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ORIGINAL ARTICLE

Proteases from *Bacillus*: a new insight into the mechanism of action for rhizobacterial suppression of nematode populations

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Abstract

Aims: The aim of this study was to investigate the role of proteases in *Bacillus* spp. of rhizobacteria in suppressing nematode populations and to understand their mechanism of action.

Methods and Results: Rhizobacteria with nematicidal activity were isolated from soil samples of five root knot nematode-infested farms. Among these strains, nematotoxicities of *Bacillus* strains were intensively analysed. Further assays of nematicidal toxins from *Bacillus* sp. strain RH219 indicated an extracellular cuticle-degrading protease Apr219 was an important pathogenic factor. The Apr219 shared high similarity with previously reported cuticle-degrading proteases from *Brevibacillus laterosporus* strain G4 and *Bacillus* sp. B16 (*Bacillus nematocida*). The cuticle-degrading protease genes were also amplified from four other nematicidal *Bacillus* strains isolated from the rhizosphere. In addition to Apr219, a neutral protease Npr219 from *Bacillus* sp. RH219 was also investigated for activity against nematodes.

Conclusions: The wide distribution of cuticle-degrading proteases in *Bacillus* strains with nematicidal activity suggested that these enzymes likely play an important role in bacteria–nematode–plant–environment interactions and that they may serve as important nematicidal factors in balancing nematode populations in the soil.

Significance and Impact of the Study: Increased understanding of the mechanism of action of *Bacillus* spp. against nematodes could potentially enhance the value of these species as effective nematicidal agents and develop new biological control strategies.

Introduction

Bacteria that colonize the rhizosphere are commonly referred to as rhizobacteria (Sikora 1992; Lugtenberg and Dekkers 1999). Rhizobacteria can play significant roles in plant health. Some rhizobacteria are significant plant pathogens, while others are beneficial mutualists. Approximately 10% of all isolated rhizobacteria affect plant growth and/or health. Consequently, the beneficial bacteria were classified as either plant growth promoting rhizobacteria (PGPR) or plant health promoting rhizobacteria (PHPR) according to their mode of action (Sikora 1992).

The rhizobacteria have been extensively studied as agents for the biological control of plant-parasitic nematodes. Among these bacteria, numerous *Bacillus* strains have been found to express activities that suppress pests and pathogens, including nematodes (Siddiqui and Mahmood 1999; Radnedge *et al.* 2003). The most thoroughly studied *Bacillus* includes *Bacillus subtilis* and

Bacillus thuringiensis (Crickmore *et al.* 1998; Krebs *et al.* 1998; Siddiqui and Mahmood 1999). *Bacillus thuringiensis* (Bt) produces one or more parasporal crystal inclusions (Cry or δ-endotoxins). These toxins are known to be toxic to a wide range of insect species (Feitelson *et al.* 1992). Some Cry proteins are also toxic to nematodes (Feitelson *et al.* 1992). To date, five Cry proteins (Cry5B, Cry6A, Cry13, Cry14A, Cry21A) known to be toxic to larvae of a number of free-living or parasitic nematodes (Crickmore *et al.* 1998; Marroquin *et al.* 2000; Wei *et al.* 2003). Additionally, a number of studies have reported direct antagonistic effects of other bacteria to pathogenic nematodes belonging to the genera *Heterodera* and *Meloidogyne*. These bacterial species include *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus mycoides* and *Bacillus pumilus* as well as isolates of unidentified species from the *Bacillus* genus (Siddiqui and Mahmood 1999; Gardener 2004). These studies also indicated that catabolic enzymes (e.g. proteases, chitinases and glucanases), peptide antibiotics or small molecules secreted by various *Bacillus* species might contribute to their activity against pathogenic nematodes (Priest 1993; Siddiqui and Mahmood 1999).

Microbial proteases have been proposed as virulence factors in their pathogenesis against nematodes. The most compelling evidences to support microbial proteases as virulence factors have come from the studies of protease-deficient mutants (Åhman *et al.* 2002; Siddiqui *et al.* 2005; Tian *et al.* 2006). In nematophagous fungi, it is believed that extracellular serine proteases are involved in several steps during the infection: releasing nutrients for microbial growth, facilitating penetration by degrading cuticle proteins and digesting the host tissue (Clarkson and Charnley 1996; Åhman *et al.* 2002; Meyer *et al.* 2004; Morton *et al.* 2004). In nematotoxic bacteria, *Bacillus laterosporus* lost 57% of its nematicidal activity because of the deletion of the extracellular alkaline protease BLG4 (Tian *et al.* 2006). Siddiqui *et al.* (2005) also demonstrated that the deletion of a major extracellular protease from *Pseudomonas fluorescens* CHA0 reduced bacterial activity against the root-knot nematode *Meloidogyne incognita*. These researches suggested extracellular proteases might play important pathogenic roles in suppressing nematodes in the soil (Siddiqui *et al.* 2005).

