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ORIGINAL ARTICLE

Cloning and phylogenetic analysis of the chitinase gene from the facultative pathogen *Paecilomyces lilacinus*

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Keywords
biological control, chitinase, entomopathogen, microbial control, mycopathogen, nematopathogen, *Paecilomyces lilacinus*.

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Abstract

Aims: To PCR-amplify the full-length genomic-encoding sequence for one chitinase from the facultative fungal pathogen *Paecilomyces lilacinus*, analyse the DNA and deduced amino acid sequences and compare the amino acid sequence with chitinases reported from mycopathogens, entomopathogens and nematopathogens.

Methods and Results: The encoding gene (designated as *PLC*) was isolated using the degenerate PCR primers and the DNA-Walking method. The gene is 1458 bp in length and contains three putative introns. A number of sequence motifs which might play a role in its regulation and function had also been found. Alignment of the translation product (designated as Plc, molecular mass of 45·783 kDa and pI of 5·65) with homologous sequences from other species showed that Plc belongs to Class V chitinase within the glycosyl hydrolase family 18. The phylogenetic and molecular evolutionary analysis using MEGA (Molecular Evolutionary Genetics Analysis) indicated that these chitinases from mycopathogens, entomopathogens and nematopathogens, the majority of which belong to glycosyl hydrolase family 18, were clustered into two well-supported subgroups corresponding to ascomycetes fungal and nonfungal chitinases (bacteria, baculoviruses).

Conclusions: Our study showed that chitinases from mycoparasitic, entomopathogenic and nematophagous fungi are closely related to each other and reaffirmed the hypothesis that baculovirus chitinase is most likely to be of a bacterial origin – acquired by gene transfer. Bacterial and baculoviral chitinases in our study are potential pathogenicity factors; however, we still cannot ascribe any specific function to those chitinases from the fungi.

Significance and Impact of the Study: To our knowledge, this is the first report describing the chitinase gene and its translation product from *Paecilomyces lilacinus*, which constitutes the largest number of formulated biological nematicides reported so far, this is also the first study to analyse and resolve the phylogenetic and molecular evolutionary relationships among the chitinases produced by mycopathogens, entomopathogens and nematopathogens.

Introduction

Paecilomyces lilacinus is a common soil hyphomycete with a cosmopolitan distribution (Dube and Smart 1987). As one of the most studied nematophagous fungi, it also constitutes the largest number of formulated biological nematicides reported so far (four of 11; Dong and Zhang 2006). To be a pathogen, the fungal spores must germinate on a host surface and the resulting hyphae must penetrate the host tissue, colonize, alter host physiology and cause disease (Sexton and Howlett 2006). The eggshell is the toughest part of nematode eggs and plays an important role in their resistance to chemical and biological nematicides (Wharton 1980). The eggshell consists of

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three basic layers, namely vitelline membrane, chitin and lipid (Bird and McClure 1976). Previously, an enzyme preparation consisting of six chitinases, semi-purified from a liquid culture of *P. lilacinus* strain 251, was applied to the eggs of the root-knot nematode *Meloidogyne javanica* to study the effect of the enzymes on eggshell structures, then transmission electron microscopic studies revealed that these chitinases drastically altered the eggshell structures when applied and the eggs treated with chitinases displayed large vacuoles in the chitinous layer and the vitelline layer was split and lost its integrity (Khan *et al.* 2004). However, the genes encoding its chitinase are unknown at present. So, the first objective of this study was to PCR-amplify the full-length genomic-encoding sequence of one chitinase.

After cellulose, chitin is the second most abundant organic source in nature; the polymer is composed of ß-(1, 4)-linked units of the amino sugar *N*-acetylglucosamine (Seidl *et al.* 2005). Chitin is also a constituent of cell walls or structural tissues of many species of fungi, insects and crustaceans (Mian *et al.* 1982). Chitinases (EC 3.2.1.14) are endoglycosidases that hydrolyse bonds that link the *N*-acetylglucosamine residues of chitin. Chitinases occur both in eukaryotic and prokaryotic organisms, including bacteria, fungi, viruses, nematodes, plants, insects and some vertebrates (Wang *et al.* 2004). There has been a considerable amount of recent research aimed at elucidating the roles of chitinase in the pathogenesis incurred by the entomopathogens (Sampson and Gooday 1999; Charnley 2003), nematopathogens (Stirling and Mankau 1979; Mian *et al.* 1982; Morgan-Jones and Rodriguez-Kabana 1985; Tian *et al.* 2000; Tikhonov *et al.* 2002; Khan *et al.* 2004; Jin *et al.* 2005) and mycopathogens (Kobayashi *et al.* 2002; Frankowski *et al.* 2001). The fungus *P. lilacinus* is highly adaptable in its life strategy in that it has been reported as an entomopathogenic (Rombach *et al.* 1986; Marti *et al.* 2006; Fiedler and Sosnowska 2007), mycoparasitic (Gupta *et al.* 1993), saprophytic (Tigano-Milani *et al.* 1995), as well as nematophagous species. So, the second objective of this study was to analyse the DNA and amino acid sequence of the chitinase from *P. lilacinus*, and more importantly, compare its amino acid sequence with those reported chitinases from mycopathogens, entomopathogens and nematopathogens. Such comparison should shed more light on the evolutionary relationships among these pathogens.

Materials and methods

Strains and plasmid

Paecilomyces lilacinus IPC used throughout this study was originally isolated from the root-knot nematode *Meloidogyne* sp. in Beijing, China and deposited in the culture collection of our laboratory. *Escherichia coli* DH5a was used for transformation and propagation of plasmid harbouring the DNA fragment of interest. Plasmid pMD19-T (Takara, Japan) was used for TA-cloning and DNA sequencing work.

