APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2005, p. 363–370 Vol. 71, No. 1
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.1.363–370.2005

Cloning of *Beauveria bassiana* Chitinase Gene *Bbchit1* and Its Application To Improve Fungal Strain Virulence

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Entomopathogenic fungi can produce a series of chitinases, some of which act synergistically with proteases to degrade insect cuticle. However, chitinase involvement in insect fungus pathogenesis has not been fully characterized. In this paper, an endochitinase, Bbchit1, was purified to homogeneity from liquid cultures of *Beauveria bassiana* grown in a medium containing colloidal chitin. Bbchit1 had a molecular mass of about 33 kDa and pI of 5.4. Based on the N-terminal amino acid sequence, the chitinase gene, *Bbchit1*, and its upstream regulatory sequence were cloned. *Bbchit1* was intronless, and there was a single copy in *B. bassiana*. Its regulatory sequence contained putative CreA/Crel carbon catabolic repressor binding domains, which was consistent with glucose suppression of *Bbchit1*. At the amino acid level, Bbchit1 showed significant similarity to a *Streptomyces avermitilis* putative endochitinase, a *Streptomyces coelicolor* putative chitinase, and *Trichoderma harzianum* endochitinase Chit36Y. However, *Bbchit1* had very low levels of identity to other chitinase genes previously isolated from entomopathogenic fungi, indicating that *Bbchit1* was a novel chitinase gene from an insect-pathogenic fungus. A *gpd-Bbchit1* construct, in which *Bbchit1* was driven by the *Aspergiullus nidulans* constitutive promoter, was transformed into the genome of *B. bassiana*, and three transformants that overproduced Bbchit1 were obtained. Insect bioassays revealed that overproduction of Bbchit1 enhanced the virulence of *B. bassiana* for aphids, as indicated by significantly lower 50% lethal concentrations and 50% lethal times of the transformants compared to the values for the wild-type strain.

Biological control agents are being considered as supplements or alternatives to synthetic chemical insecticides that are known to have toxic effects on nontarget organisms, including animals and humans (3, 31). Entomopathogenic fungi are key regulatory factors of insect populations in nature (7) and are attracting attention as biocontrol agents for insect pests (10). However, mycoinsecticides constitute a very small percentage of the total insecticide market (33), even though entomopathogenic fungi are the only practical microbial control for insects that feed by sucking plant and animal juice and for the many acridid and coleopteran pests which have no known viral or bacterial pathogens (14, 31). A major limitation to the development of mycoinsecticides has been that, compared with chemical insecticides, they require a longer time after application for insect control, during which the infected insects can cause serious damage to the crops (31). Improvements in the effectiveness of mycoinsecticides may be obtained by optimizing the preparation and application of the inoculum (24). Improving the virulence of mycoinsecticides can also be achieved through genetic modification (32, 38).

The insect cuticle, the first barrier against fungal pathogens, consists of a thin outer epicuticle, containing lipid and proteins, and a thick procuticle, consisting of chitin and proteins. Entomopathogenic fungi produce proteases, chitinases, and lipases which can degrade insect cuticle (6, 8). Overexpression of the subtilisin-like protease gene, *Pr1A*, has been shown to significantly enhance the virulence of *Metarhizium anisopliae*, suggesting that cuticle-degrading enzyme genes are candidates for genetic manipulation leading to strain improvement (31).

The entomopathogenic fungi *M. anisopliae* and *Beauveria bassiana* produce several chitinases (4, 16, 30, 27), which may have a variety of different functions. Some of these chitinases are important cuticle-degrading enzymes and act synergistically with proteases to hydrolyze insect cuticle (29). The involvement of chitinases in insect fungus pathogenesis raises the possibility that overexpression of a chitinase gene may provide a way to improve fungal virulence similar to that described for *Pr1A* in *M. anisopliae* (31). However, until now, only one insect fungus chitinase gene, *CHIT1* of *M. anisopliae*, has been characterized, and unfortunately, overexpression of this gene did not alter the pathogenicity of *M. anisopliae* for *Manduca sexta* (27). Therefore, to date, no chitinase genes have been proven to be implicated in insect fungus pathogenesis.

Here we purified an endochitinase from culture liquid of *B. bassiana* grown in a medium in which colloidal chitin was the sole carbon and nitrogen source. After cloning the endochitinase gene, we demonstrated that endochitinase overproduction can significantly enhance the virulence of *B. bassiana*.

MATERIALS AND METHODS

Fungal and bacterial strains. *B. bassiana* strain Bb0062 was isolated from a *Pieris rapae* carcass which was found on cabbage grown on the experimental farm of Southwest Agricultural University in the People's Republic of China in 1997. A single-spore isolate of Bb0062 was stored in 20% glycerol at −80°C. Cultures were grown on Sabouraud's dextrose agar supplemented with 1% (wt/vol) yeast extract (pH 7.0) for 14 days at 26°C with a daily cycle consisting of 15 h of light and 9 h of darkness. *Escherichia coli* DH5α was employed for DNA manipula-

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tion. *Agrobacterium tumefaciens* LBA4404 (2) was used for *B. bassiana* transformation.

