

Note

Chitinase gene of the dimorphic mycopathogen, *Nomuraea rileyi*

Chitinases are endoglucanases that cleave the internal β -1,4-*N*-acetyl-D-glucosamine linkages in chitin polymers. These enzymes may play a multipurpose role in the biology of insect mycopathogens. Under in vitro conditions, fungi may be induced to produce exocellular chitinases that digest available chitin substrates. Alternatively, constitutive chitinases, localized in the fungal cell wall or in the periplasmic spaces, play a role in fungal cell wall softening allowing for mycelial branch initiation or release of daughter cells (Cabib et al., 2001). The role(s) that endochitinases play in infection process is unclear. In vivo, chitinases could potentially disrupt the cuticle barrier providing the penetrant germ tubes access to nutrients. Under in vitro conditions, *Metarhizium anisopliae* ME1 produced multiple chitinase isozymes including both endochitinases (CHI1, CHI2, and CHI3) and an exochitinase (St. Leger et al., 1993). It has been proposed that a cocktail of serine-like proteases, metalloproteases, subtilisin-like proteases, and aminopeptidases act on peptides cross-linking the chitin fibrils providing available substrate for the fungal chitinases. However, Jackson et al. (1985) reported that chitinase-deficient mutants of the insect mycopathogen *Verticillium lecanii* were able to penetrate and infect aphids. Furthermore, recombinant *M. anisopliae* strains engineered to overproduce chitinase did not possess altered virulence to *Manduca sexta* larvae (Screen et al., 2001). At the late stage of infection, internal fungal cells need to emerge from the insect to produce externally borne conidiophores. At this stage the underlying insect endocuticle has been shown to be extensively digested suggesting that the exocellular chitinases linked to the mycelial phenotype play a crucial role in the egress of these mycopathogens. In addition to playing a role in the infection process, the exocellular chitinases produced during the late stage of infection may also serve as defense molecules inhibiting the development of other chitin-containing microbial competitors. For example, certain fungal endochitinases, such as those isolated from the mycoparasite *Trichoderma hazarianum*, can act as potent anti-fungal enzymes (Lorito et al., 1998). Although they are likely involved in pathogenesis, few endochitinases have been sequenced from insect mycopathogens. Kang et al. (1998) cloned and sequenced a gene coding for a

putative 58 kDa endochitinase (pCHI 11) from *M. anisopliae*. This gene, containing the characteristic site for chitinase, shared little homology with other fungal chitinase sequences. Bogo et al. (1998) reported a second, 42 kDa chitinase gene (*chit 1*) from *M. anisopliae* strain E6. A third endochitinase, sharing 90% aa homology to the CHIT 1 sequence, was detected in an EST library of *M. anisopliae* sf *acridum* (Screen et al., 2001). Additionally, two endochitinase genes (*chit1*, *chit2*) from *Beauveria bassiana* have been published in GenBank. Herein, we report the cloning of an endochitinase from the dimorphic entomopathogen *Nomuraea rileyi*, and compare its sequence to other fungal endochitinases.

The *N. rileyi* MJ strain isolated from a *Spodoptera* larvae collected in Chaing Mai Province, Thailand, was propagated in either Sabouraud dextrose broth containing 2% yeast extract (SDY) or Czapek-Dox minimal medium plus 1% chitin (crab shell, Sigma). Broth cultures were incubated on a gyrotary shaker at 250 rpm at 25 °C. Preliminary studies demonstrated that *N. rileyi* strain MJ when transferred from Sabouraud dextrose plus yeast extract broth to the Czapek-Dox (CZ) broth amended with chitin switched from budding hyphal body to the mycelial phenotype. CZ broth amended with chitin supported limited growth of *N. rileyi* but resulted in the fourfold increase of exocellular chitinase activity that was capable of digesting glycol chitin (data not shown).