Isolation of *P. lilacinus* genomic DNA

Paecilomyces lilacinus IPC was grown in potato dextrose broth (PDB) medium on a rotary shaker (170 rev min^{-1}) at 28°C for 7 days. One-tenth gram of lyophilized mycelium was ground to powder in a microcentrifuge tube with 0·1 g quartz particle. The powder was then suspended in 300 μl of cetyl triethyl ammonium bromide (CTAB) buffer [1 l contains CTAB 20 g, NaCl 81·8 g, EDTA 7·4 g, 1 mol l^{-1} Tris-HCl (pH 8·0) 100 ml, mercaptoethanol 2 ml], then incubated at 65°C for 1 h. Equal volumes of chloroform and phenol (300 μl each) were then added to the suspension, mixed well and centrifuged at 12 000 rev min^{-1} for 5 min. The upper phase was transferred to a clean tube and the preceding step was repeated. Afterwards, the upper phase was transferred to a clean tube and two volumes of ethanol were added, mixed well and centrifuged at 12 000 rev min^{-1} for 5 min. The tube was drained and added with 500 μl of prechilled 70% (v/v) ethanol, the suspension centrifuged at 12 000 rev min^{-1} for 5 min, then the ethanol-cleaning steps were repeated, after which the liquid was poured off, the pellet was vacuum-dried in the Eppendorf Vacufuge Concentrator 5301 (Eppendorf, Germany) and resuspended in 20 μl of sterile deionized water.

PCR and sequencing procedures

Initially, part of the encoding sequence was PCR-amplified using the degenerate PCR primers used for the isolation of chitinase-encoding gene (*chit1* gene) from the entomopathogenic and nematophagous fungus *Metarhizium anisopliae* (Bogo *et al.* 1998). Both taxonomically belonging to the family Clavicipitaceae, *P. lilacinus* and *M. anisopliae* can infect across the insect cuticle (e.g. Rombach *et al.* 1986; Renn *et al.* 1999; Marti *et al.* 2006; Fiedler and Sosnowska 2007) and nematode eggshell (e.g. Walters and Barker 1994; Sun *et al.* 2006). They are both potential substrates of chitinases from pathogenic fungi and show many similarities in structure and chemical composition (López-Llorca and Robertson 1992). Both are composite structures containing a fibrous material (chitin) embedded in a matrix material (protein) (St. Leger *et al.* 1997; Jansson and López-Llorca 2001). The amplification was carried out as described by Bogo *et al.* (1998). PCR mixture (10 μl) contained 10X PCR buffer

[20 mmol l^{-1} Tris-HCl (pH 8·4), 200 mmol l^{-1} KCl, 100 mmol l^{-1} $(NH_4)_2SO_4$] 1 μl, 25 mmol l^{-1} $MgCl_2$ 1 μl, dNTP mixture (10 mmol l^{-1} each) 0·2 μl, primer (100 μmol l^{-1} each) 0·2 μl, *Taq* DNA polymerase (2·5 u ml^{-1}) (Tianwei Biotech, Beijing, China) 0·2 μl and template (1–5 ng of plasmid, 10–15 ng of genomic DNA). PCR tubes were placed in the thermocycler after it had reached 94°C. PCR products were analysed on gels, the bands of interest excised, purified with a Bioteke gel extraction kit (Bioteke, Beijing, China), and the fragment was subcloned into the pMD19-T cloning vector and sequenced. The flanking 5′- and 3′-regions were PCR-amplified using the DNA-Walking SpeedUp Premix Kit (Seegene, Inc., South Korea). The PCRs and the design of the target-specific primers (TSP) were performed, according to the users' manual, except that the final volume of the first PCR was 10 μl, not the recommended value of 50 μl. Transformation of *E. coli* was performed in accordance with that described in Sambrook *et al.* (1989). Individual transformant colonies were selected and PCR-amplified using intact cells as templates. All primers used in this study, as listed in Table 1, were synthesized by Bejing Sunbiotech Co., Ltd. All primers were designed using the on-line software PRIMER 3 at http://Frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (accessed on 15 August 2006). All PCRs were performed by using the thermal cycler (MyCycler™, Bio-Rad, USA). DNA sequencing was performed by Bejing Sunbiotech Co., Ltd using the ABI 3730XL DNA Analyser (Applied Biosystems, USA).

Sequence and phylogenetic analyses

Analyses of the DNA and amino acid sequences were performed using the DNAMAN software package (Version 5.2.2, Lynnon BioSoft, USA). The putative signal peptide cleavage site was predicted using the on-line software SIGNAL P at http://www.cbs.dtu.dk/services/SignalP/ (accessed on 1 September 2006). After it has been confirmed that the chitinase of interest belongs to the glycosyl hydrolase family 18, its amino acid sequence was aligned with homologous sequences representing both classes III and V of the glycosyl hydrolase family 18. This alignment was performed as described by Wattanalai *et al.* (2004), except that MEGA version 3.1 (Kumar *et al.* 2004) was used, instead of PAUP. Then 24 other chitinase sequences from mycopathogens, entomopathogens and nematopathogens were downloaded from the National Center for Biotechnology Information (NCBI) GenBank, their relevant information was shown in Table 2. The phylogenetic and molecular evolutionary analyses were conducted using MEGA. The Neighbor-Joining method was used to gain the phylogenetic tree. Unless otherwise indicated, the numbering of amino acids throughout this study corresponds to that illustrated in Fig. 1.

Results

Isolation of the full-length genomic-encoding gene

A fragment of interest, *c.* 600 bp in length, was PCR-amplified with the primers CL and CR (data not shown). In the sequencing of the resultant plasmid clone, a 584 bp fragment, primered by the two, was identified (data not shown). In the subsequent isolation of the 3′- and 5′-flanking regions, the fragments of interest, 1003 and 692 bp in length, respectively, were PCR-amplified with the DW-ACP 4 primer (5′-ACP-CGGTC-3′) and DW-ACP 3 primer (5′-ACP-GGGTC-3′), respectively (data not shown). The splicing of the two sequences with the initial one yielded a DNA fragment of 2105 bp, comprising two untranslated regions (UTRs), 509 nucleotides (upstream) and 138 nucleotides (downstream) in length, respectively, as well as the full-length genomic-encoding gene of interest, 1458 bp in length. The encoding fragment was designated as *PLC*.

