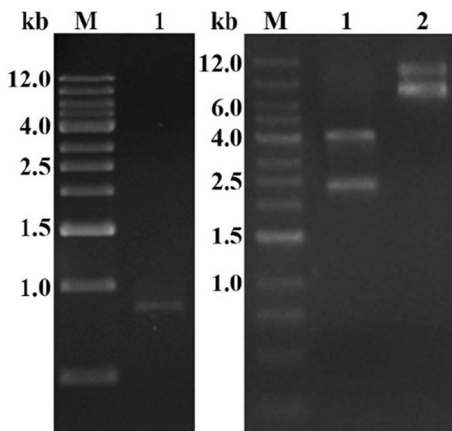


Fig. 4 The electrophoresis result of *cyt2* and the verification of pCYT-2. **a** PCR confirmation of full-length *cyt2* gene around 0.8 kb in the JW-1 (lane 1), the primers JW-2F and JW-2R were used to amplify the *cyt2* gene. **b** pCYT-2 plasmid was digested with *PvuI* around 2.4 kb and 4.0 kb (lane 1), the pCYT-2 plasmid around 6.4 kb (lane 2)

relationship between the log dose and probe mortality. The regression equation of Cyt2Ba toxin was $y = 4.49 + 1.45x$ (x means the logarithm value of concentration; y means the probability value of mortality). The LC_{50} value was 2.25 ng/mL, where 95% fiducial limit for LC_{50} was between 1.44–3.50 ng/mL and the R value was 0.9433.

Discussion

In China, the mushroom cultivation scales range from technologically assisted industrialized production to conventional field production. Because of the short reproduction cycle and the high resistance developed to pesticides, *B. difformis* damages are more severe in simple mushroom cultivation settings. Heat stress and some biological control microorganisms, such as *Beauveria bassiana* and entomopathogenic nematodes (EPNs), showed the control efficacies against *B. difformis* in soil or spawned mushroom compost [37–39]. However, these methods are not effective for controlling *B. difformis* in simple mushroom cultivation in China, because of the difficulties in adequate control of temperature, potting media, and application dose [40]. In contrary Bt strains have already been used in production of mushrooms to control *B. difformis* and are readily accessible to growers due to its broad applicability.

Bt is an effective agent to control mosquitoes due to production of multiple toxins with different modes of action [41]. Cyt toxins in Bt synergize with the toxic effect of Cry toxins. Cyt toxins have insecticidal activity and function as a receptor to synergize the Cry11 toxicity [42, 43]. Besides, at least two Cyt toxins are present in the Bt strains to control the Dipteran insects, such as *B. thuringiensis* subsp. *israelensis* and subsp. *morrisoni* [19].

In this study, the strain JW-1, as a member of *B. thuringiensis*, showed the obvious insecticidal activity against *B. difformis* larvae and did not affect the hyphal growth of

Fig. 5 Phylogenetic tree based on Cyt2 toxin sequences. The gene distances were calculated using the Jones–Taylor–Thorn-ton (JTT) model and dendro-grams were generated using the maximum likelihood method. Initial tree for the heuristic search were obtained auto-matically by applying Neighbor Joining