In this paper, we examined rhizobacteria with the ability to kill nematodes from the rhizosphere. Among these rhizobacteria, the nematotoxic factors of *Bacillus* spp. were identified and characterized. The proteases with cuticle-degrading and nematicidal activities were found to be extensively distributed in *Bacillus* spp. Such distributions suggested that extracellular enzymes from rhizobacteria might play an important role in the bacteria–nematode–plant–environment interactions.

Materials and methods

Isolation and identification of rhizobacteria with nematicidal activities

Bacteria were isolated from the rhizosphere of tobacco in five root knot nematode-infested farms in Yunnan province in China (Kaiyuan, Yuxi, Qujing, Dian Chi). To isolate these bacteria, roots were washed in 0·1 mol l^{-1} phosphate buffer, and appropriate dilutions were plated on NA agar and incubated at 28°C for 2 days (Fang 1998). Nematicidal activities of isolated strains were tested according to the methods described below using the free-living nematode *Panagrellus redivivus* as the target nematode. The nematicidal bacteria were classified at the Yunnan Institute of Microbiology based on their morphological and biochemical characteristics and their 16S rDNA sequences. After the genomic DNA of the *Bacillus* strains was extracted, 16S rRNA genes were amplified using the forward (5′-GGTTACCTTGTTACGACTT-3′) and reverse (5′-AGAGTTTGATCCTGGCTCAG-3′) primers as described by Lane (1991). The sequenced 16S rRNA genes were compared and analysed using the CLUSTALX 1.83 and MEGA version 3.1 programs (Thompson *et al.* 1997; Kumar *et al.* 2004). Candidate *Bacillus* spp. were stored in 30% glycerol at −20°C for further assays.

Bioassays

All of the isolated rhizobacterial strains were inoculated into 250 ml Erlenmeyer flasks containing 50 ml YPD (yeast, peptone, glucose) medium each and grown at 28°C with rotary shaking at 220 rev $min^{-1}$for 3 days (Dillon *et al.* 1985). After centrifugation at 8500 ***g*** for 15 min, the culture supernatants were collected for the measurement of nematicidal activity. In bioassay, approximately 200 nematodes were added to 300 μl culture supernatants in a 1·5 ml Eppendorf tube containing two antibiotics (50 μg ml^{-1} ampicillin and 30 μg ml^{-1} kanamycin). After incubating the tubes at 28°C for 2–10 h, the numbers of dead nematodes in each treatment were counted under a light microscope. The experiments were performed in triplicates and repeated at least three times. Controls were incubated with water, YPD medium and the culture supernatant boiled for 15 min. All the data were analysed by the independent samples test ($P = 0{\cdot}05$ or $P = 0{\cdot}01$), using procedures of the Statistical Package for Social Sciences (SPSS, version 10·0 for Windows, SPSS Inc., Chicago, IL, USA). Standard error (SE) was recorded.

Fragments of cuticles of the nematode *P. redivivus* were purified according to the method described by Cox *et al.* (1981). After 200 μl of the purified protease sample (the proteases Npr219 or Apr219) was mixed with nematode cuticles, the degradation process was observed under a light microscope once in every hour. For negative controls, 0·1 mol l^{-1} bovine serum albumin (BSA) and the target sample boiled for 15 min were added to the nematode cuticle.

Purification of proteases

A 500 ml bacterial culture solution for *Bacillus* sp. RH219 was pooled and bacterial cells were removed by centrifugation at 8500 ***g*** for 15 min at 4°C. The resulting supernatant was salt-out by adding ammonium sulfate to 80% saturation. After centrifugation at 8500 ***g*** for 20 min again, the precipitated protein was dissolved in 100 ml of 50 mmol l^{-1} sodium phosphate buffer (pH 7·0) with 1 mol l^{-1} ammonium sulfate.

The dissolved protein solution was applied to HiPrep 16/10 column and HiTrapTM SP FF column (Amersham Pharmacia Biotech, Uppsala, Sweden) to get purified proteases according to previously described manipulation procedures for protein purification (Tian *et al.* 2006, 2007; Huang *et al.* 2005a). The fractions were pooled and assayed for protease activity and assayed by 12% SDS-PAGE (Laemmli 1970; Huang *et al.* 2005a).

Nematicidal and cuticle-degrading activities of purified proteases Npr219 and Apr219 were analysed according to the methods described in Bioassays. The combined action of two proteases was tested using their mixtures (150 μl Npr219 and 150 μl Apr219), and its control was the two proteases tested separately (150 μl Npr219 + 150 μl water and 150 μl water + 150 μl Apr219).

The N-terminal amino acid sequences of the purified proteases were determined according to the previously described methods (Huang *et al.* 2005a). The N-terminal sequence of the first 10 amino acids was used as a query for BLAST searches in the GenBank.

