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Role of an extracellular neutral protease in infection against nematodes by *Brevibacillus laterosporus* strain G4

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Abstract Proteases have been proposed as virulence factors in microbial pathogenicity against nematodes. However, what kinds of extracellular proteases from these pathogens and how they contribute to the pathogenesis of infections against nematode in vivo remain largely unknown. A previous analysis using a strain with a deletion in an extracellular alkaline protease BLG4 gene from *Brevibacillus laterosporus* demonstrated that BLG4 was responsible for the majority of nematicidal activity by destroying host's cuticle. In recent studies, a neutral protease NPE-4, purified from the mutant BLG4–6, was found to be responsible for the majority of the remaining EDTA-inhibited protease activity. However, the purified NPE-4 and recombinant NPE-4 in a related species *Bacillus subtilis* showed little nematicidal activity in vitro and were unable to degrade the intact cuticle of the host. It is interesting to note that the addition of NPE-4 improved the pathogenicity of crude enzyme extract from wild-type *B. laterosporus* but had no effect on the BLG4-deficient mutant. This result suggests that NPE-4 functions in the presence of protease BLG4. Moreover, NPE-4 could degrade proteins from the inner layer of purified cuticles from nematode *Panagrellus redivivus* in vitro. These results indicated that the two different bacterial extracellular proteases might play differential roles at different stages of infection or a synthetic role in penetration of nematode cuticle in *B. laterosporus*. This is among the first reports to systematically evaluate and define the roles of different bacterial extracellular proteases in infection against nematodes.

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Introduction

As a pathogen, *Brevibacillus laterosporus* has been demonstrated to have a very wide spectrum of biological activities. For example, its toxicities toward the beetle, the adult mollusk, the larvae of mosquitoes, and the nematodes were recently observed (Oliveira et al. 2004). Despite showing such wide-range biological activities, *B. laterosporus* has not been seriously considered as an agent for biological control. The reasons may be due to its generally weak insecticidal activities than that of *Bacillus thuringiensis* serovar israelensis and to our lack of understanding about its pathogenic mode against invertebrates (Favret and Yousten 1985; Orlova et al. 1998). Increased understanding of *B. laterosporus* could potentially enhance the value of this species as an effective biocontrol agent (Oliveira et al. 2004; Smirnova et al. 1996; Zahner et al. 1999).

In agriculture, parasitic nematodes can cause serious crop losses worldwide and are among the most important agricultural pests (Jatala 1986; Mankau 1980). So far, it has been reported that four nematode species (including three parasitic nematodes, *Heterodera glycines*, *Trichostrongylus colubriformis*, and *Bursaphelenchus xylophilus*, and a saprophytic nematode *Panagrellus redivivus*) could be killed by different *B. laterosporus* isolates (Huang et al. 2005; Oliveira et al. 2004). Among these isolates, *B. laterosporus* strain G4, which was isolated from the soil sample as a nematode pathogen in Yunnan province in China, is being extensively studied (Huang et al. 2005).

B. laterosporus strain G4 could penetrate the nematode cuticles and eventually digest the target organism (Huang et

al. 2005). Our histopathological observation and molecular biological analysis demonstrated that its major pathogenic activity could be attributed to an extracellular alkaline serine protease, designed as BLG4 (Huang et al. 2005; Tian et al. 2006). The purified and recombinant BLG4 showed significant killing effect against nematodes (Huang et al. 2005; Tian et al. 2006). BLG4-deficient strain BLG4–6 contained only about 43% killing effects of the wild-type strain against tested nematodes and about 22% cuticle-degrading activity (not completely digested). These results suggest that BLG4 is not the only virulence factor responsible for nematicidal activities, and other pathogenic factors such as other extracellular enzymes or toxins are likely involved in the infection against nematodes by *B. laterosporus* strain G4 (Tian et al. 2006).

In a recent study, we found that most of the residue protease activity of BLG4–6 mutant could be inhibited by EDTA (Tian et al. 2006). In the present study, we purified a neutral protease (NPE-4), which was inhibited by EDTA, from the BLG4-deficient mutant BLG4–6. Its encoding gene was subsequently cloned from a genomic library and expressed in *Bacillus subtilis*. Our results indicate that the two bacterial extracellular proteases, BLG4 and NPE-4, may play differential roles at different stages of infection or a synthetic role during penetration of nematode cuticle by *B. laterosporus*.

