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Functional identification of the gene *bace16* from nematophagous bacterium *Bacillus nematocida*

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Abstract *Bacillus nematocida* is a Gram-positive bacterium capable of killing nematodes. Our recent studies identified an extracellular serine protease Bace16 in *B. nematocida* as a candidate of pathogenic factor in the infection against nematodes, which displayed a high similarity with the serine protease family subtilisin BPN', and the MEROPS ID is S08.034. To further confirm the roles that *bace16* played in the mechanism of nematocidal pathogenesis, recombinant mature Bace16 (rm-Bace16) was expressed in *Escherichia coli* strain BL21 using pET-30 vector system. Bioassay experiments demonstrated that the purified recombinant protease had the ability to degrade nematode cuticles and kill nematodes. In addition, a *bace16* knockout mutant of *B. nematocida* constructed by homologous recombination showed considerably lower proteolytic activity and less than 50% nematocidal activity than the wild-type strain. These results confirmed that Bace16 could serve as an important virulence factor during the infectious process.

Introduction

Plant parasitic nematodes have inflicted serious damages on agricultural crops and other plants, and this problem is currently depending on the use of chemical insecticides. Because of the increasing awareness by the public about the environmental side effects caused by chemical insecticides, biocontrol of plant and animal diseases has attracted a lot of attention. Effective biocontrol agents against nematodes have to recognize, attach, penetrate, and eventually, kill the parasitic nematodes. Nematode cuticle, consisting mainly of proteins including keratin, collagen, and fibers running diagonally in opposite directions from each other, is a very rigid but flexible multilayered extracellular structure exoskeleton and an effective barrier preventing nematodes from both biological and environmental damages (Maizels et al. 1993). Covering the nematode eggs is a shell containing a protein matrix (50–60% of the composition) embedded in chitin microfibrils (Huang et al. 2004). Therefore, effective degradation of cuticle or egg shell should be an important stage during the process of pathogens infecting nematodes. The extracellular enzymes from nematode pathogen, corresponding to the main chemical constituents of nematode cuticle and eggshell, have been believed to be a potential powerful virulence factor involved in either penetration of cuticle or digestion of the host.

In the 1990s, fungal pathogens were regarded as promising biocontrol agents against plant-parasitic nematodes due to their capabilities to directly penetrate the cuticles and infect the hosts (Stirling 1991; Tunlid and Jansson 1991; St. Leger 1995; Tunlid et al. 1994). In recent years, nematophagous bacteria have been used extensively as a biocontrol agent against nematodes in soil for their fast multiplication and easy cultivation and production, and furthermore, their levels of controlling nematodes were shown equivalent to those of chemical pesticides (Zhou et al. 2002). *Bacillus nematocida*, isolated from a forest soil sample from Yunnan, China, was shown to have a remarkable nematotoxic activity (Huang et al. 2005a,b). In this strain, two extracellular proteases that could degrade

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the cuticle of nematodes were cloned (Niu et al. 2006a,b). Though numerous investigations on purification, characterization, and cloning of the pathogenic extracellular proteases were reported, especially from nematophageous fungi (Tunlid et al. 1994; Åhman et al. 1996; Bonants et al. 1995; Morton et al. 2003), a few studies have been conducted to testify their roles in the infection mechanisms on the molecular level. In this study, we further confirm the nematocidal activity and the involvement of Bace16 in the infection against nematodes by heterologous expression and gene knockout. Our in vitro assay demonstrated that the recombinant protease rm-Bace16 expressed in *Escherichia coli* presented a nematotoxic activity. In addition, a *bace16* knockout mutant of *B. nematocida* decreased significantly proteolytic activity and nematotoxic activity compared with the wild-type strain under various physiological conditions. Our investigation will contribute to the better understanding of the infection mechanism and suggest that this virulence gene can be used to develop more potent nematophagous bacteria with genetic engineering to control plant parasitic nematodes.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The original isolation of *B. nematocida*, designated strain B16, has been deposited at the China General Microbiological Culture Collection Center (CGMCC, catalogue # 1128). This strain is typically grown at 37°C in Luria–Bertani (LB) medium and is used as the parent strain for the derivation of the *bace16* knockout mutant. An *E. coli* strain BL21 was employed for heterologous expression of the cuticle-degrading protease Bace16 with the pET-30a expression vector. For the construction of the *bace16* knockout mutant, pCP115, an integration vector for Gram-positive bacteria, was obtained from the Bacillus Genetic Stock Center (BGSC). Unless otherwise indicated, all *E. coli* strains were grown in LB at 37°C with shaking. Where appropriate, kanamycin (50 μg/ml), chloramphenicol (5 μg/ml), or ampicillin (100 μg/ml) was added to the medium. Chemical reagents and antibiotics were, if not otherwise specified, purchased from Sigma (St. Louis, MO).