Enzyme assay. A chitinase activity assay was performed as previously described (21). One unit of chitinase activity was defined as the amount of enzyme that released sugars equivalent to 1 μmol of *N*-acetylglucosamine per h at 37°C. The protein concentration was determined as described by Bradford (5) by using bovine serum albumin as the standard. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (17), and isoelectrophoresis focusing was performed as described by Fawcett et al. (13).

Blotting. Southern gel blot analysis was performed with about 25 μg of DNA for each sample. The DNA probes were labeled with digoxigenin according to the manufacturer's instructions (Roche, Basel, Switzerland).

For Western blot analysis, proteins were transferred from an SDS—12% PAGE gel to a nitrocellulose membrane (Bio-Rad), and immunoblotting and blot development were carried out according to the instructions provided with an Opti-4CN Western blot kit (Bio-Rad).

Induction of *B. bassiana* chitinase. *B. bassiana* conidia were inoculated into SDY medium (Sabouraud's dextrose agar supplemented with 1% [wt/vol] yeast extract without agar) at a final concentration of 10^6 conidia/ml and incubated at 26°C for 48 h with shaking at 180 rpm. The mycelia were then transferred to basal salt medium supplemented with 1% (wt/vol) colloidal chitin as the sole carbon and nitrogen source. One-milliliter portions of the culture liquid were removed for a chitinase activity assay at 12-h intervals. The values shown below for chitinase activity are means of three replicates.

Chitinase purification. Culture fluid of *B. bassiana* grown in basal salt medium containing 1% (wt/vol) colloidal chitin for 168 h was filtered through Whatman no. 5 filter paper, and the filtrate was subjected to precipitation with ammonium sulfate (75%, wt/vol). The pellet was harvested by centrifugation and dissolved in 100 mM Tris-HCl buffer (pH 8.0). After dialysis against the same buffer, the crude extract was subjected to gel filtration chromatography on Ultragel AcA54 (LKB). Fractions showing chitinase activity were pooled, concentrated against polyethylene glycol 20,000 (PEG 20,000), dialyzed against 20 mM Tris-HCl buffer (pH 7.8), and subjected to anion-exchange chromatography with a DEAE-cellulose column (Amersham). After the column was washed with 3 column volumes of 20 mM Tris-HCl buffer (pH 7.8), it was eluted with a 100-ml linear gradient of NaCl (0 to 1 M) in the washing buffer at a flow rate of 1 ml/min. Fractions with chitinase activity were collected and concentrated against PEG 20,000 for further analysis.

One hundred micrograms of purified chitinase was mixed with Freund complete adjuvant and injected at 2-week intervals into a Japanese long ear white rabbit for production of antiserum.

Gene expression analysis. To analyze the regulatory effect of glucose on the production of chitinase in the media containing colloidal chitin, 2 g of wet mycelia of *B. bassiana* was transferred from SDY medium into medium 1 (basal salt medium supplemented with 1% [wt/vol] colloidal chitin) or medium 2 (medium 1 to which 2% [wt/vol] glucose was added). After 24 h, samples were collected at 48-h intervals by filtration through Whatman no. 5 filter paper. The filtrates were lyophilized, dissolved in distilled water, desalted by using a Hiprep 16/10 desalting column (Amersham), and concentrated against PEG 20,000. Twenty micrograms of protein was then subjected to Western blot analysis.

Sequencing of the N terminus of the protein. Purified chitinase was subjected to SDS-PAGE and then electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) as described by LeGendre et al. (18). Protein bands were visualized with Coomassie blue R250 and excised from the membrane. The chitinase was N terminally sequenced at the Laboratory for Protein Chemistry of Hunan Normal University (Changsha, People's Republic of China) by using a model 491 sequencer (Applied Biosystems) and the Edman degradation method.

Gene cloning. DNA and RNA from *B. bassiana* were prepared as described by Reader and Broda (25) and Chomczynski and Sacchi (9), respectively.

Based on the N-terminal amino acid sequence of the purified chitinase and the *B. bassiana* codon bias found in a codon usage database (http://www.kazusa.or.jp/codon/), degenerate primer P1 (5′-GCCGGCACCTGCGCCAC[ATCG]AA[AG]GG-3′) was designed for cDNA cloning by using 3′ random amplification of cDNA ends (RACE) (TaKaRa). According to the 3′ RACE product, the full-length gene corresponding to the purified chitinase and its upstream regulatory sequence (URS) were subsequently cloned by PCR walking by using the Y-shaped adaptor-dependent extension (YADE) method as previously described (11, 39). Restriction enzymes EcoRV, ScaI, DraI, and SmaI were used to digest *B. bassiana* genomic DNA. Primers LP1 (5′-CCGTGCTTGCGAATGTCG-3′) and EP1 (5′-GGCACCGTCCCAGTTCTC-3′) were designed to amplify the immediate upstream sequence of the cDNA. Based on the PCR product obtained with primers LP1 and EP1, primers LP2 (5′-TTCTTTTGGGCAACAGTCCAGAGG-3′) and EP2 (5′-TCGCGGGAGGGGAGCTTAGCG-3′) were synthesized for continuous amplification of the URS. For the YADE method, linear amplification was performed at 95°C for 5 min, followed by 40 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 3 min of polymerization at 72°C and then 72°C for 3 min. Exponential amplification was performed at 95°C for 5 min, followed by 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 2 min of polymerization at 72°C and then 72°C for 10 min.