Materials and methods

Bacterial strains, plasmids, and cultivation conditions

B. laterosporus strain G4 (CCTCC, M203045) was maintained on YPD (1% yeast extract, 2% peptone, 2% glucose, pH 7.0) agar or Luria Bertani (LB) agar. *B. laterosporus* mutant BLG4–6, a wild-type strain G4 derivative but with the extracellular serine protease BLG4 disrupted, was cultured in the LB medium supplemented with 3 μg/ml chloramphenicol (Tian et al. 2006). *B. subtilis* strain WB600, a six-extracellular-protease-deficient strain, was used for heterologous expression of *B. laterosporus* neutral protease (Westers et al. 2004). The nematode *P. redivivus* was grown axenically for 4–7 days at 28°C in an oat medium (oat 20 g; water 80 ml). The plasmids used in this study are listed in Table 1.

Table 1 Plasmid used in this study

Plasmid	Description	Source/reference
pNPE-4	Cloning vector containing the NPE-4 structural gene as well as regulatory elements from *B. laterosporus*	This study
pHY300PLK	Expression vector in *B. subtilis*	Purchase from TaKaRa Bio Inc., Japan
pHYNPE-4	Expression vector containing the NPE-4 structural gene	This study

Purification of the extracellular neutral protease NPE-4 and the analysis of its N-terminal amino acid sequence

B. laterosporus mutant BLG4–6 was grown in a YPD medium at 28°C on a rotary shaker at 220 rpm for 60 h. A 300-ml culture was collected, and bacteria were removed by centrifugation at 8,500 rpm for 15 min. The supernatant solution was used for further purification. After adding ammonium sulfate to 40% saturation (*w*/*v*), the mixture was centrifuged at 8,500 rpm for 20 min at 4°C, and the supernatants was transferred to a new tube. Then, ammonium sulfate was added to 70% saturation, and centrifugation was again carried out under the same conditions. After removing supernatants, the precipitate was dissolved in 20 ml of 50 mM phosphate buffer (pH 7.0) and dialyzed thoroughly against 20-fold volumes of the 50 mM phosphate buffer (pH 7.0) (dialysis molecular size: 8,000–15,000) at 4°C. The dialyzed solution was then adjusted to pH 7.0 and applied to a HiTrap™ SP FF column (1 ml; Amersham Pharmacia Biotech) after being equilibrated with 10 mM phosphate buffer (pH 7.0). Bound proteins were eluted with a linear gradient of increasing concentrations of NaCl (0–2 M, pH 7.0). The protease activities of the fractions were measured according to previously described methods (Huang et al. 2005). The fractions containing protease activity but that could be inhibited by EDTA were pooled and applied to hydrophobic interaction chromatography, supplemented with 1 M ammonium sulfate and with pH adjusted to 7.0. The resulting sample was applied to a HiPrep™ 16/10 phenyl FF column (high sub; Amersham Pharmacia Biotech) that had been equilibrated with 50 mM phosphate buffer containing 1 M ammonium sulfate (pH 7.0). With 50 mM phosphate buffer, the bound proteins were eluted with a liner gradient of 1–0 M ammonium sulfate. The resulting fractions were assayed for protease activity and 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Huang et al. 2005).

The N-terminal amino acid sequence of the purified NPE-4 was determined according to previously described methods (Huang et al. 2005). The N-terminal sequence of the first 10 amino acids was used as a query for BLAST searches in the GenBank.

Cloning of the NPE-4 gene

A genomic library of *B. laterosporus* was used for screening to obtain the complete NPE-4 gene including its

promoter, coding sequence, and terminator (Tian et al. 2006). Primers for PCR screening were designed on the basis of the sequence of *Bacillus amyloliquefaciens* neutral protease (npr) gene (GenBank accession number K02497): I-1, 5′-GTGGGTTTAGGTAAG-3′; I-2, 5′-TTACAAGCC GACCGC-3′ (Vasantha et al. 1984). Screening of positive colonies was performed using PCR with primers I-1 and I-2 according to previously described methods (Tian et al. 2006). The inserts and partial vector sequences of the positive colonies were obtained. The nucleotide sequence was analyzed using DNAman software package, and the promoter was predicted using the BDGP Neural Network Promoter Prediction interface (http://www.fruitfly.org/seq_tools/promoter.html) (Reese 2000).