Culture of nematodes

The saprophytic nematode *Panagrellus redivius* was first cultured on oatmeal medium (oatmeal: 20 g, water: 80 ml) at 25°C for 7 days. These nematodes were separated from the culture medium by the Baerman funnel technique (Gray 1984), and an aqueous suspension of the nematode was prepared as a working stock.

Heterologous expression

The nucleotide sequence data of the gene *bace16* has been deposited in the GenBank database under the accession number AY708655 (Niu et al. 2006a). To express mature Bace16 in *E. coli* BL21, we followed the procedures described by Choi et al. (2004), with minor modifications. Briefly, the gene encoding the mature Bace16 was amplified by polymerase chain reaction (PCR) using an *Nco*I-linked sense primer 5′-C C A T G G G C C G C T G C A A C C G G A A C A G-3′ and an *Xho*I-linked antisense primer 5′-C T C G A G C A A T C C A A C T G C A T T C C A G G C-3′. PCR amplification was performed under the following conditions: 30 cycles of 95°C for 40 s, 55°C for 30 s, and 72°C for 1.5 min. The amplified 825-bp fragment was purified from agarose gel, and then, ligated into pGEM-T Easy vector (Promega) to generate the pT-mat-Bace16 plasmid. After digestion with *Nco*I and *Xho*I, the mature Bace16 fragment was inserted into the bacterial expression vector pET-30a.

Transformed cells were grown at 26°C in LB medium, supplemented with kanamycin (100 μg/ml) to a cell destiny of A660=0.4−0.6. Protein expression was induced by 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 26°C for 5 h. The recombinant proteins were purified using purification protocol of 6× His-tagged proteins by Ni-NTA affinity chromatography according to pET System Manual. Buffers used in purification were as follows: buffer A: 20 mM Tris–HCl, 5 mM Imidazole, 0.5 M NaCl, 8 M urea, pH 7.9; buffer B: 20 mM Tris–HCl, 20 mM Imidazole, 0.5 M NaCl, 8 M urea, pH 7.9; buffer C, D, and E: the same composition as buffer B, but at pH 6.5, 5.9, 4.5 respectively. Induced cells were harvested by centrifugation at 4,000 rpm for 20 min and resuspended in 30 ml buffer A and stirred for 1 h at room temperature. The lysate was centrifuged at 10,000 rpm for 15 min at 4°C. Supernatant was applied to a column containing Ni-NTA resin previously equilibrated in buffer A. Ni-NTA resin was washed with 10 column volumes of buffer A and 5 column volumes of buffer B until the A280 was <0.01. Recombinant protein was eluted with 10 ml buffer C, 10 ml buffer D, and 10 ml buffer E and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The correct conformation was regenerated according to the protocol of Yokoyama et al. (2002). The refolding trials were carried out at 4°C. The purified protein rm-Bace16 was adjusted to pH 4.0 and diluted 50-fold using renaturation buffer [50 mM Gly, 2 mM dithiothreitol (DTT), 10% glycerin, 150 mM NaCl, 50 mM H_3PO_4, 3 mM GSH, 0.3 mM oxidized glutathione (GSSG)]. The renaturation buffer was readjusted to pH 7.0 using Tris–HCl buffer and determined its proteolytic and nematocidal activities. The purified recombinant proteins were sent to the Lab for Monoclonal

Antibodies in Yunnan University to produce the mouse polyclonal antibody.