Primers Bbchit1-1 (5′-CCCTTCTACCCTTGACTTGTTC-3′) and Bbchit1-2 (5′-TATCTACAAATATGTACCAAC-3′) were used to amplify the genomic sequence of the open reading frame (ORF) of the gene.

DNA manipulation. To conveniently insert a gene into pAN52-1 (kindly provided by P. Punt, TNO Nutrition and Food Research Institute, Zeist, The Netherlands), multiple cloning sites (MCS) were introduced into the BamHI site of the vector. Oligonucleotide primers P1 (5′-GATCCGCTGTACCCGGGGGTACCACTAGTATCGATGATATCGAATTCGCTAGTG-3′) and P2 (5′-GATCCACATGCGAATTCGATATCATAGATACTAGTGGTACCCCCGGGTACAGCG-3′) were synthesized and annealed. The annealing product, which contained BamHI, EcoRI, SpeI, SmaI, and BglII sites, was cloned into BamHI-cut pAN52-1. The resulting vectors were digested with NcoI, blunt ended with mung bean nuclease to remove the ATG translation start codon in the *gpd* promoter, and religated. Sequence analysis was used to examine the orientation of the insert and to confirm that the ATG had been successfully removed. There were two orientations of the MCS within pAN52-1. One of them, pAN-ES, represented the orientation of the MCS in which 3′ to the *gpd* promoter proceeded from BamHI to BglII; the other, pAN-SE, proceeded from BglII to BamHI. The *Pgpd*/*TtrpC* released from pAN-ES was inserted into HindIII-XbaI-cut pBANF-bar (12) to form pBANF-bar-pAN-ES.

The ORF of the *Bbchit1* gene was amplified by reverse transcription (RT)-PCR with forward primer 5′-CGGAATTCATGGCTCCTTTTCTTCAAACCAG-3′, which contained an EcoRI site before the ATG codon, and reverse primer 5′-CGCCCGGGTTACGCAGTCCCCAAAGTCCC-3′, which had a SmaI site after the stop codon. The RT-PCR product was then inserted into pGEM-T by A-T cloning (Promega). After we confirmed that there was no mutant by sequence analysis, the *Bbchit1* ORF was excised from pGEM-T with EcoRI and SmaI and ligated into pBANF-bar-pAN-ES to form pBANF-bar-pAN-Bbchit1. The pBANF-bar-pAN-Bbchit1 was mobilized into *A. tumefaciens* LBA4404 (2).

Transformation and screening. The overexpression cassette located in pBANF-bar-pAN-Bbchit1was introduced into *B. bassiana* by using previously described protocols (12). Transformants were selected for resistance to 60 μg of the herbicide phosphinothricin per ml. Wet mycelia (2.5 g) of each herbicide-resistant transformant were transferred from SDY liquid medium to basal salt medium supplemented with 2% (wt/vol) glucose and 0.5% (wt/vol) $NaNO_3$ and grown at 26°C for 24 h at 100 rpm. Higher chitinase activity should have been detected in liquid cultures of the transformants which overproduced Bbchit1 than in cultures of the wild-type strain. Western blotting was employed to confirm the overproduction of Bbchit1.

Bioassay. Adult aphids (*Myzus persicae*) that were 0 to 2 days old and had been reared on cabbage in a greenhouse were obtained by the method of Vandenberg (35) and used for a bioassay. Aphids were inoculated by dipping them into a conidial suspension, fed on cabbage, and transferred into an HPG-280H artificial climate cell (Haribing Donglian Electronic Company, Haribing, People's Republic of China) kept at 22 to 24°C to monitor the course of infection. Control insects were treated with 0.05% (vol/vol) Tween 80. Six conidial suspensions (5×10^5, 1×10^6, 5×10^6, 1×10^7, 5×10^7, and 1×10^8 conidia/ml) were used for inoculation, and the concentrations were determined microscopically by using a hematocytometer (Medical Instrument Inc., Shanghai, People's Republic of China). Each treatment was replicated three times, and each replicate contained 30 aphids. Mortality was recorded at 12-h intervals. The experiments were repeated three times. A χ^2 test was used to determine the homogeneity of the variance of the repeats ($P < 0.05$). The SPSS program was then used to estimate 50% lethal concentrations (LC_{50}), 50% lethal times (LT_{50}), fiducial limits, and other regression parameters.

Nucleotide sequence accession number. The accession number of the *Bbchit1* gene is AY145440.