Heterologous expression of NPE-4

The complete NPE-4 gene was obtained from the genomic DNA by PCR using newly designed primers according to the sequenced fragment of pNPE-4: primerNPE-1, 5′-CGCAA GCTTTCGCGTCATTCGACTCATGTCTGAT-3′; primer NPE-2, 5′-CGCGGATCCTCATGGTGAAGC CACTGT GAAATGT-3′. These two primers contained *Hin*dIII and *Bam*HI restriction sites (underlined), respectively. The PCR product and plasmid vector pHY300PLK were digested by *Bam*HI/*Hin*dIII, ligated using T4 DNA ligase to form the recombined expression plasmid pHYNPE-4. This construct was then transformed into *B. subtilis* WB600 according to the method described by Spizizen (1958). Transformants with tetracycline resistance and protease activity were grown for 60 h at 37°C (220 rpm) in a super-rich medium (2% yeast extract, 2.5% tryptone, 0.3% K_2HPO_4, 3% glucose) supplemented with 100 μg/ml of tetracycline. Recombinant NPE-4 protein was purified from the medium according to the method described above. Protease activities of pHYNPE-4 transformants and the negative control transformants containing only pHY300PLK were assayed using a zymographic technique that allowed visualization of substrate-degrading zones directly in casein-containing SDS-PAGE (15%) (Lantz et al. 1991).

Bioassays

The free-living nematode *P. redivivus* was selected for the bioassays. The toxic effects of *B. laterosporus* on *P. redivivus* were tested according to previously described protocols (Huang et al. 2005). The numbers of dead nematodes were determined under a light microscopy. The bioassay experiments were performed in triple parallels and repeated at least five times. Negative controls were incubated with water, 0.1 M bovine serum albumin (BSA), and purified enzyme that had been boiled for 15 min to eliminate the enzymatic activities.

Fragments of cuticle of the nematode *P. redivivus* were purified according to the method described by Cox et al. (1981). After 200 μl of purified enzyme solution was mixed with nematode cuticle, the degradation of cuticle was observed under a light microscope once every hour. For negative controls, 0.1 M BSA and purified enzyme boiled for 15 min were added to the nematode cuticle.

Results

Purification of NPE-4

After salting out using 40–70% ammonium sulfate, cation-exchange chromatography, and hydrophobic interaction chromatography, the eluted fractions containing the EDTA-inhibited protease were pooled and analyzed (Fig. 1a,b). The homogeneity of the purified protease was confirmed by 12% SDS-PAGE (Fig. 1c). The molecular weight of the purified protease was found to be 41 kDa. The resulting EDTA-inhibited protease showed a maximum activity in pH 7.0 at 37°C. This enzyme was designed as the neutral protease NPE-4.

Cloning and analysis of NPE-4 gene

The N-terminal amino acid sequence of the purified protease NPE-4 was AAATGTGTTL, which has a 90% similarity to the N-terminal, first 10 amino acids of the *B. amyloliquefaciens* neutral protease (npr) (GenBank accession number K02497). The primers for screening the purified protease NPE-4 gene from the *B. laterosporus* genomic library were subsequently designed according to the published sequence for *B. amyloliquefaciens* neutral protease (npr). In our experiment, a 1,566-bp fragment was amplified. Thirteen positive clones were identified from a total of about 7,000 clones. After restriction analysis, three positive clones were sequenced, and a genomic clone (pNPE-4) containing a complete open reading frame (ORF), promoter, and terminator sequence was selected to use for further investigation (GenBank accession number DQ983787).

Our amplified genomic clone pNPE-4 contains the complete nucleotide sequence of NPE-4 gene as well as the 5′ and 3′ flanking regions (Fig. 2). The identified ORF within the 1,566-bp fragment begins with a translation initiation site (GTG) and ends with a translation termination codon (TAA). Several other elements homologous to previously reported prokaryotic promoter elements (GenBank accession number X61006) were also identified further upstream from the ATG start codon with the help of Internet promoter prediction programs (Nakayama et al. 1992; Vasantha et al. 1984). A putative ribosomal binding