Construction of the knockout mutant

The *bace16* mutation was constructed with the method of homologous recombination. A 295-bp fragment of the mature protease gene was firstly obtained by digesting the pT-mat-Bace16 plasmid using two restriction endonucleases *Cla*I and *Pst*I, and the fragment was then inserted into the integration vector pCP115. This recombinant plasmid, named pΔbace16, was selected and amplified in *E. coli* strain DH5α. The competent cells of *B. nematocida* were prepared and transformed with pΔbace16 according to the protocols supplied by BGSC. A clone carrying a single-crossover mutation of *bace16* was obtained by selection on LB agar medium containing 5 μg/ml chloramphenicol. The identity of this clone was confirmed by Southern hybridization and PCR analysis.

The hybridization probe for Southern hybridization here was the partial fragment of the gene *bace16* digested with restriction enzymes *Cla*I and *Pst*I. The probe was labeled with Digoxin and the subsequent Southern hybridization was then performed according to the manual provided by the manufacturer Roche. In addition, we also used PCR analysis to further confirm the knockout mutant. The upstream primer was 5′-T A A G A G C C G C A A C T G T A C C-3′ designed based on the chromosomal DNA sequence; the reverse primer was 5′-A G A A G A C A G T C A T A A G T G C G G-3′ designed based on the downstream sequence of the integration vector pCP115. Thus, the corresponding DNA fragment amplified by PCR would be obtained only if pΔbace16 had inserted into the target locus of the chromosome.

Western blot analysis

Western blot hybridization was implemented using the samples of purified native Bace16 protein, rm-Bace16, and the culture supernatant from wild strain or knockout mutants. The detailed protocols followed that described in Molecular Cloning: a Laboratory Manual, second edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA). Briefly, 20 μg sample proteins were separated on a SDS-PAGE gel and electro-transferred onto immune-blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) for Western blotting. After being blocked with 10% skim milk in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.02% Tween-20 (TBST), an anti-rm-Bace16 polyclonal antibody (prepared by the Lab for Monoclonal Antibodies in Yunnan University) was added at a 1:500 dilution. Washed three more times, the PVDF membrane was then treated with goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibody at a dilution of 1:1,000 (Bio-Rad) and washed extensively again. The position of the bounded antibodies was detected by developing in solutions containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). In Western blot analysis, the culture supernatant of the strain with only pET-30a vector was used as a negative control.

Measuring the proteolytic and nematotoxic activities

To determine the proteolytic and nematotoxic activities, we grew the wild-type *B. nematocida* strain, the *E. coli* expressional strain, and the *B. nematocida* knockout strain in yeast extract/ peptone/dextrose (YPD) medium. Crude protease extracts from these cultures were obtained using the method described by Niu et al. (2006a). These extracts were then tested for their proteolytic and nematotoxic activities.

The protease activity was assayed by a modified caseinolytic method (Elmar 1984). One unit (U) of protease activity was defined as the amount of enzyme needed to hydrolyze the substrate and produce 1 mg tyrosine in 1 min under the assay conditions. Proteolytic activity vs protein substrate was performed by using the methods reported previously by Tunlid et al. (1994). The obtaining of the substrate of nematode cuticle followed the method described by Cox et al. (1981).

The method for determining nematicidal activity was based on that described in Kwok et al. (1992) and Niu et al. (2006a). Firstly, the tested nematodes were washed thoroughly with a 10-mM phosphate-buffered saline (PBS) (pH 7.0, sterile), and a suspension containing 40–50 nematodes (20 μl) was then transferred to a sterile 1.5-ml Eppendorf tube. Finally, the sample containing protease extracts was added to the nematodes. Bovine serum albumin (BSA) and the extraction buffer were used as negative controls. After incubating the mixture at 25°C for 24–48 h, the numbers of living and dead nematodes were counted under a dissecting microscope.