Characterization of proteases

The optimal pH for the purified proteases was determined using the Britton Robinson universal buffer system with a pH range from 3 to 12. The pH stability, optimal pH and optimal temperature for the proteases were determined according to the previous literatures (Huang *et al.* 2005a; Niu *et al.* 2005).

The effects of various inhibitors [phenylmethylsufonylfluoride (PMSF), EDTA, aprotinins, leupeptin and pepstatin A] on the protease activity were examined by incubating the enzyme for 5 min at 37°C and pH 7·0 with these inhibitors. The residual proteolytic activity was measured as a percentage of that in the control without inhibitors.

PCR amplification of protease Npr219 and Apr219 genes

Genomic DNA of *Bacillus* sp. strains RH219, A29, A56, A104 and B101 was extracted, respectively, using the Wizard genomic DNA purification kit for Gram-positive bacteria (Promega, Madison, WI, USA) and stored in TE solution at −70°C for further cloning of protease genes.

Primers for the encoding gene of *Bacillus* sp. RH219 neutral protease were designed on the basis of the gene sequences of neutral proteases from *B. laterosporus* (GenBank accession no. AY867791), *B. amyloliquefaciens* (GenBank accession no. K02497) and *B. subtilis* (GenBank accession no. U30932): np1 (GGGGGATTTATTGTGGGTTT) and np2 (TACAATCCGACAGCATTCCA). PCR cycling conditions were 5 min at 94°C for the first predenaturation step before Taq polymerase was added, 30 cycles of 40 s at 95°C, 40 s at 50°C and 90 s at 72°C for amplification. After cycling, the reaction mixture was kept for 10 min at 72°C for extension.

Primers for *Bacillus* RH219 alkaline protease gene: ap1 (GCGCCTAGGGTGAGAGGCAAAAAAGGTATG) and ap2 (CGCGGATCCTTACTGAGCTGCCGCCTGTAC) were designed based on the Apr genes from *B. amyloliquefaciens* (GenBank accession no. DQ132806), *B. subtilis* (GenBank accession no. DQ241738) and *B. laterosporus* (GenBank accession no. AY720895). Cycling conditions were 5 min at 94°C, 30 cycles of 40 s at 95°C, 40 s at 60°C and 90 s at 72°C for amplification. After cycling, the reaction mixture was kept for 10 min at 72°C for extension.

To determine the distribution of the cloned cuticle-degrading protease genes in *Bacillus* spp. with nematotoxicity, PCR amplification was also conducted for the isolated bacteria using previously designed primers (ap1 and ap2). The deduced amino acid sequences from the genes were analysed using the Bioedit software package (Raleigh, NC, USA).

Results

Identification of nematicidal rhizobacteria

A total of 308 bacterial strains were isolated from the rhizosphere of tobacco using the spread-plating technique on NA medium. Of these strains, 11 showed nematicidal activity in the preliminary tests targeted towards *P. redivivus*. Among these 11, five *Bacillus* strains (A29, A56, A104, B101, RH219) were identified. The *Bacillus* spp. showed a range of nematicidal activity (Table 1). Most of the bodies and cuticles for the dead nematodes were degraded and destroyed by these strains.

Table 1 Mortality of *Panagrellus redivivus* in culture supernatants from *Bacillus* spp. The percentage of dead nematodes was determined after 2, 5, and 10 h exposures to the supernatants

Bacillus strain number	2-h mortality of *P. redivivus* % (SE)	5-h mortality of *P. redivivus* % (SE)	10-h mortality of *P. redivivus* % (SE)
RH219	80 (2·11)^{b,B}	99 (0·50)^{b,B}	100 (0)^{b,B}
A29	47 (1·67)^{b,B}	58 (3·87)^{b,B}	90 (1·41)^{b,B}
A56	61 (2·93)^{b,B}	76 (1·06)^{b,B}	100 (0)^{b,B}
A104	33 (1·19)^{b,B}	75 (2·80)^{b,B}	100 (0)^{b,B}
B101	40 (3·86)^{b,B}	82 (0·15)^{b,B}	100 (0)^{b,B}
Water	5 (0)	6 (0)	12 (0·62)
YPD medium	7 (0)	8 (0)	10 (0·10)

Death was determined by nudging nematodes with a stick under a light microscope, the immobilized nematodes were counted as dead. Mortality of nematodes = number of dead nematodes/all tested nematodes × 100%.

The mortality with the letter b indicates statistically significant difference against its corresponding water control within a column.

The mortality with the letter B indicates statistically significant difference against its corresponding YPD medium control within a column.

SE, standard error of mortality.

For all these treatments, an independent samples test showed $P < 0·01$.