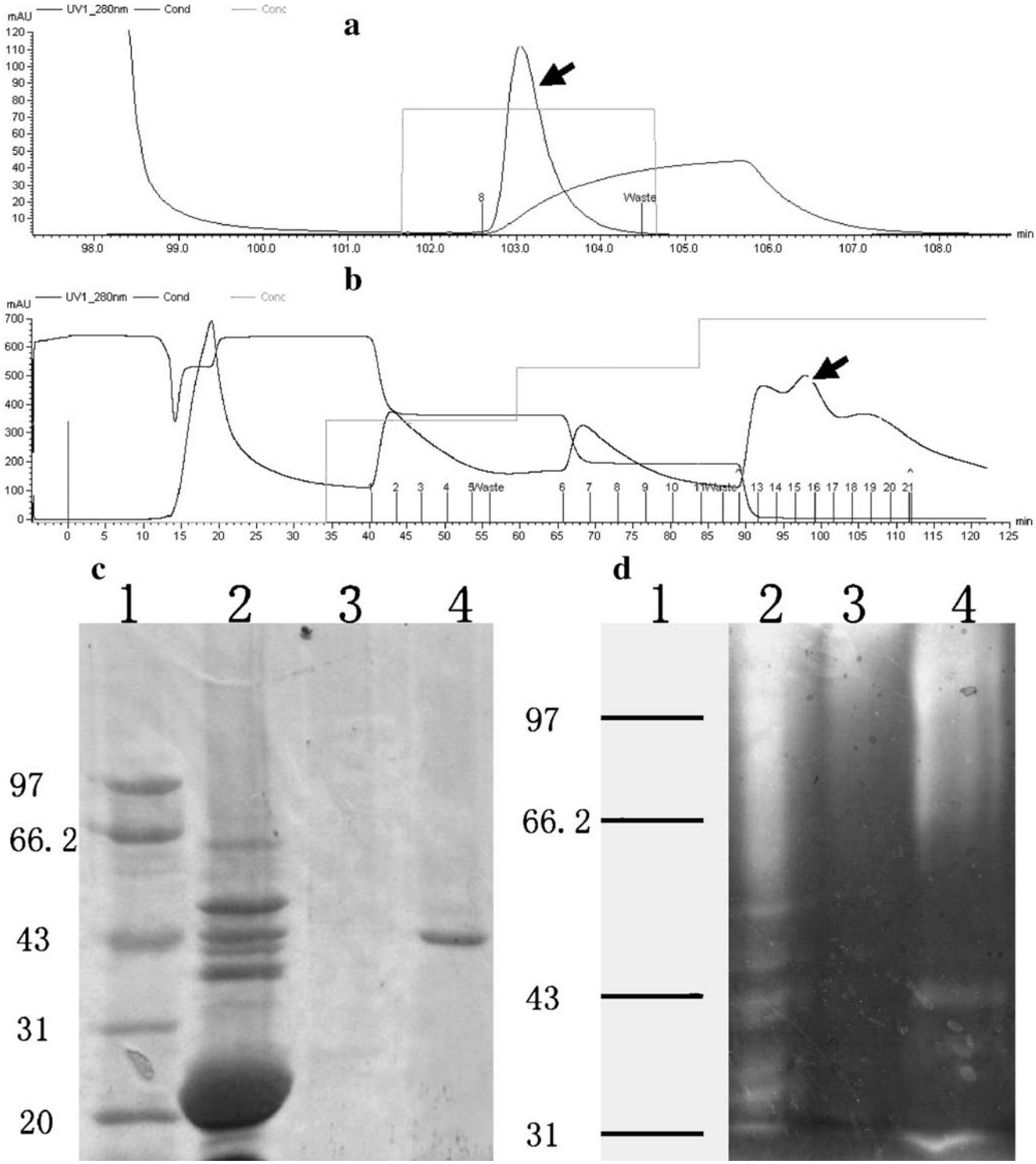


Fig. 1 Purification of extracellular neutral protease NPE-4 and SDS-PAGE analysis. **a** Cation-exchange chromatography on a HiTrap™ SP FF column after dialyzing the precipitate solution from the culture supernatant of *B. laterosporus* mutant BLG4–6. The peak with neutral protease activity inhibited by EDTA is indicated by an *arrow*. **b** Hydrophobic interaction chromatography on a HiPrep™ 16/10 phenyl FF column of fractions containing neutral protease activity from cation-exchange chromatography. The peak containing protease NPE-4 is indicated by an *arrow*. **c** SDS-PAGE (12%) of fractions containing neutral protease activity from hydrophobic interaction chromatography yielded a single protein band with a molecular mass of about 41 kDa. *Line 1* represents the protein marker; *lines 2–4* represent fraction containing neutral protease activity from cation-exchange chromatography, control, and the purified NPE-4 from hydrophobic interaction chromatography, respectively. **d** Zymographic analysis in casein-containing SDS-PAGE (15%) showed more than one proteolytic band present in the supernatant of *B. laterosporus* mutant BLG4–6 and recombinant NPE-4 band with activity present in the supernatant of pHYNPE-4 transformant. *Line 1* represents the protein marker; *lines 2–4* represent *B. laterosporus* mutant BLG4–6, pHY300PLK transformant controls, which contain only vector pHY300PLK in strain WB600, and pHYNPE-4 transformant, respectively