Nucleotide sequence

The open reading frame (ORF) is interrupted by three putative introns of 65, 56 and 68 bp long. Intron location

Table 1 Oligonucleotide primers used in this study

Designation	Sequence (5′ → 3′)	Length	Target site*	Template	Usage
CL	gctgtttatttcactaactgg	21-mer	125–145	Genomic DNA	Initial isolation of part of the encoding sequence
CR	gggtattcccagatatcaat	20-mer	688–707	Genomic DNA	Initial isolation of part of the encoding sequence
3TSP1	actgggtaggggcatttatg	20-mer	200–219	Genomic DNA	First 3′ DNA-Walking nested-PCR
3TSP3	caacttcccgtctgcctcctc	21-mer	597–619	First 3′ DNA-Walking nested-PCR product	Second 3′ DNA-Walking nested-PCR
5TSP1	cattcttgccttggtcattc	20-mer	486–505	Genomic DNA	First 5′ DNA-Walking nested-PCR
5TSP3	tgtcatcgcagtcgttctctc	21-mer	163–183	First 5′ DNA-Walking nested-PCR product	Second 5′ DNA-Walking nested-PCR

*The numbering of nucleotides starts at the predicted translational initiation ATG codon (the A in ATG being +1).

Table 2 Relevant species and their chitinases

Species	Host(s)	Accession number	Protein_ id	Identity (%)*	Reference(s)†
Nomuraea rileyi	Insect	AY264288	AAP04616	70·05	Wattanalai *et al.* (2004)
Hypocrea koningii (anamorph: *Trichoderma koningii*)	Fungi	AF188918	AAF19612	68·79	Sanz *et al.* (2004)
Aphanocladium album	Insect	X64104	CAA45468	68·63	Braga *et al.* (2002)
Metarhizium flavoviride	Insect	AJ243014	CAB44709	68·40	Fargues *et al.* (2001)
Stachybotrys elegans	Fungi	AF516397	AAM70478	68·24	Charest *et al.* (2002)
Hypocrea lixii (anamorph: *Trichoderma harzianum*	Fungi, insect and nematode	X79381	CAA55928	68·16	Santos *et al.* (1992); Ganassi *et al.* (2000); Sanz *et al.* (2004)
Metarhizium anisopliae	Insect and nematode	DQ097518	AAZ32788	68·16	Renn *et al.* (1999); Sun *et al.* (2006)
Trichoderma viride	Fungi and nematode	AF188924	AAF19618	67·69	Miller and Anagnostakis (1977); Sanz *et al.* (2004)
Torrubiella confragosa (anamorph: *Lecanicillium lecanii*	Insect and nematode	AY705927	AAV98692	64·62	Meyer (1999); Braga *et al.* (2002)
Verticillium fungicola	Fungi	AY292527	AAP45631	63·92	St. Leger *et al.* (1997)
Cordyceps bassiana (anamorph: *Beauveria bassiana*)	Insect and nematode	AY147010	AAN41260	59·43	Hegedus *et al.* (1992); Sun *et al.* (2006)
Trichoderma hamatum	Fungi	AY258898	AAP15043	58·39	Sanz *et al.* (2004)
Coniothyrium minitans	Fungi	AF285086	AAG00504	50·23	Zantinge *et al.* (2003)
Autographa californica nucleopolyhedrovirus	Insect	L22858	AAA66756	21·57	Wang *et al.* (2004)
Bacillus thuringiensis	Insect and nematode	AF424979	AAL17867	21·39	Bottjer and Bone (1987); El-Bendary (2006)
Bombyx mori nucleopolyhedrovirus	Insect	NC_001962	NP_047523	21·01	Wang *et al.* (2004)
Helicoverpa zea single nucleocapsid nucleopolyhedrovirus	Insect	NC_003349	NP_542664	20·78	Wang *et al.* (2004)
Serratia plymuthica	Fungi	AJ488913	CAD32933	20·37	Frankowski *et al.* (2001)
Stenotrophomonas maltophilia	Fungi and nematode	AF014950	AAB70917	18·60	Tian *et al.* (2000); Kobayashi *et al.* (2002)
Cydia pomonella granulovirus	Insect	NC_002816	NP_148794	18·59	Wang *et al.* (2004)
Xenorhabdus nematophila	Insect	AJ308438	CAC38398	17·59	Morgan *et al.* (2001)
Streptomyces avermitilis	Insect and nematode	NC_003155	NP_826813	16·94	Putter *et al.* (1981)
Metarhizium anisopliae	Insect and nematode	AF036320	AAC33265‡,¶	11·39	Renn *et al.* (1999); Sun *et al.* (2006)
Nepenthes khasiana§	Insect	AY618881	AAT40732¶	10·40	Eilenberg *et al.* (2006)

*Identity with Plc, the chitinase of interest, from *Paecilomyces lilacinus* IPC, as calculated by using DNAMAN.

†Those in which the pathogenicity of these organisms were reported.

‡A chitinase from *Metarhizium anisopliae*, which shares a low sequence identity (12·64%) with another chitinase (Protein_id AAZ32788) from the same species, as shown above.

§A plant with pitcher traps capable of efficient prey capture and digestion (Eilenberg *et al.* 2006).

¶Neither of them has the consensus motifs SXGG and DXXDXDXE, corresponding to a substrate-binding site and a catalytic domain, respectively, in the glycosyl hydrolase family 18; however, the remaining sequences share the conserved motifs characteristic of this hydrolase family.