Results

Heterologous expression of the Bace16 protein

SDS-PAGE analysis showed that *E. coli* BL21 cells containing the induced recombinant plasmid produced a recombinant protein (rm-Bace16) of approximately 34 kDa as inclusion body (Fig. 1a), but not a 28-kDa protein band of native Bace16 reported previously (Niu et al. 2006a). This molecular weight of rm-Bac16 was consistent with mature Bace16 fused to expressional vector pET-30a. Then, rm-Bace16 was affinity purified using a Ni-NTA column,

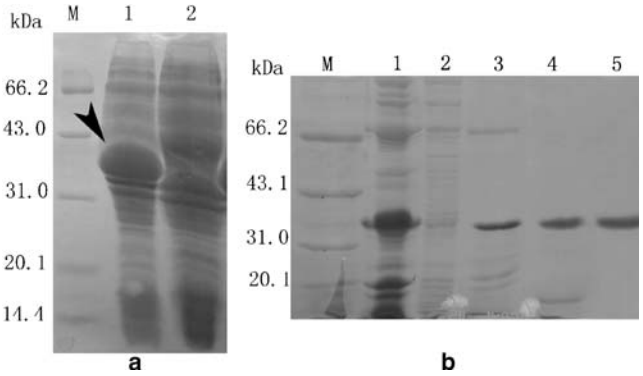

Fig. 1 **a** SDS-PAGE (12%) demonstrated the expression of the fusion protein rm-Bace16 in *E. coli* transformant. *lane 1* represented the supernatant treated with 1 mM IPTG at 26°C for 5 h from the expressional transformant; *lane 2* was the corresponding negative controls without induction; *lane M* represented the protein marker. **b** Purification of heterologously expressed bace16. *lane 1*: cleared lysate; *lane 2*: wash; *lanes 3–5*: elutions; *lane M*: marker

and the yields were shown in Fig. 1b. As the recombinant protein was produced mainly as an insoluble inclusion body and presented no significant proteolytic activity, we investigated the efficient refolding procedures of partially refolded Bace16 formed in the acidic buffer at a low temperature (4°C) to render an active protein capable of functioning in vitro. Renaturation completed, part of the insoluble inclusion body was redissolved in Tris–HCl buffer, and so, the rm-Bace16 regained proteolytic activity ($5.45\ PU\times10^{-3}$) despite of it lower than that of the native Bace16 (60.82%) (Table 1).

Construction of a knockout bace16 mutant of *B. nematocida*

An integration vector pCP115 from the BGSC was employed to inactivate the *bace16* gene of the wild-type *B. nematocida* strain through insertion mutagenesis (Fig. 2a). The insertion mutant was confirmed by PCR and Southern blot analysis. When the *Hind*III-digested genomic DNA of the wild-type strain was hybridized with the internal coding sequence probe of the *bace16* gene, two different fragments, one bigger than 1.7 kb and the other about 1.2 kb, were observed in the wild-type strain and the mutant respectively, consistent with our expectation (Fig. 2b). Homologous recombination was further confirmed by PCR where an amplicon of 0.9 kb was found in the knockout mutant of *bace16* (Fig. 2c, lane 2), as compared to the wild type (Fig. 2c, lane 1) with no amplification.

To investigate the expressional status of gene *bace16* at the protein level in the knockout mutant, anti-rm-Bace16 polyclonal antibody was produced and used for Western blot hybridization. Our experiments revealed that this polyclonal antibody could hybridize to both the native Bace16 and rm-Bace16 proteins (Fig. 3a), suggesting that the heterologously expressed serine protease Bace16 has the same epitopes as the native type. In addition, Western analysis displayed a 28-kDa protein band of the culture supernatant from wild-type strain (Fig. 3b, lane 2); but the mutant had no hybridization signal (Fig. 3b, lane 3). Taking together these results indicated that the *bace16* knockout mutant of *B. nematocida* was successfully constructed.