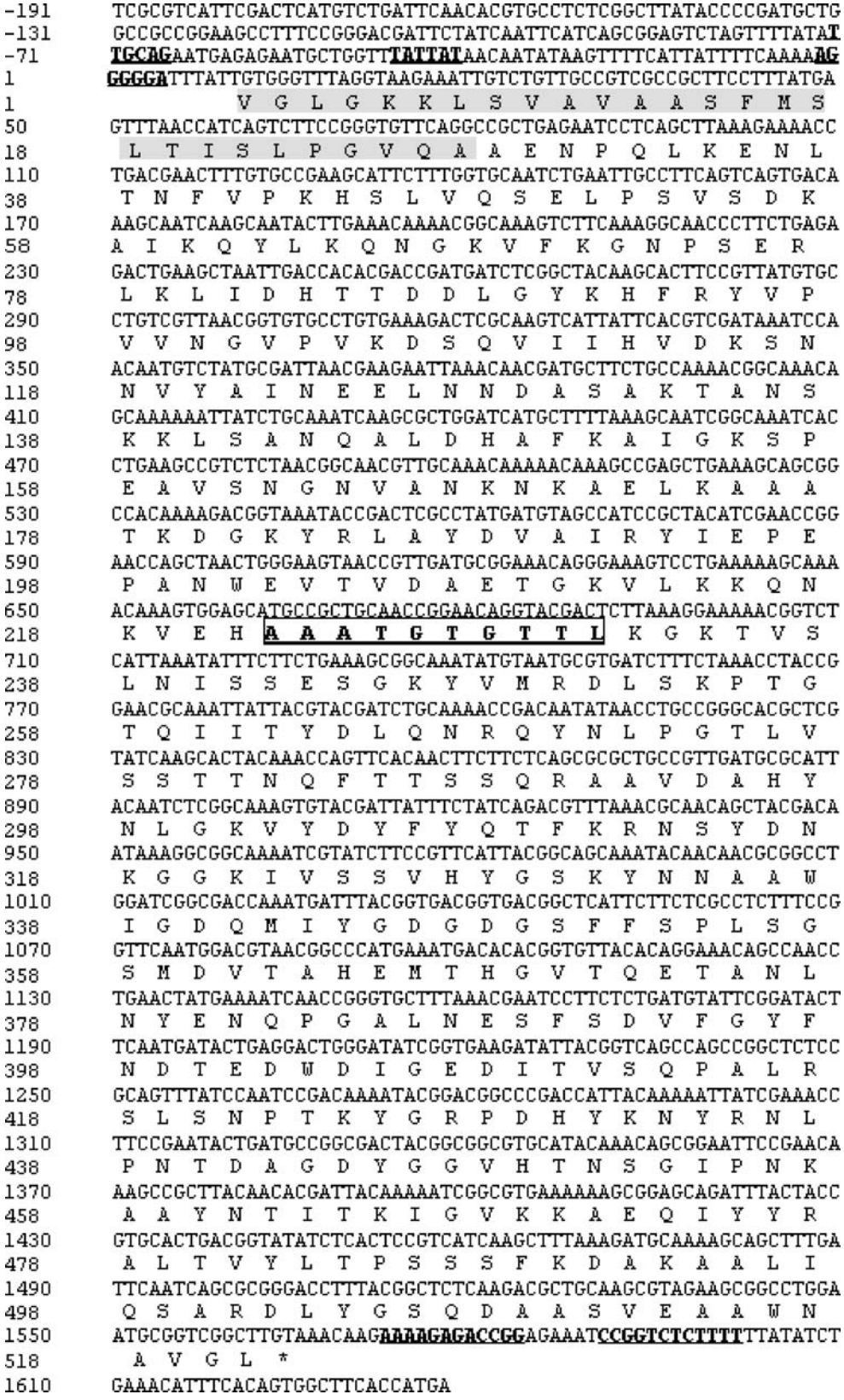


Fig. 2 The complete structure of the NPE-4 gene from *B. laterosporus*. The number of nucleotides (*left*) starts at the predicted translational initiation GTG codon (the G in GTG being +1). Putative −35 and −10 promoter regions and ribosome binding site are *underlined*. The stem-loop structure of the potential transcription terminator is *underlined* after the translation stop codon. The previously sequenced 10 N-terminal amino acids are *framed and shown in bold*