Genomic DNA was extracted from the mycelial preparations using a modified CTAB method (Boucias et al., 2000). Fourteen different fungal chitinase sequences from different genera of Hypocreales were selected and aligned using ClustalW to identify consensus regions used to design the degenerate primers ChtF (ATGAARGAYTG GGGYTTYGATGG) and ChtR (CCCARATWCCRTTCTCCAGC). *Nomuraea* genomic DNA was subjected to PCR amplification using the above-mentioned primers and the following thermal program: 94 °C for 5 min; 30 cycles of 94 °C for 30 s; 55 °C for 30 s; 68 °C for 1 min; and a 7 min final extension at 68 °C. The PCR amplified product was gel purified using QIAEX II (Qiagen) and sequenced. Based on this initial 429 bp sequence, the gene-specific primer (GSP) ChtIF was designed (ATACCCCGCCGACGATACTC). Using the same amplification conditions as

above, this GSP was combined to new degenerate primer (ChtR4- WGCYTCCCARAACATGCTGCC), and produced an additional 600 bp fragment that was purified and sequenced. The remaining of the gene sequence was obtained by rapid amplification of cDNA ends (RACE-PCR). Total RNA was isolated from *N. rileyi* cells grown in CZ+chitin using Tri-Reagent (Sigma), and poly(A) mRNA was isolated by Oligotex mRNA purification (Qiagen). Two gene-specific primers (Cht 3' RACE-GAATACCCCGCCGACGATACTCA GG, and Cht 5' RACE-GCTAGCCGGAACACCACC CTTGACG) were designed from the known chitinase sequence to match the Universal Primer Mix (UPM) of the SMART RACE cDNA amplification kit (Clontech). RACE reactions followed instruction outlined in the kit protocol. The RACE-PCR fragments were purified and sequenced directly using gene specific sequencing primers, except for the terminal 5' end fragment, which was cloned into pGEM-T easy vector and sequenced using SP6 and T7 primers.

The 3' and 5' RACE reactions produced a 1725-bp sequence that is available from the NCBI GenBank database with the Accession No. AY264288. The full-length of the chitinase cDNA contains a 120-bp untranslated sequence at the 5' end, a 1272-bp open reading frame (ORF) encoding 424 amino acids, and a 333-bp untranslated sequence at the 3' end terminating with a poly(A) tail. The sequence of the ORF derived from the 3' and 5' RACE reactions was confirmed by conducting RT-PCR with primers designed from the 3' (TTGCGGCTCTTGTCAAACG) and 5' (TCGGTA AAGAAGGCATGGCCC) termini on the original mRNA preparation using the Thermoscript RT-PCR System (Gibco-BRL). The resulting 1528 bp band was sequenced and shown to be identical to that obtained by RACE-PCR amplification. However, amplification of the genomic DNA with the same primers produced a 1710 bp band. This longer fragment not only contains the complete chitinase ORF, but also is interrupted by three short introns: a 111 bp intron at position 248, a 67 bp intron at position 401, and an 60 bp intron at position 519. These introns contained the conserved 5' GT and 3' AG ends. Gurr et al. (1987) have proposed the flexible consensus sequence PyG/ACTAAC as a putative internal splicing signal. As alternatives to this consensus sequence, the splicing signal could be AAG TAAC, AGCTAAC, and GCATAAC for the first, second, and third introns, respectively. The position and size of the three introns detected in this gene are comparable to homologous gene from the insect mycopathogen *M. anisopliae* (Bogo et al., 1998).

Analysis of the ORF using the program SignalP (Nielsen et al., 1997) predicted a 19 aa signal sequence containing a hydrophobic core preceded by positively charged residues and a signal sequence cleavage site

located between aa 19 and 20. Whether an additional cleavage site is present downstream such as that found with the *M. anisopliae* endochitinases is unknown. Analysis using ProtParam Tool revealed that the 405 aa mature chitinase (w/o signal sequence) has a molecular mass of 43.9 kDa, an aliphatic index of 68.27 and is considered to be a stable protein with an instability index (II) of 26.19 (Guruprasad et al., 1990; Ikai, 1980). Furthermore, the *N. rileyi* chitinase is an acidic protein with a calculated pI of 5.26. Analysis of this sequence with the PROSITE program revealed consensus sequences for two N-glycosylation sites, one glycosaminoglycan attachment site, two tyrosine sulfation sites, two cGMP-dependent protein kinase phosphorylation sites, two protein kinase C phosphorylation sites, six casein kinase II phosphorylation sites, and nine N-myristoylation sites (Baroch et al., 1997). Importantly, this sequence contained two highly conserved regions (S-X-G-G and D-X-D-X-E) of the active domain of the family 18 glycosyl hydrolases (Terwisscha van Scheltinga et al., 1996). The potential chitin-binding domain (VMLSIGG) where X of XXXSXGG represents hydrophobic domain was located at aa 124 and the catalytic active site (FDGIDIDWE-) was located at aa 145 where glutamic acid is the critical residue (PROSITE data base).