RESULTS

Purification of the enzyme and determination of N-terminal amino acid sequence. Chitinase activity was not observed until 36 h after mycelia were transferred from SDY medium to the basal salt medium supplemented with colloidal chitin as the

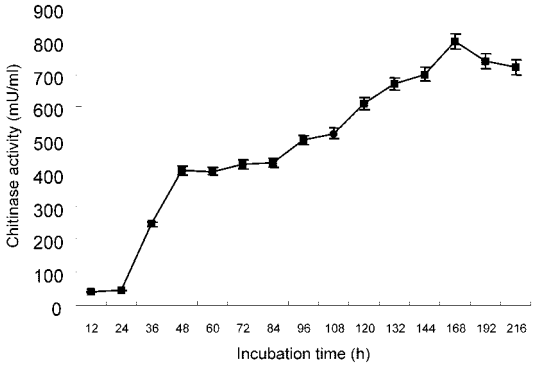

FIG. 1. *B. bassiana* chitinase production in basal salt medium supplemented with 1% (wt/vol) colloidal chitin. Wet mycelia (2 g) were transferred from SDY medium to the basal salt medium. Chitinase activity was detected at 36 h, reached a peak at 168 h, and then began to decrease.

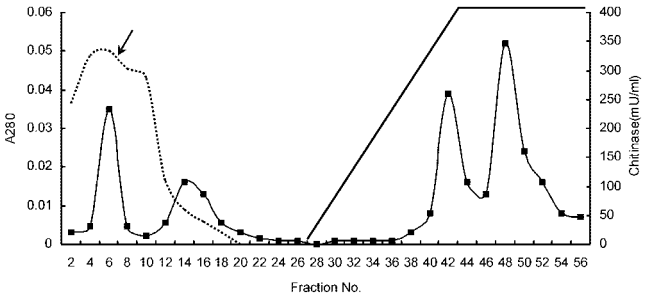

FIG. 3. Profile of elution of endochitinase activity from a DEAE-cellulose anion-exchange column. The active fractions from the Ultragel ACA54 column were concentrated against PEG 20,000, dialyzed with 20 mM Tris-Cl buffer (pH 7.8), and subjected to anion-exchange chromatography on a DEAE-cellulose column (Amersham). Elution was performed with a 100-ml linear gradient of 0 to 1 M NaCl. The flow rate was 1 ml/min. A single peak of endochitinase activity appeared in unbound fractions 2 to 12 (indicated by the arrow), which were pooled and used for further analysis.

sole carbon and nitrogen source, and it reached a maximum level 168 h after the transfer (Fig. 1). Therefore, the 168-h culture liquid was used for chitinase purification.

Only one peak of endochitinase activity was observed when Ultragel AcA54 gel filtration was used (Fig. 2). Fractions of this single peak were subjected to anion-exchange chromatography on a DEAE-cellulose column (Fig. 3). An endochitinase, designated Bbchit1, was purified to homogeneity as determined by SDS-PAGE (Fig. 4). The molecular mass of Bbchit1 was estimated to be approximately 33 kDa, which was different from the molecular masses of chitinases purified from *B. bassiana* by Peng et al. (23) and Havukkala et al. (15). Isoelectric focusing analysis showed that the pI of Bbchit1 was 5.4.

The N-terminal 22 amino acid residues of Bbchit1were determined to be AGTCATKGRPAGKVLQGYWENW. A BLASTp analysis revealed that the N-terminal amino acid sequence showed high levels of identity with the N-terminal sequences of chitinase Chit36Y (accession number AAL01372) (36) from *Trichoderma harzianum*, a putative endochitinase from *Streptomyces avermitilis* MA-4680 (accession number NP_826813), and a putative chitinase precursor from *Streptomyces coelicolor* A3 (2) (accession number NP_626743). Interestingly, the N-terminal amino acid sequence was not similar to the N-terminal amino acid sequences of other chitinases from insect-pathogenic fungi.

Cloning and sequence analysis of the chitinase gene, *Bbchit1*. To isolate the gene corresponding to Bbchit1, a degenerate primer was designed based on eight N-terminal amino acid residues. A 1,120-bp cDNA was specifically amplified by 3′ RACE. The first 22 amino acid residues of the translation product of the cDNA were identical to those of endochitinase Bbchit1, suggesting that the 3′ RACE product was the cDNA corresponding to the purified Bbchit1. To obtain the full-length gene, the YADE method was used to clone the upstream sequence of the cDNA. From the DraI-digested genomic DNA, a 1,283-bp DNA fragment was amplified with primers LP1 and EP1. Based on the YADE-generated DNA fragment, a 1,007-bp DNA fragment was obtained by PCR

