Identification and Nematicidal Characterization of Proteases Secreted by Endophytic Bacteria *Bacillus cereus* BCM2

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ABSTRACT

The endophytic bacterium *Bacillus cereus* BCM2 has shown great potential as a biocontrol organism against *Meloidogyne incognita*, which causes severe root-knot diseases in crops. In our previous study, the metabolite of BCM2 showed high nematicidal activity against the *M. incognita* second-stage juveniles. However, the mechanism employed by endophytic bacteria to infect and kill nematodes is still unclear. Here, we investigate both the endophytic bacterial extracellular proteins with nematicidal activity and their mechanism of killing nematodes. The first step was detecting the nematicidal activities of crude proteins. The results show that the nematode mortality rate reached 100% within 72 h, and the crude proteins damaged both the cuticle and eggshell, before finally destroying the targets. This suggests possible proteinaceous pathogeny in BCM2. Throughout the process, the fine-detail changes in the nematode cuticle and the intestinal structure were observed using scanning electron

microscopy and transmission electron microscopy. These images show that BCM2 extracellular proteins did not damage the internal organization of the nematode but did severely damage its cuticle, which led to content leakage. From the crude proteins, chitinase, alkaline serine protease, and neutral protease were purified and identified. The *M. incognita*–*B. cereus* BCM2 microenvironment simulation demonstrates that BCM2 adheres to the surface of nematodes and helps the metabolites that were produced by BCM2 to rapidly recognize and kill *M. incognita*. This relationship between plants, endophytic bacteria, and nematodes offers insight into the biological mechanisms that can be utilized for of nematode management.

Keywords: biocontrol strategy, disease control and pest management, endophytic bacteria, extracellular proteases, nematicidal activity, nematicidal mechanism, nematology

Plant-parasitic nematodes (PPNs) are responsible for global agricultural losses amounting to an estimated \$130 billion annually. This does not even account for the indirect losses through interactions with other pathogens (Becker 2014). Among the PPNs, the root-knot nematode (*Meloidogyne* spp.) is considered one of the most economically important pests, causing severe damage to a wide variety of crops (Trudgill and Blok 2001). The management of root-knot nematode is more difficult than other pests because nematodes mostly live underground and tend to attack plant roots (Stirling 2011; Tian et al. 2007). The physical and physiological nematode cuticle make it difficult for many organic molecules to pass through (Chitwood 2002). At present, *Meloidogyne incognita* control is largely based on chemical pesticides that are toxic to humans and degrade the environment. An estimated \$80 million is spent every year on pesticides for vegetables alone (Sikora et al. 2008). The increasing demand for environmentally friendly

products has encouraged the exploration of alternative biological control agents that can effectively and sustainably minimize *M. incognita*. Plant-derived nematicidal metabolites such as the plant volatile compound trans-2-hexenal and 4-quinolone alkaloids can be directly used as botanical nematicides (Jang et al. 2015; Lu et al. 2017). Additionally, rhizobacteria have also been shown to be a sustainable and environmentally safe alternative to chemical pesticides (Castaneda-Alvarez and Aballay 2016).

Many microorganisms can attack and prevent nematode infection through diverse processes such as capturing or parasitizing nematodes, inducing the systemic resistance of plants, or producing toxins and enzymes. Endophytic bacteria are organisms that colonize the internal tissue of a living plant without causing plant disease. Their consistent colonization in the rhizosphere leads them to directly encounter and antagonize nematodes. Several studies describing how endophytic bacteria reduce PPN populations have been published over the last decade (Hu et al. 2017; Jin et al. 2015; O'Callaghan 2016; Sikora et al. 2007). Three modes of action—niche competition, induced systemic resistance, and direct toxicity—have been suggested to be important in the biocontrol of PPNs (Hashem and Abo-Elyousr 2011; Sikora et al. 2007).

At present, rhizobacterial compounds demonstrate nematicidal activity in two ways: toxic compounds and enzymes. Many different enzymes are considered especially relevant to the biocontrol of *M. incognita* due to their ability to quickly degrade the main biochemical constituents of the nematode cuticle. Several of these enzymes, including collagenases, chitinases, and serine proteases, have been shown to be important virulence factors in the degradation of the chemical constituents of both nematode cuticles and eggshells (Chen et al. 2015; Miller and Sands 1977; Page et al. 2014; Yang et al. 2013). Endophytic bacteria may be similar to other soil living microorganisms, which also release enzymes to facilitate host defense against a pathogen.