Proteolytic activity and bioassay analysis

In the assay of proteolytic activities, the activity of native Bace16 using 0.2 M casein as substrate was $8.96\ PU\times10^{-3}$ (100%). Rm-Bace16 presented a lower protease activity (60.82%) than that of native Bace16 while they had the similar hydrolytic substrates or activity inhibitors (Table 1). For example, both of them were highly sensitive to phenylmethyl sulfonyfluoride (PMSF) (1.0 mM) and slightly inhibited by ethylenediaminetetraacetic acid (EDTA). In addition, they both could degrade a broad range of substrates including casein, denatured collagen, and nematode cuticle, but in the *bace16* knockout mutant, the protease activity decreased rapidly against casein, denatured collagen and nematode cuticle. It was also noticed that there was partial proteolytic activity retained in this mutant. This remaining activity could be enhanced by EDTA, but not inhibited by PMSF, which is suggested to be derived from the neutral protease (Niu et al. 2006b).

The bioassay (Table 2) indicated that the extracts from the wild-type strain B16, the *E. coli* transformant for

Table 1 Comparison of proteolytic activities in wild strain, recombinant strain, and *bace16* mutant

Samples	Enzyme activity as percentage (%) of control (SD)				
	Casein as substrate	Denatured collagen as substrate	Nematode cuticle as substrate	1.0 mM PMSF added	1.0 mM EDTA added
Bace16	100 (0.00)	50.24 (6.20)	4.85 (0.68)	0.00 (0.00)	88.72 (8.09)
Rm-Bace16	60.82 (8.25)	41.55 (4.33)	3.69 (0.90)	0.00 (0.00)	56.31 (7.57)
Bace16 extract	19.33 (7.50)	9.76 (4.53)	1.04 (0.72)	20.52 (8.81)	31.49 (8.64)

Protease activity was assayed at 37°C, pH 7.5 for three replicates.

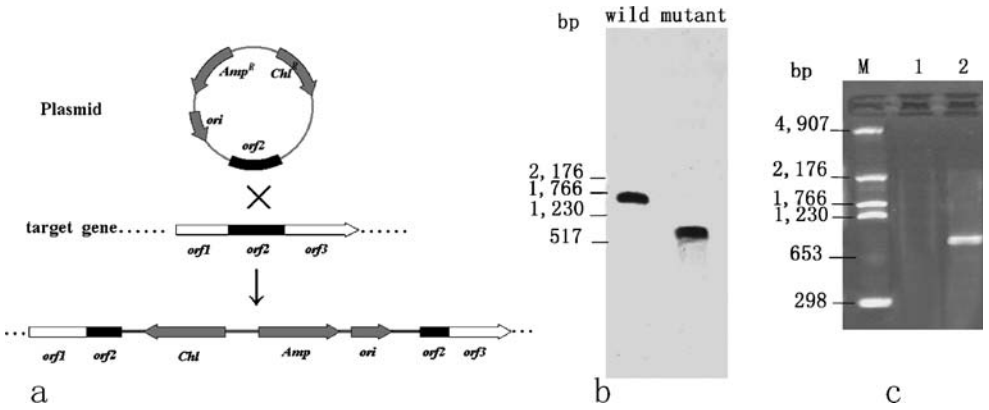

Fig. 2 **a** Use of the integration vector pCP115 to construct a knockout mutation in a hypothetical open reading frame, orf1–orf3. The frame of orf2 represents the internal 295-bp segment of the target gene. **b** Southern blot analysis of *bace16* mutant. Genomic DNAs from *B. nematocida* strain (*lane wild*) and *bace16* mutant (*lane mutant*) were digested with *Hind*III and hybridized to a DIG-labeled DNA probe consisting of a *Cla*I –*Pst*I fragment internal to the *bace16* coding sequence. **c** Agarose gel electrophoresis analysis of PCR products. *M*, Molecular marker; *lane 1*, *B. nematocida* strain B16; *lane 2*, *bace16* knockout mutant

heterologous expression and the knockout mutant B16, had remarkably different abilities to kill nematode *P. redivius*. The majority of nematodes were dead after being treated with the purified enzyme from the *E. coli* transformant for 48 h, although its nematicidal activity was weaker than that from native Bace16 (Fig. 4b). With light microscope, we also observed that the cuticle of nematode was degraded gradually after the treatment with the purified recombinant enzyme. As for the protein extract from the *bace16* knockout mutant, the experimental data showed that the nematocidal activity was only 45% of the wild-type strain, and no obvious effect could be observed on the nematode cuticle (Fig. 4c), consistent with the importance of this gene during pathogenesis.