site with the sequence AGGGGGA was identified at 7 bp upstream of GTG. At −67 bp (the first G in GTG being +1), the sequence TTGCAG indicates a potential −35 region, and a putative −10 region TATTAT was identified at −43 bp. The stem-loop structure of the potential transcription terminator was identified at 6 bp downstream from the termination codon (TAA). The deduced protease consisted of a signal peptide of 27 amino acids, a propeptide of 194

Table 2 Nematotoxic activities (*P. redivius*) of crude extracellular protein extract and neutral protease NPE-4 from *B. laterosporus* mutant BLG4-6

Samples	12 h mortality % (SD[a])	24 h mortality % (SD[a])	48 h mortality % (SD[a])
Water	3 (1.1)	7 (0.8)	13 (1.7)
NPE-4 (boiled)	5 (0.3)	8 (0.5)	15 (1.1)
Native NPE-4	7 (1.2)	10 (0.8)	17 (1.3)
Recombinant NPE-4	5 (0.2)	10 (2.4)	16 (2.3)
Crude enzyme from wild-type strain G4 (A)			
Without purified NPE-4	69 (10.9)	85 (8.6)[b]	100 (4.3)
With purified NPE-4	73 (6.2)	97 (2.3)[b]	100 (1.1)
Crude enzyme from mutant strain BLG4-6 (B)			
Without purified NPE-4	10 (0.9)	21 (3.6)	44 (4.9)
With purified NPE-4	13 (4.4)	21 (9.7)	47 (7.3)

[a] indicate the deviation range of Morality.
[b] indicate statistically significant difference between the two treatments, df=19, P<0.01.

amino acids, and the mature protease of 300 amino acid residues. The sequenced N-terminal amino acids of the mature peptide were found to be located at amino acids 222 to 231 of deduced amino acid sequence of NPE-4.

Heterologous expression of NPE-4

The *B. subtilis* expression vector pHYNPE-4 was constructed by inserting the complete gene of NPE-4 with its promoter and terminator sequences in the pHY300PLK vector. After transforming into *B. subtilis*, all the tetracycline-resistant colonies showed significant protease activity compared to pHY300PLK transformant controls, which only contained the pHY300PLK vector in strain WB600. SDS-PAGE assay for protease activity of the supernatant revealed that pHYNPE-4 transformant had an additional substrate-degrading band of approximately 41 kDa, which was not present in the control (Fig. 1d). The expressed NPE-4 was purified according to a previously described method as above.