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Despite advances in the recognition of many nematode-antagonizing compounds associated with soil and rhizobacteria microorganisms, the level of nematicidal toxicity and the identity of specific plant endophytic bacteria remains unclear. The objective of this study was to identify endophytic bacterial extracellular proteins showing nematicidal activity and then to understand their pattern of killing nematodes. *Bacillus cereus* BCM2, which was isolated from plant tissue, significantly reduced gall and egg mass indices in tomato and demonstrated high nematicidal activity against *M. incognita* second-stage juveniles (J2s), thereby damaging their bodies. Therefore, by combining research on the nematicidal compounds of endophytic bacteria and findings from our previous studies and investigating a possible ecological mechanism employed by the endophytic bacteria BCM2 against root-knot nematodes, this study offers novel insights into the biological mechanisms that can be utilized for nematode management.

MATERIALS AND METHODS

Preparation of *M. incognita* J2s. Tomato (*Solanum lycopersicon*) Hezuo903 plants were used in all experiments, and were grown from seed obtained from the Tomato Institute of Shanghai. The tomato plants were grown in 1,000 g of soil containing a 1:1 substrate of sand/soil mixture under conditions of 28 and 20°C (14-h day and 10-h night, respectively). The *M. incognita* MJIS3 population survived on the tomato plants. Egg masses harvested from the tomato root were treated with 0.5% NaClO for 3 min to sterilize the surface, then rinsed with distilled water and put on sieves with 25- μ m openings suspended over deionized water. Preparasitic J2s were then hatched at 25°C for 3 to 7 days (Hussey and Barker 1973). The J2s were collected using a modified Baerman funnel technique (Gray 1984).

Crude protein extract collection from endophytic bacteria *B. cereus* BCM2. The endophytic bacteria *B. cereus* BCM2 was originally isolated from healthy tissue of a *Fragaria ananassa* plant grown in Nanjing, Jiangsu Province, China. In our previous experiment, BCM2 was found to reduce the galls and egg masses caused by *M. incognita* on tomato roots (Hu et al. 2017). The isolate was incubated on a beef extract peptone medium (3 g of beef extract, 10 g of peptone, and 5 g of NaCl, up to 1,000 ml) at 28°C. Then, a 1% BCM2 seed solution was inoculated with 100 ml of the beef extract peptone medium in a 250-ml bottle and cultured at 28°C and 160 rpm for 60 h. The total culture volume was 1,000 ml. The cells were removed by centrifuging the medium at 5,000 rpm for 3 min; then, the remaining bacteria were removed with a 0.22- μ m cellulose acetate filter membrane. Ammonium sulfate (Guoyao Chemical Reagent Co. Ltd.) was slowly added until achieving a concentration of 80% (wt/vol) on a magnetic stirrer. It was left at 4°C overnight. The solution was centrifuged at 10,000 rpm for 5 min; then, 10 ml of 50 mM Tris-HCl was used to collect precipitate, which was transferred to a dialysis bag with an intercept molecular weight of 3 kDa. Dialysis was performed to remove residual ammonium sulfate at 4°C, and the solution was freeze-dried for later experiments.

The nematicidal activity of the crude protein extract. Crude protein extract (100 mg) was dissolved in 2 ml of sterile water and subjected to the following treatments: (i) 200 μ l of crude protein extract solution + 100 μ l of J2 suspension, (ii) 200 μ l of sterile water + 100 μ l of J2 suspension, (iii) 200 μ l of crude protein extract solution + 100 μ l of egg suspension, and (iv) 200 μ l of sterile water + 100 μ l of egg suspension. The suspensions of 100 μ l of J2s and eggs included approximately 200 J2s and 200 eggs, respectively. The experiments were performed with five repetitions and incubated at 28°C in a 96-microwell plate. The nematicidal activity of the crude protein extract was detected after 24, 48, and 72 h of incubation through observations with a light microscope (Nikon E100).

Using scanning electron microscopy to detect the hydrolytic effect of the BCM2 excretion protein on the nematode body. Excretion protein (100 mg) was dissolved in 2 ml of sterile water; then, 1,000 J2s in 1 ml of sterile water were added to

the protein suspension, which was incubated at 28°C for 48 h. The filtered nematodes were washed in Tris-HCl buffer (pH 7.0) three times and fixed in 4% glutaraldehyde for 2 h, then in osmic acid for 40 to 60 min. Next, grade dehydration was performed in ethanol (10, 30, 50, 70, 95, and 100%) and dehydration in 100% ethanol three times, each time for 15 min. The nematode material was critical point dried for 2 h and sputter coated with gold. Nematodes were observed using scanning electron microscopy (SEM) (Thermo Electron).