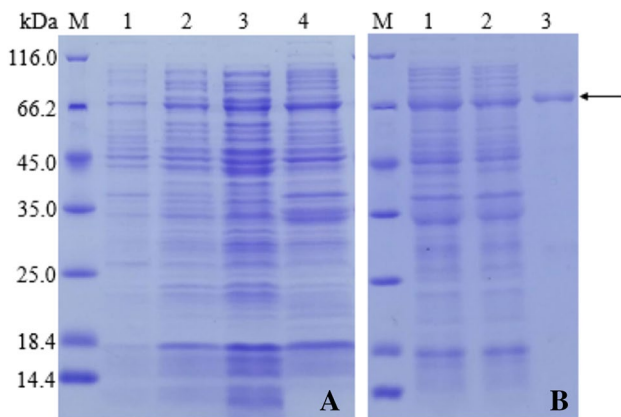
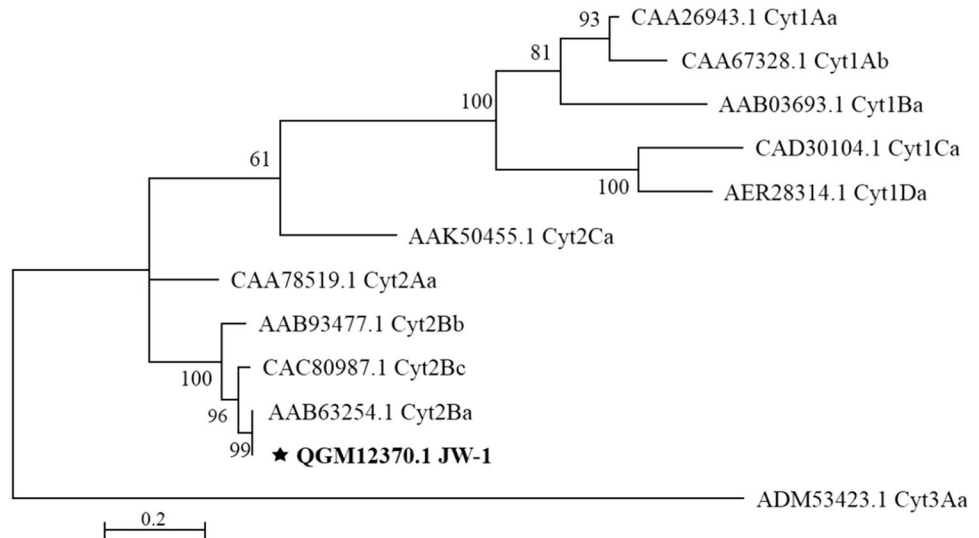


Fig. 6 SDS-PAGE analysis of expression (a) and fusion protein purification (b). **a** Protein expression before induction (lane 1), protein expression after induction (lane 2), the supernatant of induced fragmentation (lane 3), precipitation of induced fragmentation (lane 4). **b** Sample of pulverization (lane 1), effluent sample (lane 2), elution sample (lane 3)

mushroom. Cyt2Ba, composed of 263 amino acids, is a minor component within the complex of the crystal proteins expressed in the Bt strain [44]. The toxicity of the recombinant Cyt2Ba toxin from strain JW-1 in *B. difformis* larvae was confirmed by biological assay. The larvicidal activity of recombinant Bt toxin Cyt2Ba against mosquito larvae, such as *Aedes albopictus* and *Culex pipiens quinquefasciatus*, has been reported [45], but there are few research for mushroom pest control. The results from this study showed that *B. difformis* damage in mushroom cultivation could be controlled by this Bt toxin as an alternative to minimize the use of chemical insecticides. Further work on the biological activity of Cyt2 toxin and its combination with other Cyt and Cry toxins will help in our understanding of the mechanisms. This knowledge may have a significant impact on biological insect resistance-management programs.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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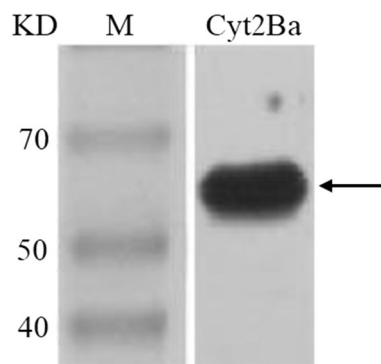