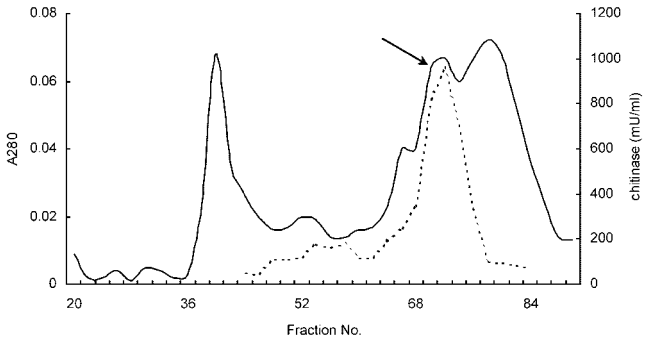

FIG. 2. Profile of elution of endochitinase activity from an Ultragel AcA54 gel filtration column. A sample of the concentrated and dialyzed crude extract from *B. bassiana* was used for gel filtration on an Ultragel AcA54 column (LKB). Elution was performed with 300 ml of 20 mM Tris-HCl buffer (pH 8.0). The flow rate was 2.5 ml/min. Only one peak of endochitinase activity was detected (indicated by the arrow); fractions of this peak were pooled and concentrated for the next purification step.

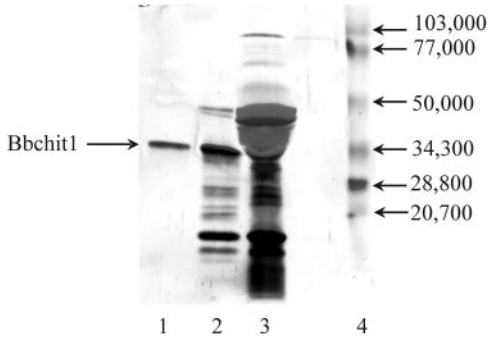

FIG. 4. SDS-PAGE of chitinase fractions from each purification step. The endochitinase Bbchit1 band is indicated by the arrow on the left. Lane 1, anion-exchange chromatography on the DEAE-cellulose column; lane 2, gel filtration chromatography on the Ultragel AcA54 column; lane 3, ammonium sulfate precipitation; lane 4, molecular weight standards (Bio-Rad), including (from top to bottom) rabbit phosphorylase *b* (molecular weight, 97,4000), bovine serum albumin (66,200), rabbit actin (43,000), bovine carbonic anhydrase (31,000), and trypsin inhibitor (20,100).