LOW RESOLUTION FIG

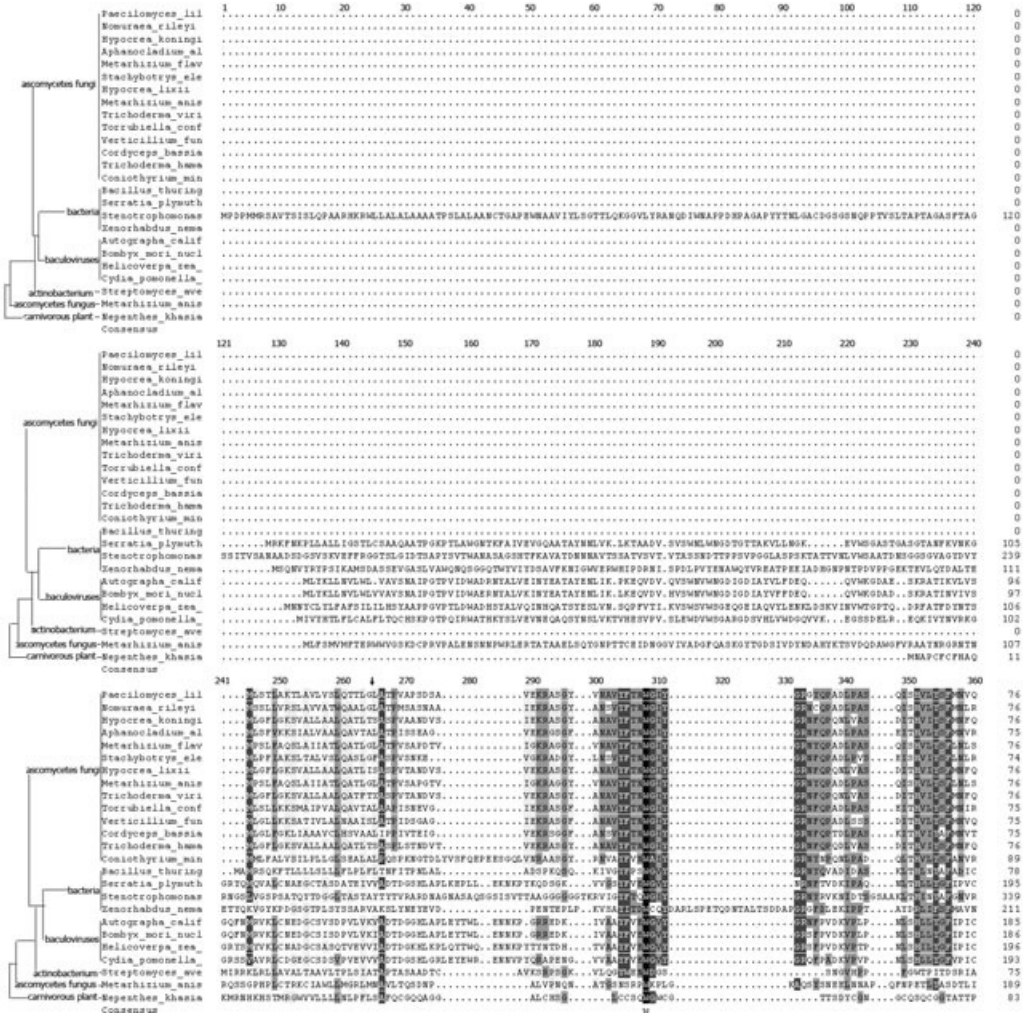

Figure 1 Multiple sequence alignment of the deduced amino acid sequence of Plc and 24 other chitinases from mycopathogens, entomopathogens and nematopathogens (120 amino acids in width), by using dnaman. Similar residues are shaded, with the highlight homology level ranging from dark black (100% identity), black (100% to 75%), grey (75% to 50%), to light grey (50% to 33%). Gaps are introduced to maximize the alignment. Multiple sequences were aligned according to the clustering in the phylogenetic analysis. The chitinase of interest, Plc from *Paecilomyces lilacinus* IPC (Protein_id ABP37997), is shown on the top row. The arrow indicates the position of cleavage of signal sequences from the proenzyme of Plc. The solid and open triangles indicate the N- and O-linked glycosylation sites in Plc, respectively. The conserved motifs SXGG and DXXDXDXE, corresponding to a substrate-binding site and a catalytic domain, respectively, in the glycosyl hydrolase family 18, are boxed. The numbers on the right side of each row indicate the exact numbering of the last amino acid residue in the respective chitinases.

was marginally supported by the presence of a consensus 5′-splice donor site (GTANGT) and a 3′-splice acceptor site (YAG) characteristic of other fungal introns (Gurr *et al.* 1987). The sequence surrounding the start of translation, 5′-TAACGATGCTT-3′, is in close agreement with higher eukaryotic consensus sequences and many fungal genes (Hayes *et al.* 1994). Several other sequences that are homologous to eukaryotic promoter elements can be identified further upstream from the start codon ATG. At −156 bp (A in ATG being +1), the sequence TATAAG indicates a potential TATA-box and five putative CAAT-boxes can be identified at −354, −323 (on the negative strand), −302, −133 (on the negative strand) and −145 bp, respectively (Gurr *et al.* 1987). The sequence AAAA, 29 bp downstream of the termination codon TAA, may serve as the polyadenylation site (Gurr *et al.* 1987). Carbon-catabolite repression in the ascomycetes is mediated by a C_2H_2-type DNA-binding binuclear zinc-finger protein CREA (carbon catabolite repressor). The CREA protein of the fungus *Aspergillus nidulans* had been demonstrated to bind to the consensus sequence 5′-SYGGRG-3′ to effect carbon-catabolite repression (Screen *et al.* 1997). Upstream of the *PLC* translation start codon, two putative CREA-binding sites, −506 to −501 and −200

