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

L.H. Lian¹,* , B.Y. Tian¹,* , R. Xiong², M.Z. Zhu¹, J. Xu¹ and K.Q. Zhang¹

¹ Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming, Yunnan, China
² School of Life Science and Technology, China Pharmaceutical University, Nanjing, Jiangsu, China

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 nematocidal 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 nematocidal activities

Bacteria were isolated from the rhizosphere of tobacco in five root knot nematode-infested farms in Yunnan province in China (Kaixuan, 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'-AGA-GTTTGCCTGGCTCAG-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 nematocidal 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 cultures 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 = 0.05 or P = 0.01), 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, 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).

Nematocidal 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 [phenylmethylsulfonyl-fluoride (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 (GGGGGATTATTGTGGGGTTT) and np2 (TCATATCGACAGCATTCCCA). PCR cycling conditions were 5 min at 94°C for the first pre-denaturation 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 (GGGCCTAGGTCAGGCGCAAAAAATGATG) and ap2 (CGCGGATCTCCTATTGGTCGCGCTGTAC) 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 nematocidal 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 nematocidal 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 nematocidal activity (Table 1). Most of the bodies and cuticles for the dead nematodes were degraded and destroyed by these strains.
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 (AQSVPGVSQ) 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
the mortality by 9% within 72 h (independent samples test: \( P < 0.05 \)). 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 \( B. \) sp. RH219 were mostly due to the extracellular serine alkaline protease Apr219.

Table 2  Characteristics of the proteases Npr219 and Apr219

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

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

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (μmol l(^{-1}))</th>
<th>Enzyme (Npr219) activity as % of control</th>
<th>Enzyme (Apr219) activity as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>1.0</td>
<td>98.1</td>
<td>0.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.0</td>
<td>3.7</td>
<td>106.3</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>10.0</td>
<td>100.0</td>
<td>99.3</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>10.0</td>
<td>109.6</td>
<td>99.5</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>10.0</td>
<td>84.4</td>
<td>101.3</td>
</tr>
</tbody>
</table>

PMSF, phenylmethylsulfonylfluoride; EDTA, ethylene diaminetetraacetic acid.

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...
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
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 extra-
cellular 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 nem-
taticidal Bacillus spp. (A104, B101, A56, A29). Further
sequencing and sequence analysis for the PCR products
demonstrated that the serine protease genes were exten-
sively distributed in the Bacillus spp. among the rhizobac-
teria 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 Paenibacillus macerans and B. amyloliquefaciens (Meyer 2003). Native populations of Bacillus and Paenibacillus 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 nematocidal 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 composed 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 protease gene 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 synergistic 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.

**Acknowledgements**

We thank Dr Xiaolong Cui for the identification of bacterial strains. This work was funded by projects from the National Natural Science Foundation of China (approved No. 30630005) and the Department of Science and Technology of Yunnan Province (approved Nos. 2005NG05 and 2004C0001Z).

**References**