Discussion

Bacteria with antagonistic effects against plant-parasitic nematodes include rhizobacteria, endophytic bacteria, rhizobia, and bacterial symbionts of entomopathogenic nematodes. These bacteria were shown to have different mechanisms to control nematodes, including suppression of root-gall development, nematode reproduction (Jonathan et al. 2000), egg hatching or juvenile survival (El-Nagdi and Youssef 2004), toxin production (e.g. Jagdale and Grewal 2002), or endoparasitism (Talavera et al. 2002). But in several recent reports, another infection mechanism related with extracellular cuticle-degrading protease was suggested (Huang et al. 2005a,b; Imran et al. 2005; Niu et al. 2006a,b).

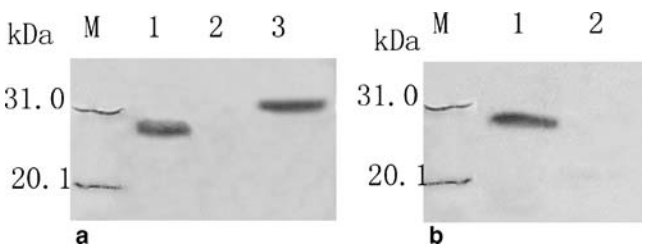

Fig. 3 Western blot analyzed the rm-Bace16 expressed in *E. coli* and *bace16* knockout mutants. **a** Western analysis of rm-Bace16. *lanes 1–4* represented protein marker, the sample of native Bace16, negative control of pET-30a vector, and rm-Bace16 as positive controls, respectively. **b** Western analysis of the expression of extracellular serine protease in *bace16* knockout mutants. *lane 1* protein marker, *lane 2* the culture supernatant of wild strain, *lane 3* the *bace16* knockout mutant

Up to now, two pathogenic proteases, the serine protease Bace16 and the neutral protease Bae16, have been purified and cloned from *B. nematocida* (Niu et al. 2006a,b). Our comparisons between Bace16 and Bae16 proteins indicated that the serine protease contributed to the majority nematicidal activity. Therefore, we first focused on *bace16* gene to validate its roles at the molecular level. Here, we described the expression of *bace16* gene in *E. coli*, the construction of the knockout mutant by homologous recombination, the detection of proteolytic activity and nematocidal ability in the wild-type strain B16, the *E. coli* transformant, and the knockout mutant of the gene *bace16*. Our experimental results confirmed that Bace16 plays an important role in the infection against nematodes.

In our experiment of heterologous expression, no obvious proteolytic or nematocidal activities could be detected when rm-Bace16 was firstly expressed in the insoluble inclusion body. However, after renaturation, while their activities were detected, they were weaker than that of the native type. Recombinant proteins expressed in *E. coli* can often be produced as inactive aggregates with incorrect protein folding, and this phenomenon is commonly believed to be associated with the high rate of protein synthesis. A series of measures, such as lowering induction temperature, shortening induction time, decreasing the concentration of IPTG, and growing the cells in minimal

Table 2 Killing of the nematode *P. redivivus* by protease extracts from the wild strain, expressional transformant, and *bace16* mutant

Extract source	Mortalities of nematodes (%) (SD)				
	12 h	24 h	36 h	48 h	60 h
Wild strain	60 (4.5)	80 (2.4)	90 (2.6)	95 (2.6)	100 (0.0)
Expressional transformant	40 (5.0)	55 (4.3)	70 (2.8)	85 (3.5)	90 (3.0)
Mutant strain	25 (3.6)	35 (2.2)	40 (2.8)	45 (1.9)	45 (2.5)
BSA	5 (0.2)	5 (0.2)	5 (0.8)	5 (0.4)	5 (0.4)
Buffer	5 (0.3)	5 (0.5)	5 (0.9)	10 (0.2)	10 (0.3)