LOW RESOLUTION FIG

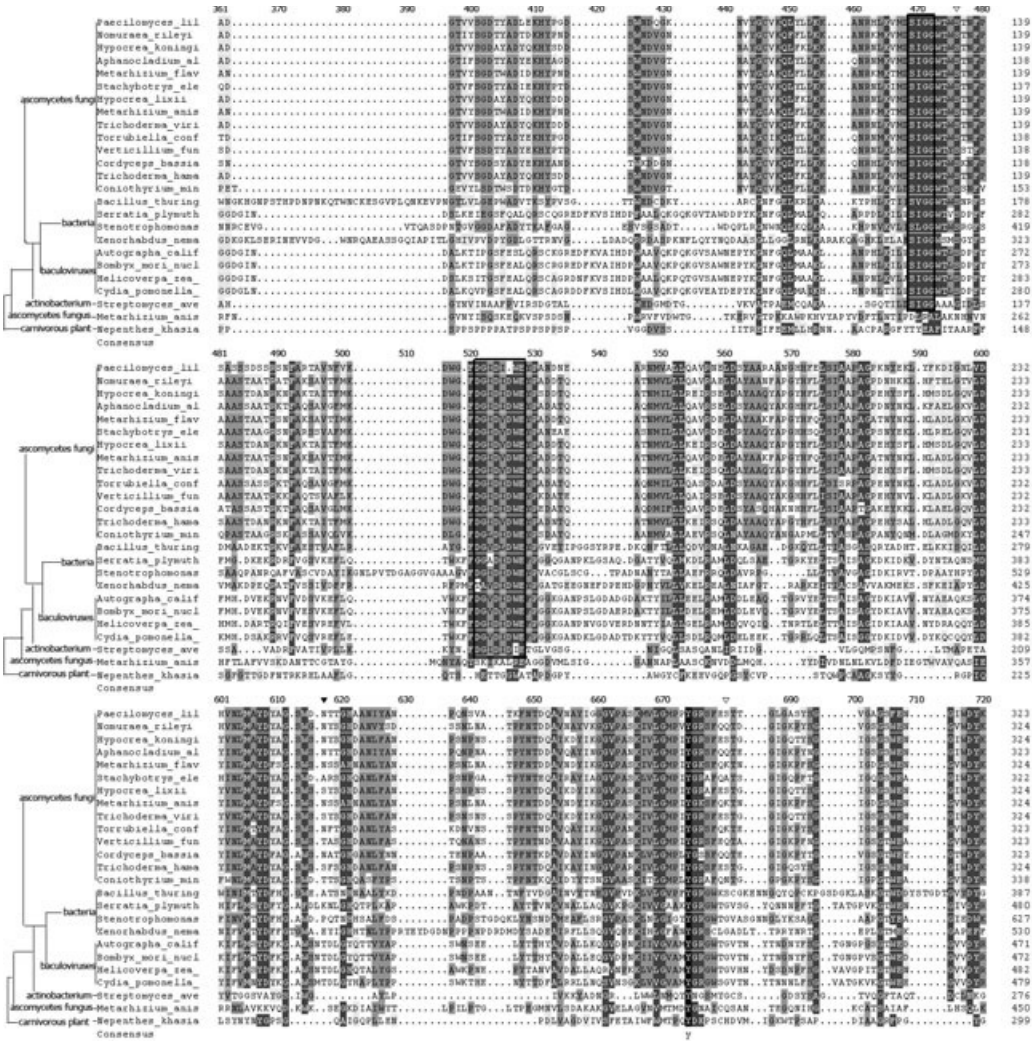

Figure 1 Continued

to −197 (on the negative strand) were identified. One stress-responsive element (STRE; CCCCT, at −315) was also observed. STRE has been shown previously to mediate transcriptional activation in response to various stresses, especially heat, osmotic stress, low pH and nutrient starvation in the yeast *Saccharomyces cerevisiae*, and has also been found in the entomopathogenic and nematophagous fungus *Beauveria bassiana* (Fang *et al.* 2005). In addition, two 5′-HGATAR-3′ elements, which correspond to the global nitrogen regulator AREA/NIT2 (Suárez *et al.* 2005), were identified at −450 to −445 and −404 to −399. The block 5′-CATGTTCT was found in nucleotide positions 1314–1321 near the poly(A) site. This sequence resembles the region 5′-CATGGTTCT described for several fungal genes and may function as a termination signal (Gurr *et al.* 1987). The third position in each codon is specified as pyrimidine, as filamentous fungal genes often exhibit this bias (Hayes *et al.* 1994). Specifically, the codon usage in the *PLC* gene shows a heavy bias towards the use of C at the third positions, 50% end with C, 17% end with T, 26% end with G and only 7% of the codons ended with A. For more detailed information concerning *PLC*, readers are referred to the NCBI GenBank accession number EF183511.

Amino acid sequence

The deduced protein is designated as Plc. The ORF translates to a polypeptide of 422 amino acid residues with a calculated molecular mass of 45·783 kDa and pI of 5·65. A signal-peptide cleavage site prediction carried out according to the SIGNAL P suggested that cleavage occurs after Gly, indicating a signal peptide of 20 amino acids long. Hydropathy analysis with DNAMAN showed that this