Fig. 7 Western Blot analysis of fusion protein purification

References

- Broadley A, Kauschke E, Mohrig W (2018) Black fungus gnats (Diptera: Sciaridae) found in association with cultivated plants and mushrooms in Australia, with notes on cosmopolitan pest species and biosecurity interceptions. *Zootaxa* 415(2):201–242. <https://doi.org/10.11646/zootaxa.4415.2.1>
- Ludwig SW, Oetting RD (2001) Evaluation of medium treatments for management of *Frankliniella occidentalis* (Thripidae: Thysanoptera) and *Bradysia coprophila* (Diptera: Sciaridae). *Pest Manag Sci* 57(12):1114–1118. <https://doi.org/10.1002/ps.404>
- San-Blas E, Luzardo M, Larreal J, Portillo E, Bastidas B (2017) Biological control of the fungus gnats *Bradysia difformis* (Diptera, Mycetophilidae) in mushrooms with *Heterorhabditis amazonensis* in tropical conditions. *Sci Hortic* 216:120–125. <https://doi.org/10.1016/j.scienta.2017.01.003>
- Zhang P, He M, Wei Y, Zhao Y, Mu W, Liu F (2016) Comparative soil distribution and dissipation of phoxim and thiamethoxam and their efficacy in controlling *Bradysia odoriphaga* Yang and Zhang in Chinese chive ecosystems. *Crop Protect* 90:1–8. <https://doi.org/10.1016/j.cropro.2016.07.028>
- Shamshad A (2010) The development of integrated pest management for the control of mushroom sciarid flies, *Lycoriella ingenua* (Dufour) and *Bradysia ocellaris* (Comstock), in cultivated mushrooms. *Pest Manag Sci* 66(10):1063–1074. <https://doi.org/10.1002/ps.1987>
- Shamshad A, Clift AD, Mansfield S (2008) Toxicity of six commercially formulated insecticides and biopesticides to third instar larvae of mushroom sciarid, *Lycoriella ingenua* Dufour (Diptera: Sciaridae), in New South Wales, Australia. *Austral Entomol* 47(3):256–260. <https://doi.org/10.1111/j.1440-6055.2008.00653.x>
- Cloyd RA, Dickinson A (2006) Effect of *Bacillus thuringiensis* subsp. *israelensis* and neonicotinoid insecticides on the fungus gnat *Bradysia* sp. nr *coprophila* (Lintner) (Diptera: Sciaridae). *Pest Manag Sci* 62(2):171–177. <https://doi.org/10.1002/ps.1143>
- Long SJ, Richardson PN, Willmott DM, Edmondson RN (2000) Infectivity of entomopathogenic nematodes (Steinernematidae, Heterorhabditidae) to mushroom phorid fly (*Megaselia halterata*) larvae. *Nematology* 2(4):451–459. <https://doi.org/10.1163/156854100509312>
- Jouzani GS, Valijanani E, Sharafi R (2017) *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. *Appl Microbiol Biot* 101(7):2691–2711. <https://doi.org/10.1007/s00253-017-8175-y>
- Sauka DH, Cozzi JG, Benintende GB (2005) Screening of *cry2* genes in *Bacillus thuringiensis* isolates from Argentina. *Antonie Van Leeuwenhoek* 88(2):163–165. <https://doi.org/10.1007/s10482-005-3368-2>
- Peng Q, Yu Q, Song F (2019) Expression of *cry* genes in *Bacillus thuringiensis* biotechnology. *Appl Microbiol Biot* 103(4):11617–11626. <https://doi.org/10.1007/s00253-018-9552-x>
- Frankenhuyzen VK (2013) Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. *J Invertebr Pathol* 114(1):76–85. <https://doi.org/10.1016/j.jip.2013.05.010>
- Kaur S (2000) Molecular approaches towards development of novel *Bacillus thuringiensis* biopesticides. *World J Microb Biot* 16:781–793. <https://doi.org/10.1023/A:1008931207374>
- Reyaz AL, Arulselvi PI (2016) Cloning, characterization and expression of a novel haplotype *cry2A*-type gene from *Bacillus thuringiensis* strain SWK1, native to Himalayan valley Kashmir. *J Invertebr Pathol* 136:1–6. <https://doi.org/10.1016/j.jip.2016.02.005>
- Hofte H, Whiteley HR (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev* 53(2):242–255