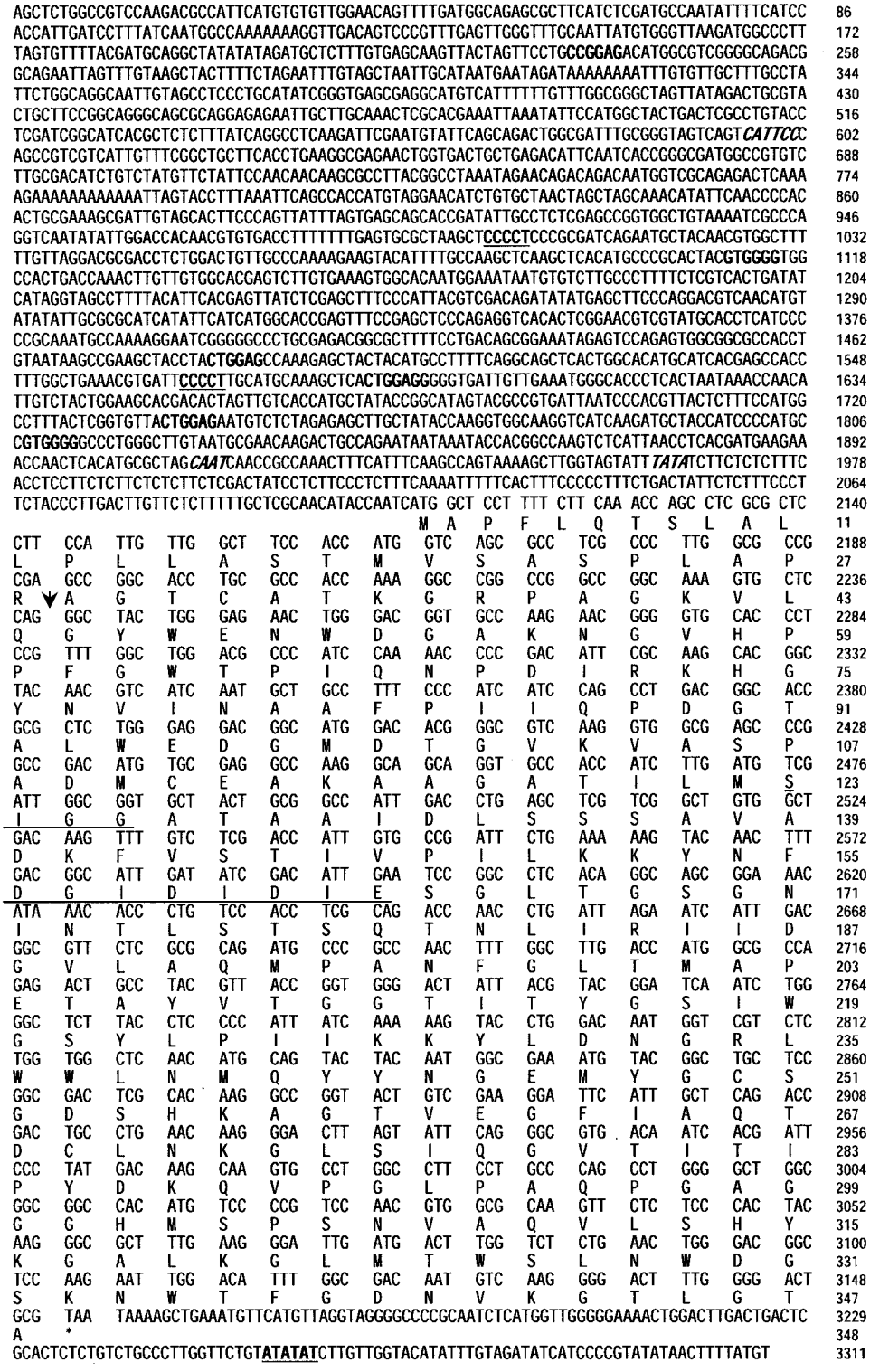

FIG. 5. Nucleotide sequence of endochitinase gene *Bbchit1*, its upstream regulatory sequence, its 3′ UTS, and its translation from an ATG start codon to a termination codon (TAA) (GenBank accession number AY145440). The deduced amino acid sequence is shown in one-letter code under the ORF. Putative TATA and CAAT boxes are indicated by boldface type and italics close to the translation start. Putative binding domains of the glucose repressor are indicated by boldface type. Two STREs are indicated by boldface type and underlined. The AbaA developmental regulator is indicated by boldface type and italics. The poly(A) tail signal sequence, TATATA, is indicated by boldface type and underlined in the 3′ UTS. The putative substrate binding site and catalytic domain of fungal chitinases are underlined. The cleavage site of the signal peptide is indicated by an arrow.

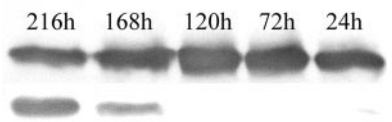

FIG. 6. Time course of Bbchit1 production. Western blots were prepared by probing lyophilized crude extracts of *B. bassiana* culture liquids in basal salt medium containing colloidal chitin without (upper panel) or with (lower panel) glucose with rabbit antibody raised against Bbchit1. Each lane contained about 20 μg of protein. In the medium without glucose, Bbchit1 was detected 24 h after inoculation, and the strongest signal was observed after 120 h. When glucose was added, the signal was not found until 168 h after inoculation, suggesting that Bbchit1 production was repressed by glucose.

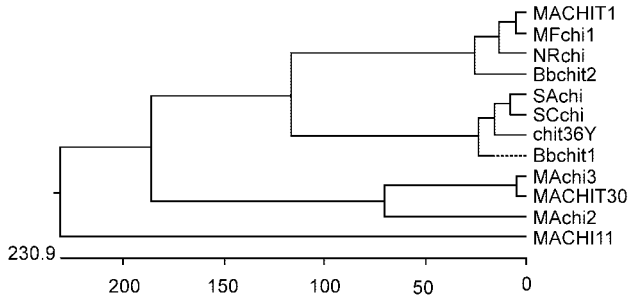

FIG. 7. Phylogenetic tree of chitinases from insect-pathogenic fungi, *Trichoderma* spp., and *Streptomyces* spp., created by using the ClustalW program with amino acid sequence data deposited in the GenBank database. The scale bar indicates the number of amino acid substitutions. The phylogenetic tree revealed that *Bbchit1* is a close homologue of chitinases from *Trichoderma* spp. and *Streptomyces* spp. and exhibits very low levels of homology with other chitinases from insect-pathogenic fungi. Abbreviations: TH, *Trichoderma harzianum*; Bb, *Beauveria bassiana*; SA, *Streptomyces avermitilis*; SC, *Streptomyces coelicolor*; MA, *Metarhizium anisopliae*; MF, *Metarhizium flavoviride*; NR, *Nomuraea rileyi*.

walking by using primers LP2 and EP2 from ScaI-digested genomic DNA. The ORF of the *Bbchit1* gene was determined by sequence analysis, and primers Bbchit1-1 and Bbchit1-2 were designed for cloning the corresponding genomic sequence. Three overlapping amplified regions resolved the *Bbchit1* gene and its regulatory elements. The entire sequence contained a 1,047-bp ORF, a 157-bp 3′ untranslated sequence (UTS), a 2,107-bp 5′ UTS, and a URS (Fig. 5). No intron was found in the *Bbchit1* gene. Southern blotting with the ORF as a probe showed that there was a single copy of *Bbchit1* in the *B. bassiana* genome.

The ORF encoded a 348-amino-acid enchitinase precursor, and the first 28 amino acid residues were determined to be a signal sequence by comparing the precursor to the N-terminal 22 amino acid residues of the purified Bbchit1. The deduced signal peptide formed a hydrophobic structure predicted by the DNAstar program. In the deduced amino acid sequence of *Bbchit1*, the consensus motifs SXGG and DXXDXDXE, corresponding to a substrate binding site and a catalytic domain, respectively, in family 18 chitinases (22), were identified, indicating that Bbchit1 was a member of glycosyl hydrolase family 18.