LOW RESOLUTION FIG

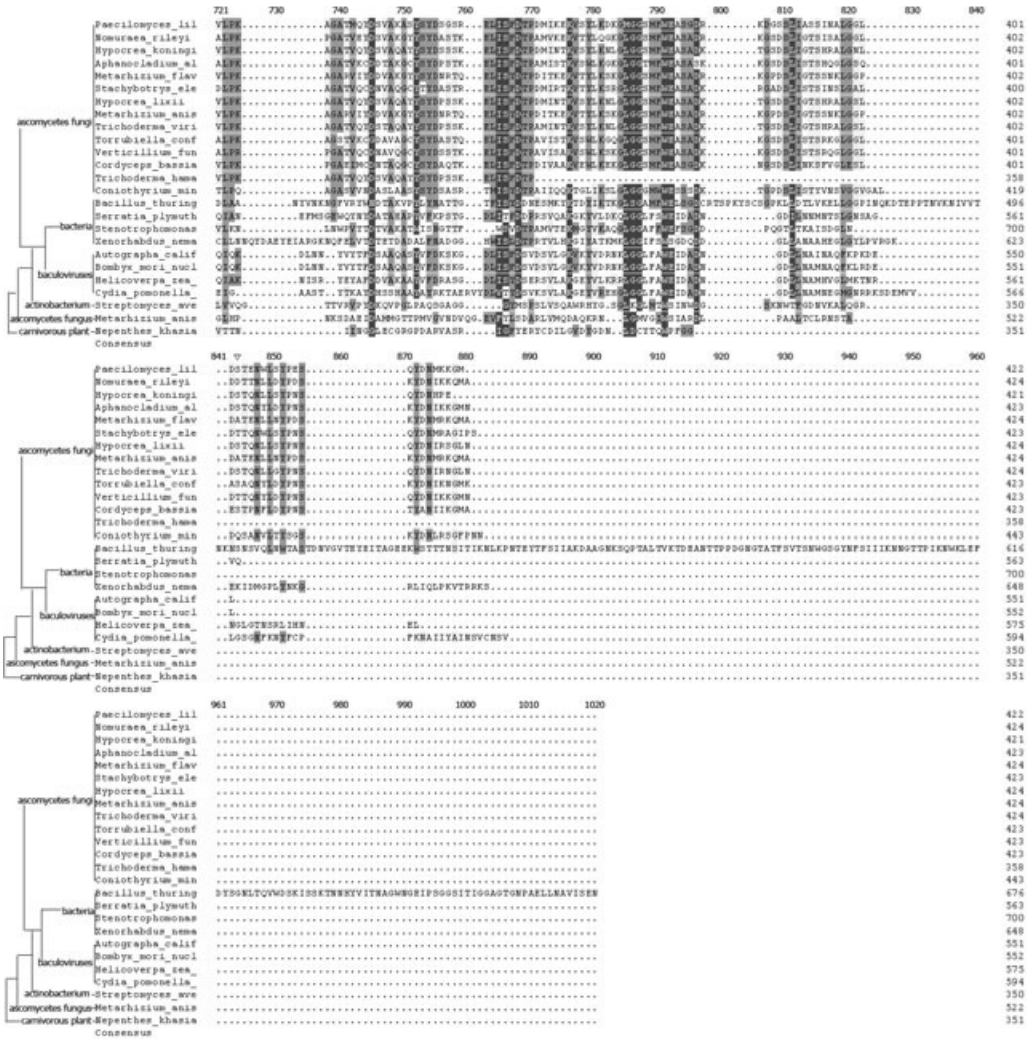

Figure 1 Continued

area is also highly hydrophobic, suggesting that the protein is translocated to the endoplasmic reticulum. After processing of the 20 N-terminal amino acids, the calculated size and pI of Plc are 43·741 kDa and 5·41, respectively. The deduced primary sequence of the enzyme contains one putative N-linked glycosylation sites (Asn_{617}), which follows the general rule of Asn-X-Thr/Ser, where X is any residue except perhaps aspartate, glutamic acid or proline (Mononen and Karjalainen 1984). Three Ser-Thr base pairs (Ser_{476}, Ser_{680} and Ser_{844}) are potential sites for *O*-glycosylation (St. Leger *et al.* 1992). The consensus motifs SXGG and DXXDXDXE, corresponding to a substrate-binding site and a catalytic domain, respectively, in glycosyl hydrolase family 18 (Pfam database; Falquet *et al.* 2002), were identified, indicating that Plc is a member of this family. However, the catalytic domain consensus motif ($D^1XXD^2XD^3XE$), highly conserved among fungal chitinases (Screen *et al.* 1997), has one amino acid deletion at D^3 in the Plc sequence. Also, for more detailed information concerning Plc, please see Fig. 1 or NCBI Protein_id ABP37997. Further, the conceptual translation of the *PLC* was aligned with the same homologous sequences representing both classes (III and V) of the glycosyl hydrolase family 18, same as those used in the characterization of the chitinase from the entomopathogenic fungus *Nomuraea rileyi* (Wattanalai *et al.* 2004). It was shown that family 18 chitinases could be divided into two groups based on their amino acid sequences. The two groups corresponded to the classes III and V fungal chitinases. Additionally, the dendrogram shows that Plc belongs to the class V, as illustrated in Fig. 2. Additionally, Khan *et al.* (2003) had found six active forms of chitinase after isoelectric focusing of proteins from *P. lilacinus* strain 251. From these,

LOW RESOLUTION FIG

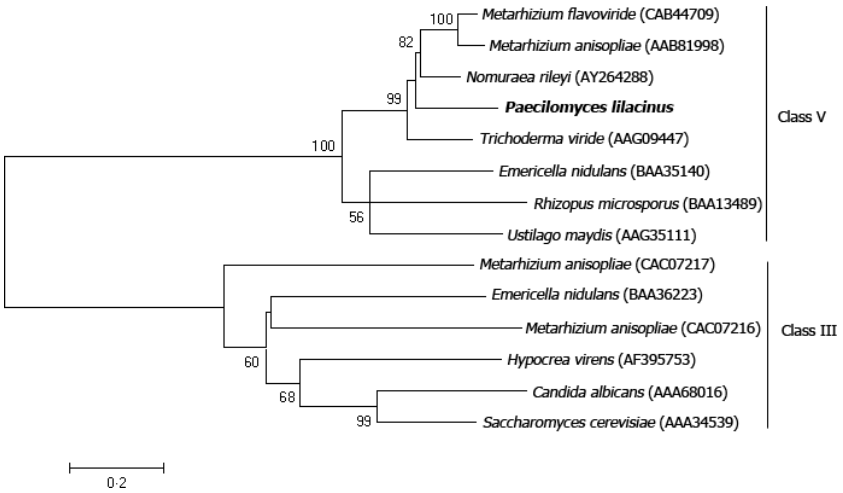

Figure 2 An unrooted Neighbor-Joining tree derived from the sequences of Plc and 13 other fungal chitinases representing either class III or V of the glycosyl hydrolase family 18. The tree was constructed using programs in the mega. Accession numbers for these sequences are indicated after each species name. The chitinase of interest, Plc from *Paecilomyces lilacinus* IPC (Protein_id ABP37997), is in bold. Bootstrap confidence levels (1000 replicates, nodes supported with more than 50%) are shown at the nodes. The bootstrap frequency lower than 50% is not shown. The sequences used were the same as used by Wattanalai *et al.* (2004) to further classify the chitinase (Protein_id AAP04616) from the entomopathogenic fungus *Nomuraea rileyi*, after it has been confirmed that the chitinase of interest belongs to the glycosyl hydrolase family 18.

two proteins were confirmed as a chitinase by Edman sequencing and database matching; however, the N-terminal sequences of these two proteins do not match their counterpart in Plc.