- Osman GEH, Already R, Assaedi ASA, Organji SR, El-Ghareeb D, Abulreesh HH, Althubiani AS (2015) Bioinsecticide *Bacillus thuringiensis* a comprehensive review. *Egypt J Biol Pest Control* 25(1):271–288
- Cantón PE, Reyes EZ, De Escudero IR, Bravo A, Soberón M (2011) Binding of *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba to Cyt1Aa has an important role in synergism. *Peptides* 32(3):595–600. <https://doi.org/10.1016/j.peptides.2010.06.005>
- Khasdan V, Ben-Dov E, Manasherob R, Boussiba S, Zaritsky A (2001) Toxicity and synergism in transgenic *Escherichia coli* expressing four genes from *Bacillus thuringiensis* subsp. *israelensis*. *Environ Microbiol* 3(12):798–806. <https://doi.org/10.1046/j.1462-2920.2001.00253.x>
- Soberón M, López-Díaz JA, Bravo A (2013) Cyt toxins produced by *Bacillus thuringiensis*: a protein fold conserved in several pathogenic microorganisms. *Peptides* 41:87–93. <https://doi.org/10.1016/j.peptides.2012.05.023>
- Ben-Dov E (2014) *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins* 6(4):1222–1243. <https://doi.org/10.3390/toxins6041222>
- Palma L, Munoz D, Berry C, Murillo J, Caballero P (2014) *Bacillus thuringiensis* toxins: an overview of their biocidal activity. *Toxins* 6(12):3296–3325. <https://doi.org/10.3390/toxin6123296>
- Koni PA, Ellar DJ (1993) Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic delta-endotoxin. *J Mol Biol* 229(2):327. <https://doi.org/10.1006/jmbi.1993.1037>
- Yu J, Xu W, Zeng S, Zhang XQ, Liu JY, Xie RY, Pang Y (2002) Cloning and expression of *cyt2Ba7* gene from a soil-isolated *Bacillus thuringiensis*. *Curr Microbiol* 45(5):309–314. <https://doi.org/10.1007/s00284-002-3757-y>
- Nisnevitch M, Cohen S, Ben-Dov E, Zaritsky A, Sofer Y, Cahan R (2006) Cyt2Ba of *Bacillus thuringiensis israelensis*: activation by putative endogenous protease. *Biochem Biophys Res* 344(1):99–105. <https://doi.org/10.1016/j.bbrc.2006.03.134>
- Wu Y, Gao M, Dai S, Yi D, Fan HY (2008) Investigation of the *cyt* gene in *Bacillus thuringiensis* and the biological activities of Bt isolates from the soil of China. *Biol Control* 47(3):335–339. <https://doi.org/10.1016/j.biocontrol.2008.08.020>
- Wirth MC, Delecluse A, Walton WE (2001) Cyt1Ab1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and *B. thuringiensis* subsp. *israelensis* synergize *Bacillus sphaericus* against *Aedes aegypti* and resistant *Culex quinquefasciatus* (Diptera: Culicidae). *Appl Environ Microb* 67(7):3280–3284. <https://doi.org/10.1128/Aem.67.7.3280-3284.2001>
- Wang FF, Qu SX, Lin JS, Li HP, Hou LJ, Jiang N, Luo X, Ma L, Han JC (2019) Screening of *Bacillus thuringiensis* and identification of insecticidal crystal protein gene against *Bradysia difformis* in mushroom cultivation. *J Zhejiang Univ* 45(2):189–195. <https://doi.org/10.3785/j.issn.1008-9209.2018.08.141>
- Finney D (1971) *Probit Analysis*. Cambridge University Press, Cambridge, pp 50–80
- Ma L, Wang XQ, Deng P, Baird SM, Liu YZ, Qu SX, Lu S-E (2017) The *pafr* gene is required for antifungal activity of strain MS82 against *Mycogone perniciosa*. *Adv Microbiol* 7:217–230. <https://doi.org/10.4236/aim.2017.74018>
- Chen Y, Gao H, Zhang Y, Deng M, Wu Z, Zhu L, Duan Q, Xu B, Liang C, Yue Z, Xiao X (2012) Analysis of the bacterial diversity existing on animal hide and wool: development of a preliminary PCR-restriction fragment length polymorphism fingerprint database for identifying isolates. *J AOAC Int* 95(6):1750–1754. <https://doi.org/10.5740/jaoacint.11-482>
- Lalitha S (2000) Primer Premier 5. Biotech Softw Internet Rep 1(6):270–272. <https://doi.org/10.1089/152791600459894>
- Tamura K, Stecher G, Peterson D, Filipiński A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0.