Extracts were incubated with nematodes in Eppendorf tubes. After 12–60 h, the numbers of live and dead nematodes were counted in a light microscope; mortality = number of dead nematodes/number of ones tested. The bioassays were performed with 10 to 15 parallels and were repeated at least twice.

media, were tried to increase the percentage of target protein in soluble form, but all failed. The complex steps of renaturation, i.e., isolation, solubilization, and refolding were then performed, and a lower activity was regained in rm-Bace16 probably for the halfway renaturation. In addition, pET-30a carrying additional N- and C-terminal sequences was used to construct the heterologous expressional vector. When the target protein was fused with extra amino acids (His-Tag of N- and C-terminal, plus thrombin, S-Tag and so on) from pET-30a, the protease conformation might be different contributing to its lower proteolytic and nematocidal activities.

Here, it was also demonstrated that the disruption of gene *Bace16* did not eliminate *B. nematocida* of all the capability to infect nematodes, suggesting the existence of other pathogenic factors such as toxic peptides, intermediate metabolic products, and potentially other virulence protease. The neutral protease Bae16, another candidate of pathogenic factors, has been reported in our earlier work, and its half lethal concentrations (LC50) were 1.69 and 2.26 μg/ml for *P. redivius* and *Bursaphelenchus xylophilus*, respectively (Niu et al. 2006b). Thus, it is possible that the intact Bae16 takes partial responsibility for the retained activity when gene *bace16* is knocked out. However, to support this speculation, further evidences are required in which the extracts from *B. nematocida* with only serine protease apart from Bae16 will be prepared.

That serine proteases serve as the important pathogenic factors was firstly described in detail in nematophageous fungi. In 1990, the first serine protease of P32 was purified from a strain of *Verticillium suchlasporium* (Lopez-Llorca 1990). Two years later, P32 was localized in infection process with immunocytochemistry, and it was shown that this virulence protease was involved in egg penetration by *V. suchlasporium* (Lopez-Llorca and Roberson 1992). Up to now, several kinds of serine proteases have been detected and partly characterized from nematophagous fungi or nematode-trapping fungi, including *Arthrobotrys oligospora*, *Pochonia suchlasporia* (syn. *V. suchlasporium*), *Pochonia chlamydosporia* (syn. *Verticillium chlamydospo-*