Multiple sequence alignment

A protein–protein BLAST (Basic Local Alignment Search Tool) search using the deduced amino acid sequence Plc demonstrated similarities to a number of fungal chitinases classified in family 18 of the glycosyl hydrolases, such as those of *Nomuraea rileyi* (Protein_id AAP04616, 70·05% identity as calculated using DNAMAN), *Metarhizium flavoviride* (CAB44709, 68·40%), *Stachybotrys elegans* (AAM70478, 68·24%) and *Trichoderma viride* (AAF19618, 67·69%). Among the 100 sequences producing the most significant alignments, 52 sequences belong to endochitinase, as presented in their sequence reports, so the Plc in our study might be endochitinase too.

Because a number of reports have suggested the role of chitinases in the pathogenesis of many microbial pathogens, in this study, we compared the amino acid sequence of Plc with those of chitinases from mycopathogens, entomopathogens and nematopathogens. As illustrated in Fig. 1, conserved regions were detected for consensus motifs SXGG and DXXDXDXE, corresponding to a substrate-binding site and a catalytic domain, respectively, in the glycosyl hydrolase family 18, The exceptions were for the chitinases from the fungus *M. anisopliae* (Protein_id AAC33265) and the carnivorous plant *Nepenthes khasiana* (AAT40732). Also, the entire amino acid sequence of Plc exhibits high identity with deduced amino acid sequences from other fungal chitinases except the one from the entomopathogenic and nematophagous fungus *M. anisopliae*. The sequence identities between Plc and these chitinases vary between 70·05% and 50·23% (pairwise alignments of the amino acids in the preproenzyme). The collective sequence identity in the fungal group is 74·94% (as illustrated in Fig. 3). The Plc amino acid sequence is most similar to the sequence from the entomopathogenic fungus *N. rileyi*. As mentioned above, these fungi included mycopathogens, entomopathogens and nematopathogens. Like *P. lilacinus*, some have also been found capable of infecting multiple hosts, i.e. *Hypocrea lixii* (anamorph: *Trichoderma harzianum*), *M. anisopliae*, *Trichoderma viride*, *Torrubiella confragosa* (anamorph: *Lecanicillium lecanii*), *Cordyceps bassiana* (anamorph: *Beauveria bassiana*) and *Trichoderma hamatum*. In contrast, Plc has lower sequence identities to those from bacterial and baculoviral pathogens. Further, the number of amino acid residues for fungal chitinases ranging from 358 to 443 in our study. For bacteria, the lengths are 563–700. For baculoviruses, the range is 551–594. The chitinases from bacteria (excluding one from the entomopathogenic and nematopathogenic actinobacterium *S. avermitilis*) have a low degree of sequence similarity – 26·15% identity. Their viral counterparts share a value as high as 73·18%. Among all the chitinases from glycosyl hydrolase family 18, the one from the actinobacterium is the least similar to Plc, having only 16·94% identity in

LOW RESOLUTION FIG

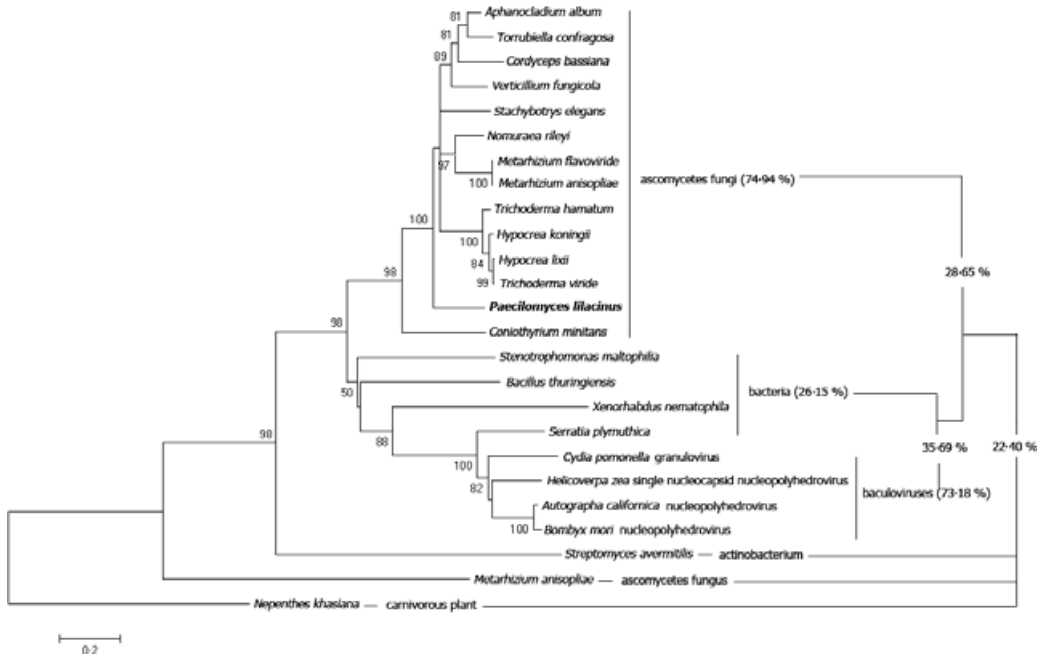

Figure 3 An unrooted Neighbor-Joining tree derived from 25 chitinases representing mycopathogens, entomopathogens and nematopathogens. The tree was constructed using programs in the mega. Bootstrap values (1000 replicates, nodes supported with more than 50%) are given. The bootstrap frequency lower than 50% is not shown. The chitinase of interest, Plc from *Paecilomyces lilacinus* IPC (Protein_id ABP37997), is in bold. The chitinase (Protein_id AAT40732) of the carnivorous plant, *Nepenthes khasiana*, was used as the outgroup. The percentages on the right side are the identity values for the respective group. Sequence accession numbers, their respective identity values with Plc and the pathogenicity of these species are indicated in Table 2.

sequence and sharing the least conserved features in amino acid sequence. For the chitinase from the fungus *M. anisopliae* (AAC33265), its amino acid residue number is beyond the range of 358–443, and its sequence has negligible conserved features with other sequences, such is the case with the carnivorous plant *N. khasiana* (AAT40732). The overall amino acid sequence identity for the 25 sequences is 22·40%.