A BLAST search of the deduced amino acid sequence demonstrated similarities to a number of fungal chitinases classified in family 18 of the glycosyl hydrolases such as *Metarhizium flavoviride* (74% identity), *Aphanocladium album* (71% identity), *M. anisopliae* (69% identity), *Trichoderma viride* (67% identity), *Trichoderma hamatum* (66% identity). Furthermore, the conceptual translation of the *Nomuraea* chitinase was aligned with homologous sequences representing both classes (III and V) of the glycosyl hydrolase family 18. This alignment was performed using ClustalX with default parameters (Thompson et al., 1997). Dendograms were calculated from the alignment by the unweighted pair group method using arithmetic averages (UPGMA) as implemented in PAUP* version 4.0 beta 10 (Swofford, 2000). The UPGMA analysis (Fig. 1) shows that family 18 chitinases can be divided into two groups based on their amino acid sequences. The two UPGMA groups correspond to the classes III and V fungal chitinases. Additionally, the dendrogram shows that the cloned *Nomuraea* chitinase belongs to the class V. Its sequence is most similar to class V chitinase sequences previously cloned from other entomopathogenic fungi of the genus *Metarhizium*. The alignment of the *N. rileyi*, *M. flavoviride*, and *M. anisopliae* class V chitinases is presented in Fig. 2. The dendrogram, as well as additional distance and parsimony analyses inferred from the alignment (data not shown), suggest that *Nomuraea* and *Metarhizium* chitinases are closely related.

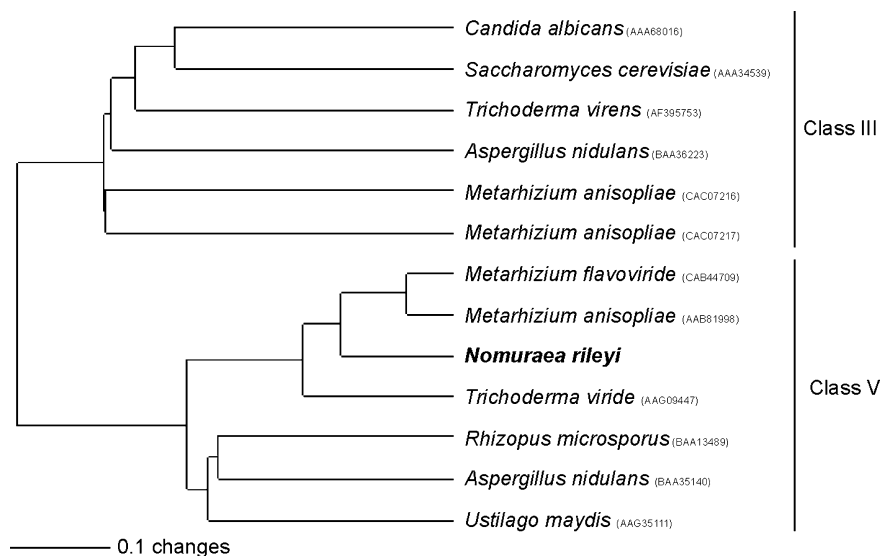


Fig. 1. UPGMA tree based on fungal chitinase sequences. The *N. rileyi* chitinase is grouped within the class V and is associated with *Metarhizium* class V chitinases. Accession numbers for the fungal sequences are indicated after each species name.