In the upstream regulatory sequence, TATAA and CAAT sites were deduced. In addition, six consensus motifs were found for the CreA/Crel carbon catabolic repressor (SYGG RG) (1, 26). One consensus motif was also identified for the AbaA developmental regulator (CATTCY). Two stress-responsive elements (STREs) (CCCCT) were also observed. STREs have been shown previously to mediate transcriptional activation in response to various stresses, especially heat, osmotic stress, low pH, and nutrient starvation in *Saccharomyces cerevisiae* (28). Potentially, the STREs performed a similar function in *B. bassiana*.

Western blotting was employed to investigate the effect of glucose on Bbchit1 production in different cultures. Cultures were concentrated (from 100 to 0.5 ml) and then used for Western blot analysis. In basal medium supplemented with colloidal chitin, *Bbchit1* was detected 24 h after inoculation, and the strongest signal was observed after 120 h (Fig. 6). Glucose in the colloidal chitin medium suppressed Bbchit1 production, in accordance with the presence of CreA/Crel carbon catabolic repressor binding domains in the *Bbchit1* promoter.

Homology analysis of *Bbchit1*. The amino acid sequence encoded by *Bbchit1* showed high levels of identity with the sequences encoded by *Chit36Y* from *T. harzianum* (accession number AAL01372) (75%) (36), a putative endochitinase gene from *S. avermitilis* MA-4680 (accession number NP_826813) (74%), and a putative chitinase gene from *S. coelicolor* A3 (2) (75%) (accession number NP_626743). Besides *Bbchit1*, the following nine chitinase genes have been isolated from insect-pathogenic fungi: *CHIT42* (accession number AF027497) (4), *chi11* (accession number AF036320) (16), *chi2* (accession number AJ293217), *chi3* (accession number AJ293218), *ChiA* (accession number X89212), and *CHIT30* (accession number AY545982) from *M. anisopliae*; *chi1* (accession number AJ243014) from *Metarhizium flavoviride*; *Bbchit2* (accession number AY147011) from *B. bassiana*; and *chi* from *Nomuraea rileyi* (accession number AY264288.) (37). Interestingly, *Bbchit1* showed very low levels of similarity to all of them. In the phylogenetic tree constructed by using the ClustalW program (Fig. 7), *Bbchit1* was a close homologue of *ChitY36* and two chitinase genes from *Streptomyces* spp., but there was a large phylogenetic distance between *Bbchit1* and other chitinase genes from insect-pathogenic fungi. Therefore, *Bbchit1* is a novel chitinase gene isolated from entomopathogenic fungi.

Construction and characterization of *B. bassiana* transformants overproducing endochitinase Bbchit1. *B. bassiana* wild-type strain Bb0062 was transformed with the binary plasmid pBANF-bar-pAN-Bbchit1 (Fig. 8), in which the *Bbchit1* gene was placed downstream of the constitutive *gpd* promoter, which was mediated by *A. tumefaciens*, and transformants were selected on the basis of herbicide resistance. Fifty herbicide-resistant colonies were obtained and analyzed. In basal salt medium supplemented with glucose that repressed native Bbchit1 production, three transformants, transformants 1–4, 1–8, and 1–22, showed significantly higher chitinase activity than wild-type strain Bb0062. Western blotting confirmed that there was overproduction of Bbchit1 in these three transformants. The results showed that Bbchit1 was successfully overproduced in transformants 1–4, 1–8, and 1–22 (Fig. 9). Single-spore strains of these three transformants were isolated and tested for Bbchit1 production again (Table 1). The 1–4, 1–8,