Bioassays for strain RH219

As shown in Table 1, strain RH219, which was identified as *Bacillus* sp. RH219, showed the most nematicidal activity in our tests targeting *P. redivivus*. In the bioassays, the mortality of the nematode was 80%, 99% and 100% within 2, 5, and 10 h, respectively. After 12 h, all dead nematodes were completely destroyed and digested. In all the controls: water, YPD medium and culture supernatant boiled for 15 min, the mortalities were below 25% up to 10 h. In addition, the cuticles of the dead nematodes were intact after 10 h.

Purification of proteases

After salting out using 80% ammonium sulfate and through hydrophobic interaction chromatography, the eluted fractions containing protease activity were pooled and analysed. Twelve per cent SDS-PAGE assays indicated fractions 22–26 contained a single protein band with a molecular mass about 41 kDa (Fig. 1a,c). The resulting protein showed a maximum protease activity in pH 6·0 at 50°C. The protease was designed as Npr219. Fractions 14–20 also showed a protease activity with a maximum protease activity in pH 10·0, but included several protein bands by SDS-PAGE. After further purification by cation-exchange chromatography, the fraction containing protease activity was only detected in one peak (Fig. 1b). The homogeneity of the purified protease was confirmed by 12% SDS-PAGE (Fig. 1d). The molecular weight of the enzyme was found to be about 33 kDa, designated as Apr219.

Properties of Npr219 and Apr219

As shown in Table 2, proteases Npr219 and Apr219 showed maximum proteolytic activity at 50°C, pH 6·0 and 60°C, pH 10·0, respectively. The effects of different inhibitors chosen on the basis of their selective inhibition are summarized in Table 3. Similar inhibitory effects were observed with PMSF on Apr219 and with EDTA on Npr219. Other inhibitors tested did not significantly influence proteolytic activity.

The BLAST result for the first 10 N-terminal amino acid sequences (AQSVPYGVSQ) of Apr219 indicated 100% similarity to serine proteases (subtilisin BPN′) from *B. amyloliquefaciens* (GenBank accession no. AAZ66858), *B. subtilis* (GenBank accession no. ABB92698) and *B. laterosporus* (GenBank accession no. AAU81559). The first 10 N-terminal amino acid sequences (AAATGTGTTL) of Npr219 showed 90–100% similarity with the neutral metalloprotease from *B. laterosporus* (GenBank accession no. AAW59490), *B. amyloliquefaciens* (GenBank accession no. AAB05346) and *B. subtilis* (GenBank accession no. AAA82609). Npr219 was markedly inhibited by the metalloprotease inhibitor EDTA.

Bioassays for Apr219 and Npr219

In the bioassay, Apr219 (protease activity units: 930 U ml^{-1} at 37°C, pH 7·0) killed 73% of the tested nematodes within 24 h; after 48 h, 97% of the tested nematodes were killed. Under the microscope, we noticed that the cuticles of nematodes had been degraded and destroyed by the proteolytic action. Npr219 (protease activity units: 870 U ml^{-1} at 37°C, pH 7·0) and all controls showed that <20% of the nematodes were killed (Fig. 2). However, the addition of neutral protease Npr219 (150 μl Npr219 and 150 μl Apr219, protease activity units: 905 U ml^{-1} at 37°C, pH 7·0) in the treatment of Apr219 (150 μl water and 150 μl Apr219, protease activity units: 510 U ml^{-1} at 37°C, pH 7·0) increased