Using transmission electron microscopy to detect the hydrolytic effect of the BCM2 excretion protein on the nematode internal structure. To study the active site in the BCM2 crude protein extract during infection, we used transmission electron microscopy (TEM) (Hitachi H7650-1010) to detect the intestinal construction of the nematode. The nematode treatment method and the preparation for TEM before graded dehydration in acetone was the same as for SEM. The nematode samples were soaked with different ratios of acetone/resin (vol/vol = 1/1, 1/2, and 0/1) for 1 h in vol/vol = 1/1 and 1/2 and overnight with vol/vol = 0/1 acetone/resin. Then, the nematodes were embedded in epoxy resin 618 and polymerized at 30, 45, and 60°C. Ultrathin sections (80 nm) were cut with a microtome using a diamond knife. The sections on grids were stained with aqueous uranyl acetate for 25 min. The sections were observed using TEM and photographed.

Purification of the crude proteins and the effect of purified protease on the nematode. *Ion exchange chromatography.* Crude protein (100 mg) was diluted in 1 ml of sterile water and applied to a diethylaminoethyl (DEAE) Sephadex column and a carboxymethyl (CM) Sephadex column (GE Healthcare), each of which had been previously equilibrated with 50 mM Tris-HCl (pH 7.0). Unbound nematicidal proteins were detected in two ion exchange chromatography assays across three different treatments: (i) 200 μ l of unbound protein in the CM column + 100 μ l of J2 suspension, (ii) 200 μ l of unbound protein in the DEAE column + 100 μ l of J2 suspension, and (iii) 200 μ l of sterile water + 100 μ l of J2 suspension. The suspension of 100 μ l of J2s included approximately 200 J2s. Nematicidal activity was detected 24, 48, and 72 h after treatment, while being cultured at 28°C. Three repetitions of each treatment were performed and nematodes were observed with a light microscope.

Sephadex gel filtration. Crude protein (500 mg) was diluted in 2 ml of 50 mM Tris-HCl and filtrated through a cellulose acetate membrane (0.45 μ m). The solution was loaded on a 1.2-m Sephadex gel column (Sephadex G-75), which was connected to the protein chromatography system (AKTA pure; GE Healthcare). The column had been previously equilibrated with 20 mM Tris-HCl (pH 7.0). The buffer flow rate was maintained at 0.2 ml/min, and one tube was collected every 5 min. The purified protein samples were detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were stained with Coomassie brilliant blue G-250. This purified protein was used for further analysis.

*Effect of purified protein on *M. incognita*.* Single protein components detected the nematicidal activity in different treatments: (i) 200 μ l of purified protein + 100 μ l of J2 suspension and (ii) 200 μ l of sterile water + 100 μ l of J2 suspension. The suspension of 100 μ l of J2s include approximately 200 J2s. Nematicidal activity was detected after 24, 48, 72, 96, and 120 h of treatment, while being cultured at 28°C. The corrected mortality rate was calculated as the (mortality rate of nematodes in treatment – mortality rate in control)/(1 – mortality rate in control) $\times$ 100%. Three repetitions were performed of each treatment, and the nematode status was observed with a light microscope.

In-gel tryptic digestion of proteins. The protein spots were manually excised from the Coomassie Brilliant Blue G-250-stained gels, then transferred to EP tubes loaded with 200 μ l of 30% acetonitrile (ACN) per 100 mM NH_4HCO_3 solution per tube. After being destained for 1 h, gel plugs were dehydrated with 100 μ l of 100% ACN for 20 min, then thoroughly dried in a freezer dryer (CHRIST) for 30 min. The dried gel particles were rehydrated at 4°C for 45 min with trypsin (10 ng/ μ l) at 5 μ l/tube (Promega Corp.); then, 30 μ l of 25 mM NH_4HCO_3 was added and the solution was

incubated at 37°C for 20 h. After trypsin digestion, the peptide mixtures were extracted with 100 µl of extraction solution (60% ACN per 0.1% trifluoroacetic acid [TFA]) per tube for 15 min, with ultrasound at 37°C. Finally, the extracts were freeze dried again.