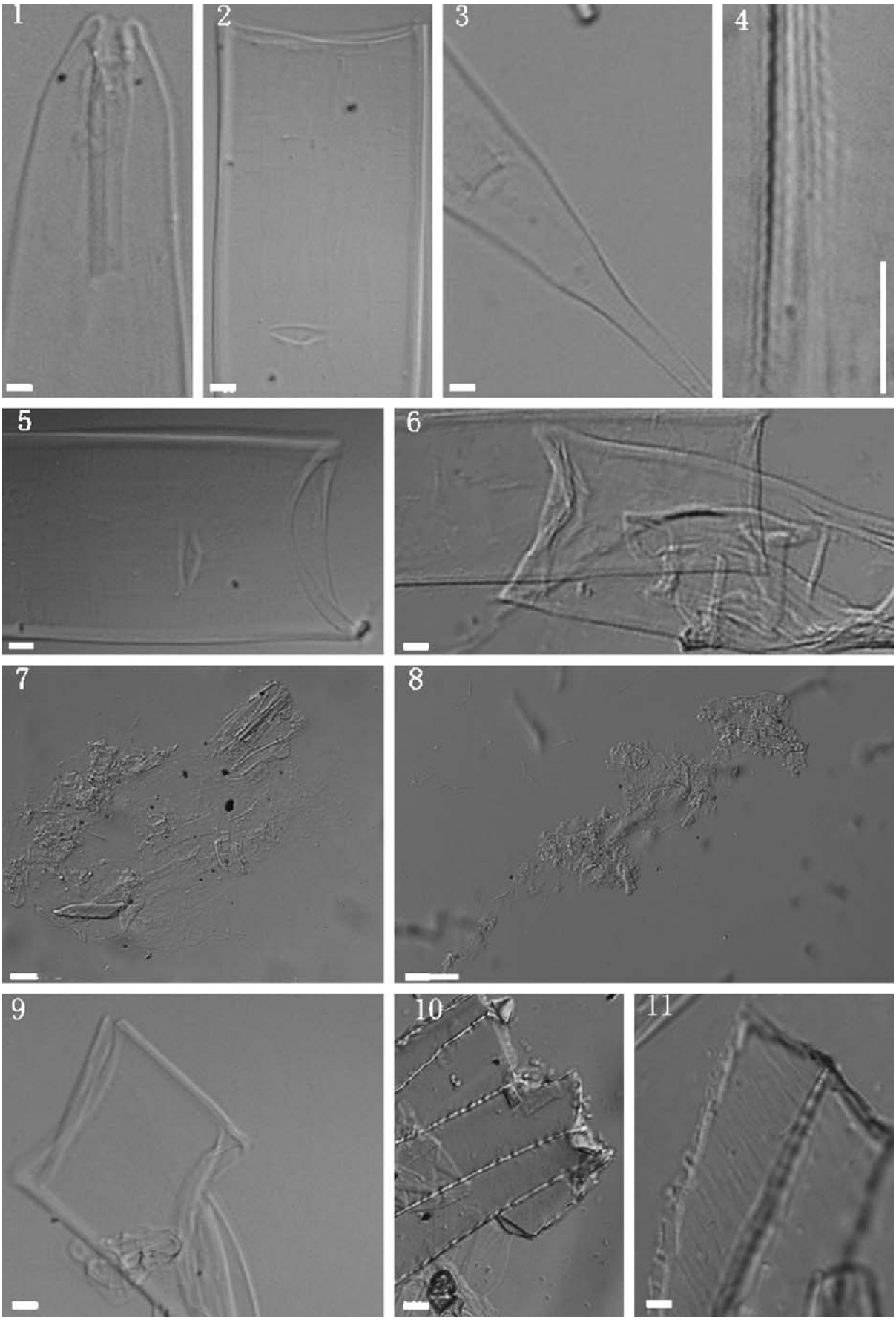
Bioassays

After treatment with BLG4 (protease activity units: 1,080 U/ml at 37°C, pH 7.0), purified NPE-4 (protease activity units: 980 U/ml at 37°C, pH 7.0), recombinant NPE-4 (protease activity units: 830 U/ml at 37°C, pH 7.0), and the negative control (protease K activity: 1000 U/ml at 37°C, pH 7.0), the mortalities of tested nematodes were checked under a light microscope within 12, 24, and 48 h, respectively. Bioassay results indicated that except BLG4, other treatments, including native or recombinant NPE-4, showed little nematicidal activity (independent samples test: $P>0.05$). However, in another assay, when NPE-4 (protease activity units: 420 U/ml at 37°C, pH 7.0) was added to the crude protein extracts (protease activity units: 2,300 U/ml at 37°C, pH 7.0) from wild-type *B. laterosporus* strain G4, we observed higher mortality and faster killing than the treatment with only the crude protein extracts from the wild-type *B. laterosporus* strain G4. Specifically, the mortality in the treatment with NPE-4 was about 4% higher (73 vs 69%) within 12 h (independent samples test: $P>0.05$) and 12% higher (97 vs 85%) within 24 h (independent samples test: $P<0.01$) than those without (Table 2). No significant effects were observed when similar amounts of NPE-4 were added to the crude protein extracts from *B. laterosporus* mutant BLG4–6 (independent samples test: $P>0.05$) (protease activity units: 710 U/ml at 37°C, pH 7.0).

Furthermore, the purified cuticles from *P. redivivus* were compared among treatments with BLG4, native, and recombinant NPE-4 to evaluate the mechanisms of action of between the two proteases. The purified nematode cuticles generally maintain a rigid, tube-like appearance and largely keep intact even after treatment by sonication and SDS. SDS treatment can remove tightly adhering muscle fragment and other components of the body wall (Cox et al. 1981). Most extracted cuticles were destroyed, and only incomplete and minor fragments were observed after treated with BLG4 for 2 h. However, no significant degradation was found in NPE-4 treatments and controls. After 24 h of incubation, the outer cuticle layer in the treatments of NPE-4 was still intact, and striates were clear. In contrast, compared to the control group, the lateral sections of cuticle became irregular, and the whole body of the cuticle appeared faint in samples treated with NPE-4 (Fig. 3).