Phylogenetic analysis

The primary sequences of all the 25 chitinases were aligned and analysed using the software MEGA (Fig. 3). The analysis showed that these chitinase sequences were clustered into two well-supported subgroups corresponding to those from ascomycetes fungi and those of nonfungal origins (bacteria and baculoviruses). As expected from the preceding multiple sequence analysis, the Plc protein is closely related to ascomycetes fungal chitinases belonging to the glycosyl hydrolase family 18, receiving 98% bootstrap confidence support. In contrast, the bacterial chitinases, except the one from the actinobacterium, were more related to chitnases from baculoviruses. The baculoviral chitinases were clustered exclusively within the bacteria cluster (82% bootstrap confidence support). Phylogenetically distant from these two clusters are chitinases from the entomopathogenic and nematophagous fungus *M. anisopliae* (AAC33265), the entomopathogenic and nematopathogenic actinobacterium *S. avermitilis* and the carnivorous plant *N. khasiana*.

Discussion

Our study elucidated the relationships among chitinases from mycopathogens, entomopathogens and nematopathogens. The analysis showed that these chitinase sequences, the majority of which belong to the glycosyl hydrolases family 18, were clustered into two well-supported subgroups corresponding to those from ascomycetes fungi and those of nonfungal origins (bacteria and baculoviruses). These phylogenetic relationships can be interpreted in terms of phylogenetic distance and ecological divergence among the species. Potential microbial control agents against fungi, nematodes and insects include fungi, bacteria and viruses. Fungi are of special interest for a number of reasons. They are known for their diversity and remarkable ability to degrade complex and persistent natural materials (Atagana *et al.* 2006). Fungi also characteristically attack their hosts in a unique, aggressive manner. Bacteria and viruses are by comparison passive

invaders; insects must directly ingest these agents. Fungal pathogens attack insects directly by breaching the host integument, assisted or enabled by cuticle-degrading enzymes. Target insects need not be actively feeding, or even capable of 'eating' (e.g. aphids; Leathers *et al.* 1993). Fungal spores are capable of germinating and penetrating a healthy insect and do not require a compromised host defence (Jackson 1997). In contrast to bacteria, fungi are able to extend the location of their biomass through hyphal growth (Atagana *et al.* 2006). To our knowledge, fungi are often the best known natural pathogens of insects, nematodes and other fungi. However, among all the domains of organisms, bacteria comprise the most biologically and phylogenetically diverse one (Adams *et al.* 2006). In terms of biomass, metabolic activity and number, bacteria are the most abundant micro-organisms in soil (Sturz and Kimpinski 1999). The lytic activity of bacteria is one of a number of mechanisms that has been implicated in biocontrol for several years (Kobayashi *et al.* 2002). Baculoviruses are insect-specific viruses with an established application as insecticides against agricultural and forestry pest (Lihoradova *et al.* 2007). The Baculoviridae have been divided into two genera, the nucleopolyhedroviruses (NPVs) and the granuloviruses, based on occlusion body morphology. Infection of silkworm larvae with the *Bombyx mori* NPV induces a characteristic terminal liquefaction of insect tissue after death; liquefaction of the host cadaver is believed to facilitate the dispersal of the progeny virus into the environment (Daimon *et al.* 2007). Previous studies have identified two gene products encoded by many baculoviruses that are essential for this liquefaction process: VCHIA, a chitinase, and V-CATH, a cysteine protease (Daimon *et al.* 2007). In Wang *et al.*'s (2004) work, through Neighbor-Joining and maximum parsimony analyses, their results clearly indicated a monophyletic relationship of the baculovirus chitinases to the chitinases of a sub-branch of bacteria. Their results strongly supported the hypothesis that baculovirus chitinase is most likely to be of a bacterial origin and was acquired by gene transfer. In this study, the baculoviral chitinases were clustered exclusively within the bacteria cluster (82% bootstrap confidence support), reaffirming their hypothesis.

In terms of the specific function of these chitinases in our study, only a few have reported biological activity. Those included the antifungal chitinases from bacteria *Serratia plymuthica* (Frankowski *et al.* 2001), *Stenotrophomonas maltophilia* (Kobayashi *et al.* 2002) and insecticidal chitinase from the bacterium *Xenorhabdus nematophila* (Morgan *et al.* 2001). Chitinases have been found in chitin-containing organisms, such as insects and fungi, and in organisms that do not contain chitin, such as bacteria and plants; chitinases in nonchitin-containing organisms may be involved in the defence against chitin-containing pathogens and pests (Shen and Jacobs-Lorena 1997). So, these bacterial chitinases could be involved in pathogenesis process. However, at present, the roles of these chitinases from fungi in their pathogenesis remain undetermined. In all filamentous fungi, like those in our study, chitin is one of the major components of the cell wall and is thought to be important for maintaining cell wall integrity. Proper chitin metabolism may be essential for hyphal growth and morphology; chitinases are thought to be involved in processes requiring cell wall digestion, viz. spore germination, hyphal tip growth, hyphal branching, hyphal autolysis and differentiation into spores, assimilation of chitin (Takaya *et al.* 1998), as well as mycoparasitism (Kobayashi *et al.* 2002; Frankowski et al. 2001), entomopathogenicity (Charnley 2003) and nematopathogenicity (Stirling and Mankau 1979; Mian *et al.* 1982; Morgan-Jones and Rodriguez-Kabana 1985; Tian *et al.* 2000; Tikhonov *et al.* 2002; Khan *et al.* 2004; Jin *et al.* 2005). In one study, five chitinase genes, representing members of groups A–C of the glycoside hydrolase family 18, were cloned from the mycoparasitic fungus *Hypocrea atroviridis* P1 (ATCC 74058) (anamorph: *Trichoderma atroviride*; Seidl *et al.* 2005). So, one fungal species might have more than one chitinase-encoding gene, whose products might belong to different chitinase families, such is the case of *M. anisopliae* in our study, while chitinase itself might have diverse functions. In insects, chitin is the major component of the exoskeleton (Shen and Jacobs-Lorena 1997). The liquefaction of cadavers is probably pivotal to the release of progeny virus and its dissemination into the environment; chitinase is considered responsible for the liquefaction process (Wang *et al.* 2004; Daimon *et al.* 2007).

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