Matrix-assisted laser desorption-ionization time of flight mass spectrometry and tandem mass spectrometry analysis. Tryptic peptides dissolved in 1 µl of 0.1% trifluoroacetic acid (TFA) were mixed with 1 µl of α -cyano-4-hydroxycinnamic acid in 80% ACN containing 0.1% TFA. Peptide samples were then transferred to wells of targeted plates, dried at room temperature,

and analyzed on the UltrafleXtreme TOF/TOF (Bruker Daltonics). Mass spectrometry (MS) calibration was automatically performed by a peptide standard kit (Bruker Daltonics) containing des-Arg1-bradykinin (m/z 904), Angiotensin I (m/z 1296.6851), Glu1-fibrinopeptide B (m/z 1570.6774), ACTH (1-17, m/z 2903.0867), ACTH (18-39, m/z 2465.1989), and ACTH (7-38, m/z 3657.9294). The tandem MS (MS/MS) calibration was performed by MS/MS fragment peaks of Glu1-fibrinopeptide B. All MS mass spectra were recorded in the reflector positive mode using a laser operating at a 200-Hz repetition rate with a wavelength of 355 nm. The accelerated voltage was operated at 2 kV. The MS/MS mass spectra were acquired by a data-dependent acquisition method, where the 10 strongest precursors were selected from one MS scan. All MS and MS/MS spectra were obtained as a conglomerate of at least 1,000 and 3,000 laser shots, respectively. Neither baseline subtraction nor smoothing was applied to recorded spectra. MS and MS/MS data were analyzed and peak lists were generated using FlexAnalysis 3.1 (Bruker Daltonics). MS peaks were selected from 850 to 3,700 Da and filtered with a signal-to-noise ratio > 20. A peak intensity filter was used with no more than 50 peaks per 200 Da. MS/MS peaks were selected based on a signal-to-noise ratio > 10 over a mass range of 60 to 2,000 Da below the precursor mass. MS and MS/MS data were analyzed using the MASCOT 2.4 search engine (Matrix Science) to search against the NCBI database. Searching parameters were as follows: trypsin digestion with one missed cleavage, variable modifications (oxidation of methionine and carbamidomethylation of cysteine), and the mass tolerance of precursor ion and fragment ion at 0.3 Da. For all proteins successfully identified by the Peptide Mass Fingerprint or MS/MS, a Mascot score > 59 (the default MASCOT threshold for such searches) was accepted as significant ($P < 0.05$).

Fig. 1. Expression of the BCM2 crude protein extract activity against *Meloidogyne incognita* second-stage juveniles. CK = control. Asterisks indicate a significant difference ($P < 0.01$).

Fig. 2. *Meloidogyne incognita* body and eggs, after treatment with the BCM2 crude protein extract, bars = 20 µm, **A**, *M. incognita* second-stage juvenile (J2) in the rigid state; **B**, *M. incognita* J2 in the seepage state; **C**, contents of *M. incognita* J2 are missing; **D**, live *M. incognita* J2; **E**, degradation of the *M. incognita* egg wall; **F**, content seepage from hatching *M. incognita*; **G**, entirely broken egg; and **H**, live egg.

Microenvironment simulation and SEM detection of *M. incognita*-*B. cereus* BCM2. To detect the adsorption of the endophytic bacteria *B. cereus* BCM2 onto the nematode body, we constructed a BCM2 Δ tr'-pBCgfp-1 marked with a green fluorescence protein and simulated the distribution of *M. incognita* and *B. cereus* BCM2 in the rhizosphere. The following experiments were designed: (i) BCM2 Δ tr'-pBCgfp-1 and *M. incognita* J2 suspension were cocultured in microwell plate at 28°C for 24 h, and the nematode's ability to adsorb the bacteria was detected by using fluorescence microscopy; and (ii) the microenvironment of *M. incognita*-*B. cereus* BCM2 was simulated; 2,000 J2s were inoculated with 10 ml of *B. cereus* BCM2 suspension (optical density at 600 nm = 0.563) in sterilized soil. After culturing at 28°C for 2 days, the J2s were separated by using the 25% sucrose centrifugal flotation method (Zhao et al. 2015). SEM was used to detect whether *B. cereus* BCM2 had adsorbed on the nematode body. This experiment was performed twice.

Data statistics. SPSS Statistics 17 was used in all data analysis for this study. The results are represented as mean $\pm$ standard deviation. One-way analysis of variance and Tukey's multiple comparison test were used for mean analysis, with $P < 0.05$ considered statistically significant.