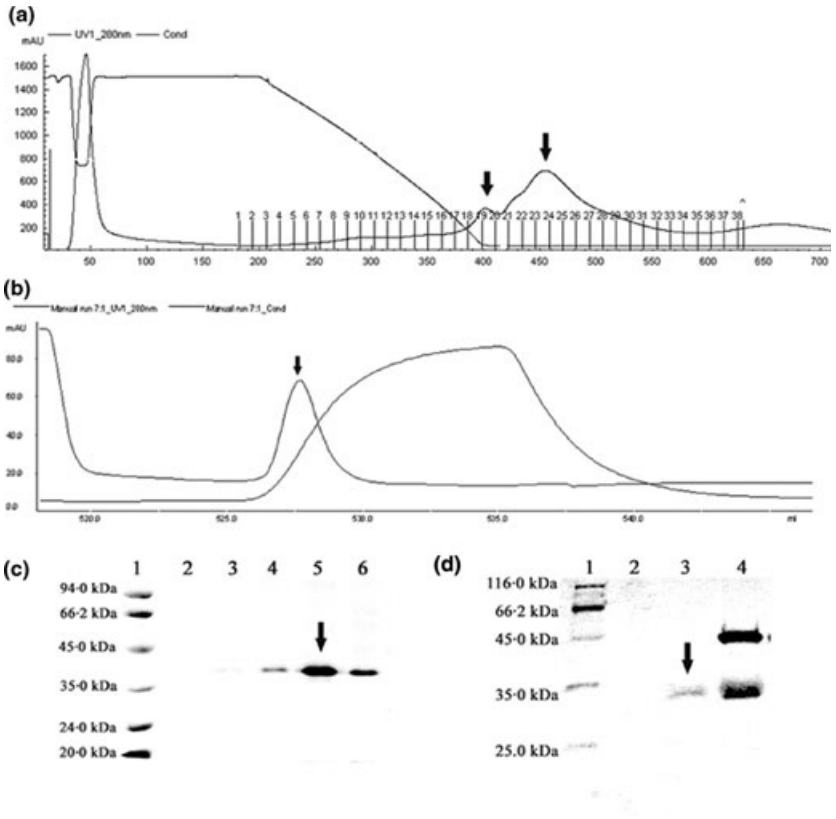

Figure 1 Purification of extracellular proteases Apr219 and Npr219 from the culture supernatant of *Bacillus* sp. RH219. (a) Result of hydrophobic interaction chromatography (HIC). The protease activities were detected in tubes (14–30); (b) Result of cation-exchange chromatography. The fractions with protease activity were obtained from the elution peak. The arrow indicated the peaks with the protease activities. (c) Twelve per cent SDS-PAGE assays indicated fractions 22–26 contained a single protein band with a molecular mass about 41 kDa, which was designed as Npr219. Line 1 represents protein marker, the arrow indicates purified Npr219. (d) After purification by cation-exchange chromatography with tubes 14–20, the fraction containing protease activity only detected one peak. The homogeneity of the purified protease with a molecular mass about 33 kDa was confirmed by 12% SDS-PAGE, designed as Apr219. Line 1 represents protein marker, the arrow indicates purified Apr219.

Table 2 Characteristics of the proteases Npr219 and Apr219

Parameter	Npr219	Apr219
Protease class	Metalloprotease	Serine protease
Molecular mass (kDa)	41	33
Optimum pH	6	10
Optimum temperature (°C)	50	60
pH stability (≥60%)	6–10	6–10
Thermostability (°C)	55	60
N-terminal amino acid sequence	AAATGTGTTL	AQSVPYGVSQ

Table 3 Effect of inhibitors on the activities of the proteases Npr219 and Apr219

Inhibitors	Concentration	Enzyme (Npr219) activity as % of control	Enzyme (Apr219) activity as % of control
PMSF	1·0 mmol l^{-1}	98·1	0·0
EDTA	1·0 mmol l^{-1}	3·7	106·3
Aprotinins	1·0 μg ml^{-1}	100·0	99·3
Leupeptin	10·0 μg ml^{-1}	109·6	99·5
Pepstatin A	10·0 μg ml^{-1}	84·4	101·3

PMSF, phenylmethylsufonylfluoride; EDTA, ethylene diaminetetraacetic acid.

the mortality by 9% within 72 h (independent samples test: $P < 0{\cdot}05$). Furthermore, the purified cuticles from nematode *P. redivivus* were treated with the purified proteases Apr219 and/or Npr219 to verify the hydrolysis of nematode cuticles *in vitro*. When treated with Apr219 alone, within 2 h, many incomplete and degraded fragments were observed. In contrast, the cuticles were intact in the negative controls and in the Npr219 treatment (Fig. 3). The results suggested nematicidal and cuticle-degrading activities for *Bacillus* sp. RH219 were mostly due to the extracellular serine alkaline protease Apr219.

PCR amplification of protease Npr219 and Apr219 genes

Protease Npr219 gene was amplified by PCR with previously designed primers. A 1577-bp fragment was obtained (GenBank accession no. DQ983789). The deduced protease (Npr219) consisted of a signal peptide of 27 amino acids, a propeptide of 194 amino acids and the mature protein of 300 amino acid residues. The sequenced N-terminal amino acids of the mature peptide were found to be located at amino acids 226–236 of the deduced amino acid sequence of Npr219.

After PCR amplification with the primers for Apr219, the nucleotide sequence of an amplified 1149-bp fragment