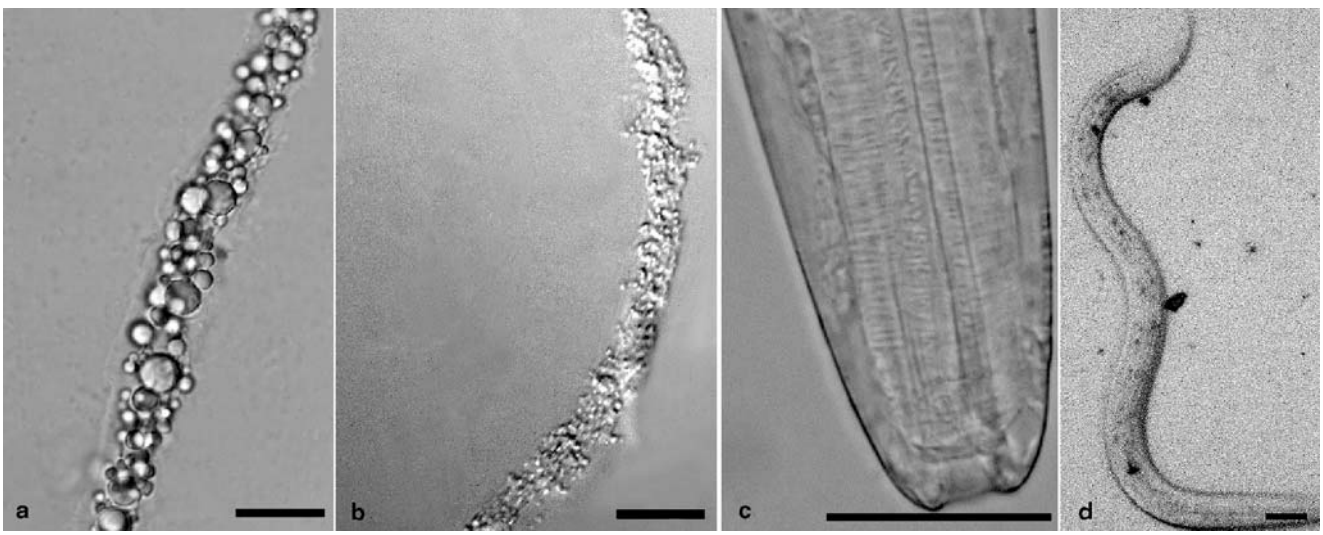

Fig. 4 The action of purified protease Bace16, Rm-Bace16, and crude enzyme of bace16 mutant against cuticle observed in a light microscope. **a** The cuticle was degraded when treated with purified enzyme Bace16 for 60 h; **b** the cuticle was gradually degraded when treated with the recombinant protease Rm-Bace16 for 60 h; **c** the cuticle was complete when treated with crude enzyme of bace16 mutant within the same time; **d** the nematode was still alive using BSA under the same conditions

rium), *Paecilomyces lilacinus*, and *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*). These extracellular serine proteases can destroy the cuticle or eggshells of nematodes. At the same time, some experiments also supported that serine protease could also initiate or trap nematophagous fungi. For example, it has been shown that deletion mutants of PII had a lower number of traps, while the multicopy mutants had a larger number in *A. oligospora* (Åhman et al. 2002). However, researches on the virulence proteins from bacteria, which might also target the invertebrate phylum Nematodes, has been mostly ignored. Wei and Hale expressed seven different crystal toxin proteins from two largely unstudied *Bacillus thuringiensis* (*Bt*) crystal protein subfamilies. Their study demonstrated that four members among them were active against multiple nematode species and that each tested nematode species was susceptible to at lease one toxin (Wei et al. 2003). It was then concluded that *Bt* crystal proteins could be employed as the important pathogenic factors and have a potential for controlling nematode pests infecting animals and plants. In another invertebrate pathogen *Brevibacillus laterosporus*, its toxic activity was thought to be related with the parasporal crystals (Smirnava et al. 1996, 1997). However, most *B. laterosporus* strains did not produce the parasporal crystalline as *B. thuringiensis* did. Huang et al. suggested that the extracellular serine protease, rather than the reported parasporal crystal, be employed in the infection against invertebrate by the G4 strain (Huang et al. 2005a,b). For *Pseudomonas fluorescens* strain, it was also reported that the strain CHA805 of nonpolar *aparA* (encoding the major extracellular protease) mutation decreased the inhibition of egg hatching and mortality of nematodes *Meloidogyne incognita* (Imran et al. 2005). In our present study, the serine protease Bace16 from *B. nematocida*, sharing a high similarity with the protease from *B. laterosporus* G4 strain in a variety of characters and the gene sequences, was confirmed to have similar functions in infection. All these data supported the hypothesis that virulence proteases may be distributed widely in nematophagous bacteria and contribute directly or indirectly to the infectious process, just like those in nematophagous fungi.

In the soil, bacteria are among the most abundant microorganisms in the root zone. Their presence can significantly modify the rhizosphere environment and affect the nematode or the host–parasite interrelationship (Neipp and Becker 1999). As a nematode pathogen, *B. nematocida* certainly has the potential for being an excellent biological agent. In a previous research on serine protease PII from the nematophagous fungi *A. oligospora*, the over-expression mutant of PII displayed higher toxic activities than the wild-type strain, implying that pathogenic microbes may improve the pathogenicity of proteases by modification of the enzyme after their translations (Åhman et al. 2002). Therefore, the extracellular serine protease Bace16 identified here as an important pathogenic factor in *B. nematocida* could supply an alternative gene to construct strains with a greater toxicity.

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