RESULTS

Observing nematocidal activities of the crude protein extract and symptoms of nematode death under the light microscope. The nematocidal activity of crude protein extract is shown in Figure 1. The fatality rate of *M. incognita* within 48 h of treatment with crude proteins was 82.3%, and 100% within 72 h. This differs significantly from the sterile water treatment ($P < 0.01$). The dead body of a nematode, as seen under a light microscope, is shown in Figure 2A to D. Nematodes were rigid in death (Fig. 2A), with seepage observed within 48 h of treatment (Fig. 2B) and severe content loss within 72 h (Fig. 2C).

Nematodes in the egg state were examined under a light microscope (Fig. 2E to H). Most eggs did not appear abnormal after undergoing treatment with the BCM2 extraction protein for 24 h (Fig. 2H). In the 48-h treatment, the egg surface degraded and the egg wall bulged (Fig. 2E). In the 72-h treatment, multiple broken eggs were observed. Overall, eggs ranged from having degraded walls to being completely broken (Fig. 2G). In addition, we also found that hatching eggs were degraded (Fig. 2F). The degradation in both the nematode body and egg wall constitution suggests that

Fig. 3. Scanning electron microscopy detection of the degradation of the *Meloidogyne incognita* body by the BCM2 crude protein extract. **A**, *M. incognita* somatic with holes, bar = 10 μ m; **B**, coarse *M. incognita* body with holes, bar = 10 μ m; **C**, further degradation of the *M. incognita* body, bar = 30 μ m; **D**, local amplification of panel E, where the nematode body is almost falling off, bar = 50 μ m; **E**, further degradation of the *M. incognita* body results in cortex eversion, bar = 100 μ m; **F**, local amplification of panel E, showing nematode cortex eversion, bar = 20 μ m; **G**, nematode body becomes unidentifiable after additional degradation, bar = 30 μ m; **H**, live *M. incognita*, bar = 50 μ m; and **I**, skin of a live *M. incognita* shown with a smooth grain, bar = 10 μ m.

there is more than one compound in BCM2 crude extracellular protein that attacked and killed the *M. incognita*.

Effect of BCM2 crude protein extract on the *M. incognita* J2 cuticle. Under SEM, fine-scale changes were observed in the nematode cuticle after being treated with crude protein extract (Fig. 3). Following the BCM2 crude extracellular protein extract treatment, a hole formed in the body wall of the worm first, which exposed the internal structure (Fig. 3A). The nematodes displayed different degrees of hydrolysis and cuticle roughness (Fig. 3B, indicated by arrows). Next, the worm body degraded further (Fig. 3C), until other parts of the worm were lost to cortical exfoliation (Fig. 3D to F). Eventually, the worm body was almost completely destroyed and unidentifiable (Fig. 3G). The nematode in Figure 3H has not been treated with crude protein extract before SEM preparation. This worm body is in perfect shape, with a smooth surface and clear lateral striae, lying in a curved state after glutaraldehyde fixation (Fig. 3I). These results indicate that the presence of BCM2 crude proteins degrades the bodily composition of nematodes.

Internal structure damage of *M. incognita* J2 treated with BCM2 crude protein extract. The internal structures of the nematode under TEM are shown in Figure 4. For both the BCM2 crude-protein-extract-treated (Fig. 4A) and sterile-water-control (Fig. 4B) nematodes, the internal organs were arranged tightly, with clear texture near the body surface. BCM2 extracellular protein and control treatments are shown in Figure 4C and Figure 4D, respectively. In the healthy nematode (Fig. 4D), the gut was intact, organization interval regular, and gap edge smooth. There was no obvious difference between the two treatments. Therefore, based on the slow reaction rate of the BCM2 crude protein extract, we conclude that the BCM2 extracellular protein severely damages the nematode cuticle but not through intake. The crude protein extract first degrades the nematode's body wall, which leads to content leakage and leaves the nematode vulnerable to further degradation.

Fig. 4. Effects of BCM2 crude protein extract on the interior of *Meloidogyne incognita* as observed with transmission electron microscopy detection, bars = 2 μ m. **A**, Structure of *M. incognita* tissue after treatment with BCM2 crude protein extract; **B**, structure of *M. incognita* tissue after treatment with sterile water; **C**, intestinal structure of *M. incognita* after treatment with BCM2 crude protein extract; and **D**, intestinal structure of *M. incognita* intestinal after treatment with sterile water.