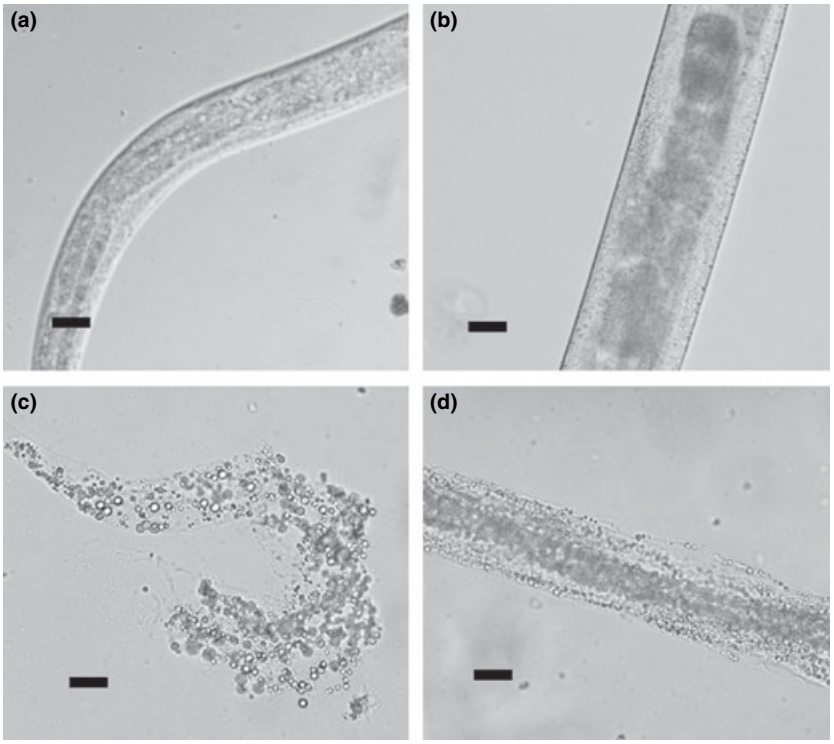

Figure 2 The action of the proteases Apr219, Npr219 and culture supernatant of *Bacillus* sp. RH219 against *Panagrellus redivivus*. (a) The cuticles of nematodes in the control were intact and smooth within 24 h. (b) The cuticles of nematodes in the Npr219 treatment were also intact within 24 h. (c) The nematode cuticles were degraded and their bodies destroyed after 24 h in the culture supernatant treatments. (d) The nematode cuticles were degraded after 24 h in the Apr219 treatments (bar = 10 μm).

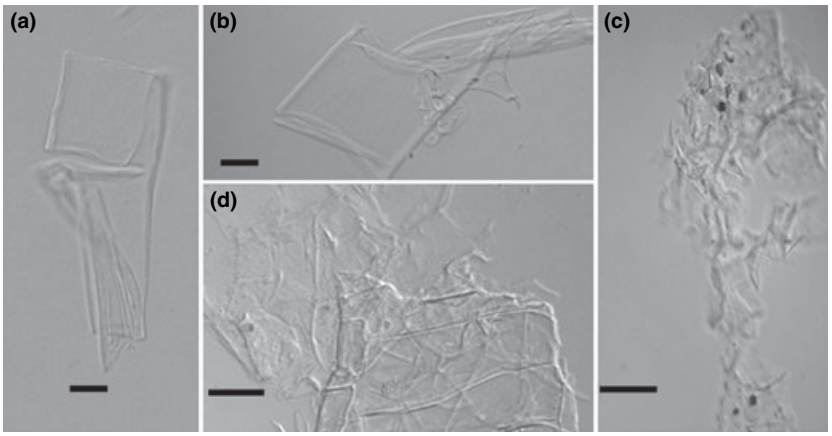

Figure 3 The action of Apr219, Npr219 and culture supernatant of *Bacillus* sp. RH219 against purified nematode cuticles. (a) The purified cuticle of *Panagrellus redivivus*. (b) The cuticle was intact in the Npr219 treatment for 2 h. (c) and (d) The cuticle was treated with culture supernatant and Apr219 for 2 h, respectively. Most extracted cuticles were destroyed and only incomplete and minor fragments were observed (bar = 10 μm).

was determined (GenBank accession no. DQ983786). The deduced protein (Apr219) consisted of a signal peptide of 32 amino acids, a propeptide of 75 amino acids and a mature protein of 275 residues. The previously sequenced N-terminal amino acids were found to be located at amino acids 108–118 of the deduced protein (Apr219). Biochemical experiments have indicated previously that Apr219 is a serine protease. The deduced amino acid sequence of Apr219 has a serine protease catalytic triad centre containing Asp32, His64 and Ser221 and shows 99% similarity with previously reported cuticle-degrading proteases BLG4 (*B. laterosporus* strain G4) and an extracellular protease (*Bacillus* sp. B16) (Huang *et al.* 2005a; Niu *et al.* 2005). Moreover, the 1149-bp fragments of Apr219 were also amplified from other four isolated nematicidal *Bacillus* spp. (A104, B101, A56, A29). Further sequencing and sequence analysis for the PCR products demonstrated that the serine protease genes were extensively distributed in the *Bacillus* spp. among the rhizobacteria with nematicidal activities.

Discussion

At present, a number of commercial biocontrol products from rhizobacteria have been developed (http://www.oardc.ohio-state.edu/apsbcc/productlist.htm) and

many plant disease biocontrol products that contain *Bacillus* spp. have been used (Gardener 2004; Schisler *et al.* 2004). Among these agents, a commercial *Bacillus*-based formulation contains both *Paenobacillus macerans* and *B. amyloliquefaciens* (Meyer 2003). Native populations of *Bacillus* and *Paenobacillus* spp. occur abundantly in most agricultural soils. Multiple *Bacillus* spp. can promote crop health in a variety of ways. For example, they can suppress plant pathogens and pests by producing antibiotic metabolites, or can directly stimulate plant host defences prior to infection and promote plant growth and health. Despite a wealth of new information on the genetics and physiology of *Bacillus* and related species, our understanding of the microbial mechanism of action against nematode populations remains very limited. Increased understanding of the nematotoxic mechanism of antagonist populations in the soil could potentially enhance the value of these species as effective biocontrol agents (Morton *et al.* 2004).