<i>M. flavoviride</i>	MPSLFAQSLAI IATLQATLGLATPV SAPDTVIGKRAGGYVNAVYFTNWGIYGRNYQPADL
<i>M. anisopliae</i>	MPSLFAQSLAI IATLQATLGLATPV SAPDTVIGKHAGGYVNAVYFTNWGIYGRNYQPADL
<i>N. rileyi</i>	<u>MSLLVRS LAVVATWQAALGL</u> ATPMSASNA AIEKRASGYANSVYFTNWGIYGRNCQPADL
<i>M. flavoviride</i>	PASQISHVLYSFLNLSANGTVYS GDTWADIDKHYPNDSWNDVGNVYGC AKQLFLLKKAN
<i>M. anisopliae</i>	PASQISHVLYSFLNLSNNGTVYS GDSWADIDKHYPNDSWNDVGTNVYGCVKQLYLLKKAN
<i>N. rileyi</i>	PASQISHVLYSFMNLRADGTI YSGDTYADTDKHYPNDSWNDVGNVYGCVKQFLLKKAN
<i>M. flavoviride</i>	RKMKTMLSIGGWTWSTNFPAAASTAATRSNFAKSAVTIMKDWGFDGIDVDWEYPADDTQA
<i>M. anisopliae</i>	RNMKTMLSIGGWTWSTNFPAAASTAATRSNFAKSAVTIMKDWGFDGIDVDWEYPADDVQA
<i>N. rileyi</i>	RKMKVMLSIGGWTWSTNFPAAASTAATRAFSAKSAVTFMKDWGFDGIDVDWEYPADDTQA
<i>M. flavoviride</i>	TNMVLLLQAVRDEL DAYAAKFAPGYHFQLSIAAPAGATNYNKLHLADLGKVL DYINLMAY
<i>M. anisopliae</i>	TNMVLLLQAI REELDAYAAKFAQGYHFQLSIAAPAGPANYNKLHLGDLGKVL DYINLMAY
<i>N. rileyi</i>	TNMVLLLQAVRAEL DAYAANFAPGYHFLLSIAAPAGPDNHKKLHFTLGVLDYINLMAY
<i>M. flavoviride</i>	DFSGSWSNSSAHNANLYANPSNLNATPFNTDDAVNDYIKGGVPASKIVLGMPIYGKSFQK
<i>M. anisopliae</i>	DFSGSWSNSSAHNANLYA I RANLNA - PFNTDHAVNDYIKGGVPASKIVLALPIYGN SFQK
<i>N. rileyi</i>	DYAGSWGNYSGHDANVYSDSSNLNATPFNTDDAVNAVYKGGVPASKIVLGMPIYGRSFQK
<i>M. flavoviride</i>	TNGIGKPFSGIGDGSWENGVDYKVL PKAGARVIYDDVAKGYYSYDNR TQELISYDTPDI
<i>M. anisopliae</i>	TNGIGKPFSGAGDGSWENG IWDYKVH SKAGADGIYDDGDKGYYSYDPSVKELISIDTPDI
<i>N. rileyi</i>	TDGIGKPYSGVSGSWENGVDYKALPKPGATVEYDSVAKGYYSYDASTKELISFDTPAM
<i>M. flavoviride</i>	TKEKVTYLKS KGLGGSMFWEASADRKGPDSLIGTSSNKLGGPDATENLLNYPDSKYDNMR
<i>M. anisopliae</i>	TKDKVTYLKS KGLGGSMFWEASDRSGS QSLIGTSSNKLGGPDSTENLLNYPDSKYDNMR
<i>N. rileyi</i>	VKEKVTYLQKGLGGSMFWEASADRKGS DSLIGTSSALGGLDDTTNLLDYPDSKYDNMR
<i>M. flavoviride</i>	KQMA
<i>M. anisopliae</i>	KQMA
<i>N. rileyi</i>	KQMA

Fig. 2. Alignment of *N. rileyi* 424 aa chitinase sequence with the Class V chitinases of *M. anisopliae* and *M. flavoviridae*. Approximately, $\geq 70\%$ homology was noted among these three sequences. The signal sequence of *N. rileyi* chitinase is bold and underlined. Highlighted regions are the potential chitin-binding domain (VMLSIGG) and the catalytic active site (FDGIDIDWE).

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