Purification of BCM2 crude proteins and their observed nematocidal activities. *Ion exchange chromatography.* The nematocidal activity of crude protein extract purified with either DEAE or CM was detected 24 and 48 h after treatment (Fig. 5). The nematode mortality rates for the two purified protein treatments were 21.5 and 25.6% ($P < 0.05$) after 24 h and 90.4 and 87.7% ($P < 0.05$) after 48 h for DEAE or CM, respectively, and 100% after 72 h for both. As these results indicate, nematocidal activity did not differ significantly between the two treatments, which indicates that the BCM2 crude extracellular protein extract has two or more components that similarly and independently attack nematodes.

Sephadex gel filtration. A GE Healthcare AKTA system was used to purify and analyze the nematocidal proteins. Three peaks were observed (Supplementary Fig. S1) with three single components (marked as peaks 1, 2, and 3) detected. The fractions were assayed with SDS-PAGE (Supplementary Fig. S2). Three purified proteins were collected and marked as samples 1, 2, and 3. The molecular weights were 45 to 35 kDa, approximately 35 kDa, and 35 to 25 kDa, respectively. The purified proteins were then freeze-dried and their nematocidal activity was assayed.

*Effect of purified proteins on *M. incognita*.* Sample 2 showed the strongest nematocidal activity (Table 1). After 48 h, the corrected mortality rate of nematodes exposed to sample 2 reached 90.0%. All treated nematodes were dead within 96 h. In contrast, the sample 1 corrected nematode mortality rate was 66.1% after 120 h (at the end of the test). The corrected nematode mortality rate was 86.5% for sample 3, which was significantly

Fig. 5. Mortality rate of *Meloidogyne incognita* second-stage juveniles treated with BCM2 crude protein extract supernatant using diethylaminoethyl (DEAE) and carboxymethyl (CM) ion-exchange chromatography. Different letters indicate significant differences according to Tukey's multiple-range test ($P < 0.05$).

TABLE 1. Nematicidal activity of three Sephadex gel filtration purified protein samples from endophytic bacteria *Bacillus cereus* BCM2

Sample	Corrected nematode mortality rate (%) ^z				
	24 h	48 h	72 h	96 h	120 h
1	0 c	13.3 $\pm$ 1.5 b	21.4 $\pm$ 4.2 c	36.3 $\pm$ 8.1 c	66.1 $\pm$ 12.1 b
2	22.3 $\pm$ 10.3 a	90.0 $\pm$ 9.4 a	98.8 $\pm$ 0.3 a	100 a	100 a
3	9.0 $\pm$ 4.1 b	24.9 $\pm$ 4.7 b	63.3 $\pm$ 1.8 b	73.3 $\pm$ 4.3 b	86.5 $\pm$ 5.6 a

^z Data are represented as mean $\pm$ standard deviation based on three repetitions. Different letters indicate significant differences according to Tukey's multiple-range test ($P < 0.05$).

lower than in the sample 2 ($P < 0.05$) (Table 1). This result suggests that protein 2 may play an important role in the process of killing nematodes.

Matrix-assisted laser desorption-ionization tandem time of flight analysis. According to the results of MS and MS/MS, three purified proteins were identified in the NCBI nr database of the MASCOT 2.4 server. The results are listed in Table 2 and the search results are shown in Supplementary Fig. S3. The three proteases were chitosanase, alkaline serine protease, and neutral protease.

Microenvironment simulation of *M. incognita*–*B. cereus* BCM2. The amount of BCM2 that adhered to the nematode body during the microenvironment simulation is shown in Figure 6. BCM2 $_{str'}$ -pBCgfp-1 adhered to the nematode after BCM2 $_{str'}$ -pBCgfp-1 and *M. incognita* J2 were cocultured (Fig. 6A and B). SEM figures of BCM2 and *M. incognita* J2 cocultured in a microenvironment are shown in Figure 6C to E. BCM2 can still be detected on the nematode after multiple rounds of cleaning, centrifugation, and dehydration (Fig. 6C and D, from two distinct experiments), which indicated that the bacterial strain can adhere to

TABLE 2. Matrix-assisted laser desorption-ionization time of flight tandem mass spectrometry analysis of three nematocidal proteases from endophytic bacteria *Bacillus cereus* BCM2

Sample	Calculated molecular weight (Da)	Amino acid number	Protein family	Accession number	Name
1	50,795	453	Glycosyl hydrolases family 8	gi 446936326	Chitosanase
2	42,175	397	Peptidase S8 family domain in thermitase like	gi 446713583	Alkaline serine protease
3	29,182	317	Peptidase M4 family	gi 76364030	Neutral protease