In this study, we concentrated on investigating the action mode for *Bacillus* spp. from among the nematicidal rhizobacteria. Five *Bacillus* spp. isolated from root knot nematode-infested rhizosphere soil were identified. Among these strains, *Bacillus* sp. strain RH219 showed a remarkable nematicidal activity. RH219 strain killed 80% tested nematode within 2 h and completely destroyed and digested all tested targets after 12 h. It was well known that the cuticle of nematodes is rigid and composes of proteins and chitins (Cox *et al.* 1981; Åhman 2000). The results suggested that the hydrolytic enzymes might be involved in the penetration process to help bacteria kill the hosts (Åhman 2000).

Our further analysis about nematotoxic factors indicated that an extracellular cuticle-degrading protease designed Apr219 served as an important nematicidal factor. The deduced amino acid sequence of the cloned protease gene was 99% identical to the previously reported cuticle-degrading proteases from *B. laterosporus* strain G4 and *Bacillus* sp. B16 (Niu *et al.* 2005; Tian *et al.* 2006). The consistency of these nematotoxic proteases from the different nematicidal bacterial strains suggested that these proteases must be highly conservative in this group of bacteria.

Subtilisin has long been proposed as a nematotoxic factor in nematophagous fungi (St Leger 1995; Huang *et al.* 2005b). The identified bacterial nematotoxic proteases, Apr219 from *Bacillus* sp. RH219, BLG4 from *B. laterosporus* strain G4 and extracellular protease from *Bacillus* sp. B16 (*B. nematocida*), were found to belong to this family of enzyme (Niu *et al.* 2005; Tian *et al.* 2006). Our PCR amplification and sequence analysis for the protease gene demonstrated that the gene was distributed in all other four isolated nematicidal *Bacillus* strains (A29, A56, A104, B101). The wide distribution of the serine cuticle-degrading protease in nematophagous fungi and *Bacillus* suggested that they must serve as an important nematicidal factor in balancing nematode populations in the soil (Huang *et al.* 2005a,b; Åhman 2000; Niu *et al.* 2006; Tian *et al.* 2006).

Other than the extracellular alkaline protease, the role of a neutral protease from RH219 during the infection of nematodes was also investigated. It was reported that an EDTA-inhibited neutral protease could degrade nematode cuticle in the presence of protease BLG4 and the two different bacterial extracellular proteases might play a synergetic role in the penetration of nematode cuticle (Niu *et al.* 2006; Tian *et al.* 2007). Our current results are consistent with this conclusion. The neutral protease Npr219 from *Bacillus* sp. RH219 had little effect. However, its addition to Apr219 increased the nematode mortality rate by 9% compared with that of nematodes treated with Apr219 alone.

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References

Åhman, J. (2000) Extracellular serine proteases as virulence factors in nematophagous fungi: molecular characterization and functional analysis of the PII protease in *Arthobotrys oligospora*. *Doctoral Dissertation*, Lund University.

Åhman, J., Johansson, T., Olsson, M., Punt, P.J., van den Hondel, C.A.M.J.J. and Tunlid, A. (2002) Improving the pathogenicity of a nematode-trapping fungus by genetic engineering of a subtilisin with nematotoxic activity. *Appl Environ Microbiol* **68**, 3408–3415.

Clarkson, J.M. and Charnley, A.K. (1996) New insights into the mechanisms of fungal pathogenesis in insects. *Trends Microbiol* **4**, 197–203.

Cox, G.N., Kusch, M. and Edgar, R.S. (1981) Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. *J Cell Biol* **90**, 7–17.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Rie, J.V., Lereclus, D., Baum, J. and Dean, D.H. (1998) Review of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**, 807–813.

Dillon, J.A.R., Nasim, A. and Nestman, E.R. (1985) *Recombinant DNA Methodology*. New York: John Wiley & Sons.

Fang, Z.D. (1998) *Methods of Plant Pathology*. Beijing: China Agriculture Press.

Feitelson, J.S., Payne, J. and Kim, L. (1992) *Bacillus thuringiensis*: insects and beyond. *Nat Biotechnol* **10**, 271–275.

Gardener, B.B.M. (2004) Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. *Phytopathology* **94**, 1252–1258.

Huang, X.W., Tian, B.Y., Niu, Q.H., Yang, J.K., Zhang, L.M. and Zhang, K.Q. (2005a) An extracellular protease from *Brevibacillus laterosporus* G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes. *Res Microbiol* **156**, 719–727.