Discussion

Microbial proteases have been proposed as virulence factors in the pathogenesis of nematodes by microorganisms. Previous studies have suggested that microbial proteases may contribute to infection of hosts by degrading the host's protective barriers (Åhman et al. 2002; Huang et al. 2004).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◀ **Fig. 3** The action of protease against purified nematode cuticle observed under a dissecting microscope. *1–4* The purified cuticle of *P. redivivus*. *5–6* The cuticle was intact in negative controls for 2 and 24 h, respectively. *7–8* The cuticle was treated with extracellular alkaline protease BLG4 for 2 and 24 h, respectively. Most extracted cuticles were destroyed, and only incomplete and minor fragments were observed. After 24 h, the cuticle was completely degraded. *9–11* The cuticle was treated with extracellular neutral protease NPE-4 for 2 and 24 h, respectively. No significant degradation was found in NPE-4 treatments for 2 h. However, the lateral sections of cuticle became irregular, and the whole body of the cuticle appeared faint after being treated with NPE-4 for 24 h

The most compelling evidences to support microbial proteases as virulence factors derived from studying protease-deficient mutants (Åhman et al. 2002; Tian et al. 2006). In nematophagous fungi, it is believed that extracellular serine proteases are involved in several steps of the infection: releasing nutrients for pathogenic growth, facilitating penetration by degrading proteins of the cuticle, and digesting the host tissue. It has also been shown that bacterial proteases can degrade and digest nematode cuticle or even kill hosts (Åhman et al. 2002; Clarkson and Charnley 1996; Meyer 2003; Morton et al. 2004). However, what kind of extracellular proteases from these nematophagous microorganisms and how they contribute to the pathogenesis in infections of nematodes in vivo remain largely unknown.

B. laterosporus strain G4 shows high toxicity to the tested nematodes, with activities similar to nematophagous fungi. Bacteria can also directly penetrate cuticles of nematodes to kill their hosts (Huang et al. 2005). An extracellular alkaline protease BLG4 was first identified from *B. laterosporus* as a pathogenic factor. Histopathological observations and the analysis of a strain with a deletion of BLG4 in *B. laterosporus* demonstrated that BLG4 was responsible for the majority of nematicidal activity by destroying the host's cuticle (Huang et al. 2005; Tian et al. 2006).

In a previous study, some protease and cuticle-degrading activities were still detectable after deletion of BLG4 in mutant BLG4–6, suggesting that other enzymes were probably involved in nematode infection by *B. laterosporus*. The results of zymographic analysis indicated that there was more than one proteolytic band (Fig. 1d) present in the supernatant of mutant BLG4–6. However, the remaining protease activity of BLG4–6 mutant could be almost completely inhibited by EDTA (Tian et al. 2006). A neutral protease NPE-4, purified from mutant BLG4–6, was found to be responsible for the majority of the remaining protease activity. However, this purified enzyme showed little nematicidal activity in vitro and could not degrade the intact cuticle of the host. It is interesting to note that NPE-4 improved the pathogenicity of crude enzyme from wild-type *B. laterosporus* but had no effect on BLG4-deficient mutant (BLG4–6). Taken together, these results suggest that NPE-4 functions in infection against nematodes in the presence of protease BLG4 (Table 2). Moreover, the purified cuticles became loose, and the lateral sections became irregular after being treated by NPE-4 for 24 h, suggesting that NPE-4 could degrade the proteins of the inner layer (Fig. 3). Thus, it is reasonable to conclude that the two different bacterial extracellular proteases may play different roles at different stages of infection or a synthetic role in penetration of nematode cuticle by *B. laterosporus*. Our current study thus contributes to our understanding of the interaction between the nematophagous bacterium and its host.

Various proteases (including bacterial protease, collagenase, and elastase) have been examined for their effects on purified cuticles to measure the composition and regional differentiation of cuticle components (Cox et al. 1981; Katznelson et al. 1964; Niu et al. 2006). These studies showed that protein components of the inner and outer cuticle layers and the pharynx cuticle are different, suggesting that different proteases from bacteria may function differently during infection of nematodes. Clearly, identification and localization of more enzymes involved in infection and penetration of nematode cuticles by *B. laterosporus* need to be investigated further in light of our current results.

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