Fig. 6. Microenvironment simulation of *Meloidogyne incognita*–*Bacillus cereus* BCM2 and detection of nematode body attachment. **A**, Micrograph of BCM2 $_{str'}$ -pBCgfp-1 and *M. incognita* second-stage juvenile (J2) after coculture, bar = 20 µm; **B**, fluorescence micrograph of BCM2 $_{str'}$ -pBCgfp-1 and *M. incognita* J2 after coculture, bar = 20 µm; **C**, first scanning electron microscopy (SEM) result of the BCM2 and *M. incognita* J2 coculture in soil, bar = 30 µm; **D**, second SEM result of the BCM2 and *M. incognita* J2 coculture in soil, bar = 10 µm; and **E**, nematode without *B. cereus* BCM2, bar = 20 µm.

nematodes in rhizospheric microenvironments. This ability helps the secondary metabolites of the bacteria to rapidly find and kill pathogens such as nematodes.

DISCUSSION

The enzymes of the endophytic bacteria BCM2 helped kill the *M. incognita* J2 in vitro. At first, the dead nematode body was rigid but, over time, it began to leak and degrade. The extracellular protease of BCM2 also affected nematode eggs. As Niu et al. (2006, 2012) reported, serine proteases and neutral protease from *B. nematocida* showed the ability to degrade the main chemical compositions of both the nematode cuticles and eggshells. Several other types of extracellular enzymes, including chitinases, glucanases, and collagenases, have also been widely considered to control PPNs (Huang et al. 2004; Krechel et al. 2002; Page et al. 2014; Yang et al. 2007, 2013). Thus, the extraction of BCM2 may have similar proteases that degrade the cuticles and eggshells of nematodes.

Most nematophagous fungi and pathogenic bacteria (*Microbacterium nematophilum*, *Xenorhabdus nematophila*, and *Yersinia pestis*) infect nematodes by adhering to their cuticles. In contrast, most bacterial pathogens, and several yeasts such as *Candida albicans*, infect *Caenorhabditis elegans* by colonizing its intestine (Li et al. 2015). Thus, both innate epidermal and intestinal immune responses are important for defending against microbial pathogens. Zhang et al. (2012) labeled the nematocidal crystal protein (Cry55Aa, Cry6Aa, and Cry5Ba) produced by *B. thuringiensis* by using NHS-Rhodamine. Confocal microscopic observation showed that nematocidal crystal proteins were initially detected in the stylet and esophageal lumen of *M. hapla*, and later in its gut. In this study, SEM and TEM were used to detect the degradation of *M. incognita*. The results show that the BCM2 crude proteins entered the nematode by destroying its wall, instead of entering via the stylet,

and destroying its internal structure. Our results suggest that protease exists in the metabolites of BCM2 and that it degrades cuticles and egg walls of nematodes, as previous studies have suggested.

In addition to the chitosanase, alkaline serine protease and neutral protease involved with nematocidal activity were purified here by Sephadex gel filtration. Thus far, only one collagenolytic or proteolytic enzyme from *B. cereus* is known to have demonstrated nematocidal activity against PPN *M. javanicus* (Sela et al. 1998). There have been no reports concerning endophytic bacterial nematocidal proteases. Chitosanases are enzymes capable of catalyzing the hydrolysis of chitin or chitosan into smaller N-acetylchitooligosaccharides or chitooligosaccharides (Wang et al. 2012). Chitin is one of the most widespread biopolymers in nature, and it is an essential structural component of both fungi and nematode eggs (Wharton and Jenkins 1978; Xu et al. 2011). Chitinases degrade chitin and play key roles in the life cycles of pathogenic fungi and nematodes alike. Collagens and lipids are especially important PPN structures for mobility. However, chitin, proteins and lipids were also detected during the sedentary stage. The changing nematode body structures make PPNs susceptible to a broad spectrum of bacteria and fungi during various stages of their life cycle (Castaneda-Alvarez and Aballay 2016). Chitinases from *Lysobacter capsici* YS1215 can kill the larvae and eggs of nematodes (Lee et al. 2013, 2014). The chitosanase purified from BCM2 is also capable of degrading the nematodes.