Huang, X.W., Zhao, N.H. and Zhang, K.Q. (2005b) Extracellular enzymes serving as virulence factors in nematophagous fungi involved in infection of the host. *Res Microbiol* **115**, 811–816.

Krebs, B., Höeding, B., Kübart, S., Workie, M.A., Junge, H., Schmiedeknecht, G., Grosch, R., Bochow, H. *et al.* (1998) Use of *Bacillus subtilis* as biocontrol agent. I. Activities and characterization of *Bacillus subtilis* strains. *Z Pflanzenkr Pflanzenschutz* **105**, 181–197.

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Research in Bacterial Systematics* ed. Stackebrandt, E. and Goodfellow, M. pp. 115–175. Chichester: Wiley.

Lugtenberg, B.J.J. and Dekkers, L.C. (1999) What makes *Pseudomonas* bacteria rhizosphere competent? *Environ Microbiol* **1**, 9–13.

Marroquin, L.D., Elyassnia, D., Griffitts, J.S., Feitelson, J.S. and Aroian, R.V. (2000) *Bacillus thuringiensis* (Bt) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. *Genetics* **155**, 1693–1699.

Meyer, S.L.F. (2003) United States Department of Agriculture-Agriculture Research Service research programs on microbes for management of plant-parasitic nematodes. *Pest Manag Sci* **59**, 665–670.

Meyer, S.L.F., Huettel, R.N., Liu, X.Z., Humber, R.A., Juba, J. and Nitao, J.K. (2004) Activity of fungal culture filtrates against soybean cyst nematode and root-knot nematode egg hatch and juvenile motility. *Nematology* **6**, 23–32.

Morton, C.O., Hirsch, P.R. and Kerry, B.R. (2004) Infection of plant-parasitic nematodes by nematophagous fungi: a review of the application of molecular biology to understand infection processes and to improve biological control. *Nematology* **6**, 161–170.

Niu, Q.H., Huang, X.W., Tian, B.Y., Yang, J.K., Liu, J., Zhang, L. and Zhang, K.Q. (2005) *Bacillus* sp. B16 kills nematodes with a serine protease identified as a pathogenic factor. *Appl Microbiol Biotechnol* **69**, 722–730.

Niu, Q.H., Huang, X.W., Zhang, L., Li, Y.X., Li, J., Yang, J. K. and Zhang, K.Q. (2006) A neutral protease from *Bacillus nematocida*, another potential virulence factor in the infection against nematodes. *Arch Microbiol* **185**, 439–448.

Priest, F. (1993) Systematics and ecology of *Bacillus*. In *Bacillus subtilis and Other Gram-Positive Bacteria* ed. Sonenshein, A.L., Hoch, J.A. and Losick, R. pp. 3–16. Washington, DC: American Society for Microbiology Press.

Radnedge, L., Agron, P.G., Hill, K.K., Jackson, P.J., Ticknor, L.O., Keim, P. and Andersen, G.L. (2003) Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. *Appl Environ Microbiol* **69**, 2755–2764.

Schisler, D.A., Slininger, P.J., Behle, R.W. and Jackson, M.A. (2004) Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology* **94**, 1267–1271.

Siddiqui, Z.A. and Mahmood, I. (1999) Role of bacteria in the management of plant parasitic nematodes: a review. *Bioresour Technol* **69**, 167–179.

Siddiqui, I.A., Haas, D. and Heeb, S. (2005) Extracellular protease of *Pseudomonas fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode. *Meloidogyne incognita*. *Appl Environ Microbiol* **71**, 5646–5649.

Sikora, R.A. (1992) Management of the antagonistic potential in agriculture ecosystems for the biological control of plant parasitic nematodes. *Annu Rev Phytopathol* **30**, 245–270.

St Leger, R.J. (1995) The role of cuticle-degrading proteases in fungal pathogenesis of insects. *Can J Bot* **73**, 1119–1125.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Tian, B.Y., Li, N., Lian, L.H., Liu, J.W., Yang, J.K. and Zhang, K.Q. (2006) Cloning, expression and deletion of the cuticle-degrading protease BLG4 from nematophagous bacterium *Brevibacillus laterosporus* G4. *Arch Microbiol* **186**, 297–305.

Tian, B.Y., Yang, J.K., Lian, L.H., Wang, C.Y., Li, N. and Zhang, K.Q. (2007) Role of neutral protease from *Brevibacillus laterosporus* in pathogenesis of nematode. *Appl Microbiol Biotechnol* **74**, 372–380.

Wei, J.Z., Hale, K., Carta, L., Platzer, E., Wong, C., Fang, S.C. and Aroian, R.V. (2003) *Bacillus thuringiensis* crystal proteins that target nematodes. *Proc Natl Acad Sci USA* **100**, 2760–2765.