- Mol Biol Evol 30:2725–2729. <https://doi.org/10.1093/molbev/mst197>
33. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791. <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>
 34. Jin X, Yao JM, Fan HL, Che YC, Pan JR, Zhang LL, Pan XH, Gelbič I, Huang TP, Guan X (2019) Heterologous expression and purification of BtCspB, a novel cold-shock protein-like bacteriocin from *Bacillus thuringiensis* BRC-ZYR2. *World J Microbiol Biotechnol* 35:23. <https://doi.org/10.1007/s11274-019-2595-z>
 35. Delécluse A, Charles JF, Klier A, Rapoport A (1991) Deletion by in vivo recombination shows that the 28-kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. *J Bacteriol* 173(11):3374–3381. <https://doi.org/10.1128/jb.173.11.3374-3381.1991>
 36. Yun DK, Lee J, Keum YS (2013) Finasteride increases the expression of hemoxygenase-1 (HO-1) and NF-E2-related factor-2 (Nrf2) proteins in PC-3 cells: implication of finasteride-mediated high-grade prostate tumor occurrence. *Biomol Ther* 21(1):49–53. <https://doi.org/10.4062/biomolther.2012.080>
 37. Andreadis SS, Cloonan KR, Bellicanta GS, Paley K, Pecchia J, Jenkins NE (2016) Efficacy of *Beauveria bassiana* formulations against the fungus gnat *Lycoriella ingenua*. *Biol Control* 103:165–171. <https://doi.org/10.1016/j.biocontrol.2016.09.003>
 38. Katumanyane A, Ferreira T, Malan AP (2018) Greenhouse application of *Steinernema yirgalemense* to control fungus gnats, *Bradysia impatiens*. *Biocontrol* 63:729–738. <https://doi.org/10.1007/s10526-018-9895-3>
 39. Zhu GD, Yin L, Ming X, Zhao HP, Xia NN, Wang XH (2018) Effects of high-temperature stress and heat shock on two root maggots, *Bradysia odoriphaga* and *Bradysia difformis* (Diptera: Sciaridae). *J Asia-Pac Entomol* 21(1):106–114. <https://doi.org/10.1016/j.aspen.2017.11.001>
 40. dos Santos LK, Soares-da-Silva J, da Silva MC, Tadei WP, Polanczyk RA, Pinheiro VCS (2018) Isolation and molecular characterization of *Bacillus thuringiensis* found in soils of the Cerrado region of Brazil, and their toxicity to *Aedes aegypti* larvae. *Rev Bras Entomol* 62(1):5–12. <https://doi.org/10.1016/j.rbe.2017.11.004>
 41. Regis L, Silva SBD, Melosantos MAV (2000) The use of bacterial larvicides in mosquito and black fly control programmes in Brazil. *Mem Inst Oswaldo Cruz* 95(suppl. 1):207–210. <https://doi.org/10.1590/s0074-02762000000700035>
 42. Pérez C, Fernandez LE, Sun J, Folch JL, Gill SS, Soberón M, Bravo A (2005) *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. *PNAS* 102(51):18303–18308. <https://doi.org/10.1073/pnas.0505494102>
 43. Vidal-Quist JC, Castañera P, González-Cabrera J (2010) Cyt1Aa protein from *Bacillus thuringiensis* (Berliner) serovar *israelensis* is active against the Mediterranean fruit fly, *Ceratitidis capitata* (Wiedemann). *Pest Manag Sci* 66(9):949–955. <https://doi.org/10.1002/ps.1965>
 44. Cohen S, Dym O, Albeck S, Ben-Dov E, Cahan R, Firer M, Zaritsky A (2008) High-resolution crystal structure of activated Cyt2Ba monomer from *Bacillus thuringiensis* subsp. *Israelensis*. *J Mol Biol* 380:820–827. <https://doi.org/10.1016/j.jmb.2008.05.010>
 45. Deng S, Deng M, Chen J, Zheng L, Peng H (2017) Larvicidal activity of recombinant *Escherichia coli* expressing scorpion neurotoxin AaIT or B.t.i toxin Cyt2Ba against mosquito larvae and formulations for enhancing the effects. *J South Med Univ* 37(6):750–754. <https://doi.org/10.3969/j.issn.1673-4254.2017.06.06>

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