Among the pathogenic microorganisms, alkaline serine protease has been widely reported. It is deeply involved in host–pathogen interactions. Inhibiting the activity of alkaline serine protease is an effective means to deter pathogen infection (Ribeiro-Guimarães et al. 2007; Serrano-Luna et al. 2007). The extracted crude proteins from *Brevibacillus laterosporus* G4 killed all nematodes within 12 h. The purified protease, 30 kDa of serine protease, showed strong

Fig. 7. Ecological mechanism of the endophytic bacteria *Bacillus cereus* BCM2 attack on *Meloidogyne incognita*.

hydrolytic activity against denatured casein and skimmed milk, and could also moderately hydrolyze gelatin, bovine serum albumin, denatured collagen, and native collagen. Further, the serine protease also showed activity toward the purified nematode cuticle (Huang et al. 2005a). In previous studies, *Bacillus nematocida* B16 was isolated from the soil and purified alkaline serine protease B16. The protease killed both the free-living nematode *Panagrellus redivivus* and the plant-parasitic nematode *Bursaphelenchus xylophilus*. Overexpression of the coding gene for alkaline serine protease B16 significantly increased the nematocidal activity in the mutant compared with the wild-type *Bacillus nematocida* B16 (Huang et al. 2005a; Niu et al. 2012). In this study, the alkaline serine protease was obtained from endogenous bacteria *B. cereus* BCM2 and was significantly more nematocidal than the other two proteases.

Neutral protease is a metalloproteinase and hydrolyzes the polypeptide chain from the hydrophobic structure of imino and the residues of aromatic groups (Stark et al. 1992). Here, a neutral protease, Bae16, was purified from the strain *B. nematocida*. The in vitro experiment demonstrated that this protease could destroy the nematode cuticle and its hydrolytic substrates included denatured casein, skimmed milk, gelatin, bovine serum albumin, denatured collagen, and native collagen (Huang et al. 2005b; Niu et al. 2006). A nematocidal neutral protease was purified from BCM2 but it was not as effective as the alkaline serine protease while slightly more efficient than the chitosanase, which was also a potential nematotoxin in the endophytic *B. cereus* infection administered to PPN.

Bacteria release enzymes that facilitate attacks against a broad host range, as do other soil-living microorganisms. Previously, several types of extracellular proteases of bacteria have been reported to degrade the main biochemical constitutions of the nematode cuticle and eggshell (Castaneda-Alvarez and Aballay 2016). However, this study was the first to investigate the extracellular enzymes of endophytic bacteria that harm nematodes. Recording initial nematode contact is the first important step when studying the effect of *B. cereus* BCM2. According to our previous studies, BCM2 can colonize the roots of plants, thereby competing in the same ecological niche as PPNs. The bacteria remain sedentary in plants and the rhizosphere for a long time, with $3.39 \log \text{CFU g}^{-1}$ of root and $6.14 \log \text{CFU g}^{-1}$ in the rhizosphere soil at 90 days postinoculation (Wang et al. 2018). Additionally, the endogenous bacteria is found in large quantities in both the root knot and the gap in the intrusion caused by root-knot nematodes, thus increasing their chance of direct contact with nematodes (Hu et al. 2017) (Fig. 7). In addition, preinoculation with BCM2 can effectively induce systemic resistance of tomato against *M. incognita*. In all, 34 defense-related genes were identified, which represent the targets for priming BCM2 resistance against *M. incognita* (Hu et al. 2018).

Endophytic bacterial *B. cereus* BCM2 adheres to the surface of nematodes and effectively exerts the function of its metabolites, as has been demonstrated in this study. Furthermore, the three proteases produced by the same endophytic bacterial strain utilize their different characteristics to coordinate attacks against PPN. Thus, based on the evidence, we suggest that the endophytic bacteria BCM2 ecologically antagonizes root-knot nematodes (Fig. 7). When a plant is infected with root-knot nematodes, the nematodes first encounter a very strong physical barrier due to a large amount of sedentary BCM2 in the rhizosphere. There is a short time where the root-knot nematodes live freely in soil. Then, infective J2s penetrate the root and migrate between cells to reach the plant vascular cylinder (Abad et al. 2008). If BCM2 is successfully adsorbed on the nematode body at this stage, then the secondary metabolites produced by the bacteria BCM2 rapidly invade the nematode and degrade its body wall. Otherwise, BCM2 colonization induces systemic resistance in the plant and mediates the expression of defense-related genes to resist the infecting nematode. Even if the nematode invasion succeeds, the secondary metabolites produced by BCM2, which accumulate at the feeding

site, can kill the adult nematode or affect the next generation of its offspring. In this way, BCM2 effectively wards off a nematode infection.

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