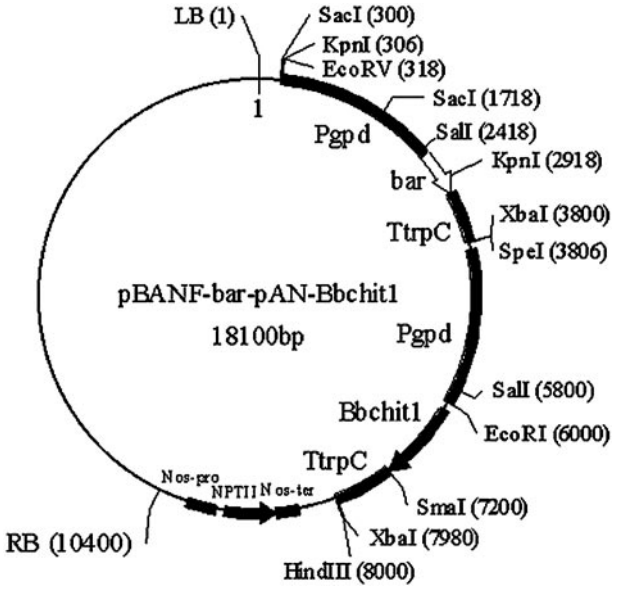

FIG. 8. Diagram of pBANF-bar-pAN-Bbchit1. The RT-PCR-generated cDNA corresponding to the ORF of the *B. bassiana* chitinase gene *Bbchit1* was inserted 3′ to the *Aspergillus nidulans* constitutive *gpd* promoter sequence and 5′ to the terminator sequence of the *A. nidulans TrpC* gene. Abbreviations: Pgpd, *A. nidulans gpd* promoter; bar, the sequence encoding the phosphinothricin acetyltransferase gene; TtrpC, terminator sequence of the *A. nidulans TrpC* gene; Nos-pro, *A. tumefaciens Nos* gene promoter; Nos-ter, *A. tumefaciens Nos* gene terminator; NPTII, kanamycin resistance gene; LB, left border; RB, right border.

and 1–22 single-spore strains were all morphologically stable and showed wild-type levels of growth and conidiation.

Insect bioassay. We tested the virulence of the wild-type strain and the genetically modified strains by inoculating aphids with conidia at concentrations ranging from 5×10^5 to 1×10^8 conidia/ml. The LC_{50}s of wild-type strain Bb0062 and transformants 1–4 and 1–8 were successfully calculated at 84 and 96 h after inoculation (Table 2). At both 84 and 96 h after inoculation, the LC_{50}s of 1–4 and 1–8 were significantly lower than that of wild-type strain Bb0062, as indicated by the lack of overlap of the 95% fiducial limits. Because the LC_{50} is based on the efficiency of infection (38), overproduction of endochitinase Bbchit1 can promote the infection efficiency of *B. bassiana* in aphids. The LT_{50}s with 1×10^7 and 5×10^7 conidia/ml were also determined (Table 2); however, the LT_{50}s with other concentrations could not be calculated because the standard errors were too high. The LT_{50}s of transformants 1–4 and 1–8 were also significantly lower than that of wild-type strain Bb0062. At 1×10^7 conidia/ml, the LT_{50}s of 1–4 and 1–8 were 19.8 and 17.5% lower than that of Bb0062, respectively, and at 5×10^7 conidia/ml, the LT_{50}s of these two transformants were 14.6 and 21.2% lower than that of Bb0062, respectively. Taken together, the insect bioassay results showed that overproduction of the novel endochitinase Bbchit1 can promote the infection efficiency of *B. bassiana* and accelerate infection.

TABLE 1. Chitinase production by the *B. bassiana* wild-type strain and transformants in basal salt medium containing glucose[a]

Strain	Chitnase activity (mU/ml)
Bb0062 (wild type)	5.65 ± 1.25
1–4	42.97 ± 5.61
1–8	97.20 ± 4.52
1–22	39.56 ± 6.52

[a] Wet mycelia (2.5 g) were transferred from SDY medium to basal salt medium supplemented with 2% (wt/vol) glucose and 0.5% (wt/vol) $NaNO_3$ and grown at 26°C for 24 h at 100 rpm. The chitinase activities are means ± standard errors of the means based on three replicates.

DISCUSSION

St. Leger et al. (31) found that protease Pr1, a cuticle-degrading enzyme, was also toxic to insects and activated the insect prophenoloxidase cascade. The combined effect of the toxicity of Pr1 and the reaction products of prophenoloxidase caused a 25% reduction in the time to death but did not cause any reduction in the LC_{50} (31). We found that overproduction of another cuticle-degrading enzyme, endochitinase Bbchit1, not only reduced the time to death but also increased the infection efficiency of *B. bassiana*. Increased Bbchit1 production in the transformants resulted in a lower LC_{50}, indicating that there was a dosage effect of Bbchit1 on infection efficiency (Tables 1 and 2). Nevertheless, transformant 1–8 showed higher chitinase activity but did not have significantly a lower LT_{50} than transformant 1–4, indicating that there was no significant dosage effect for Bbchit1 toxicity. Further experiments should be conducted to elucidate the effect of chitinase Bbchit1 overproduction on fungal virulence.

In many fungi, plants, and animals, several classes of chitinases have been found, and the physiological roles of these chitinases vary. Mycoparasitic *Trichoderma* species produce several chitinases, some of which are involved in fungal infection; however, only *ech42* (20) and *chit33* (19) can be used for biological activity enhancement. RSC-a and RSC-c, two chitinases from rye seed, have different chitin binding characteristics. RSC-a can bind to hyphal tips, lateral walls, and septa, while RSC-c can only bind to hyphal tips; the result of this is that RSC-a inhibits fungal growth more effectively than RSC-c (34). The situation is similar in entomopathogenic fungi. *B. bassiana*, *M. anisopliae*, and *Metarhizium flavoviride* all have several chitinases with different roles (31). Screen et al. (27) investigated the role of chitinase in insect fungus pathogenesis and showed that *CHIT1*, a chitinase gene showing high homology to *ech42*, did not alter the virulence of *M. anisopliae*. Similar to *CHIT1*, *Bbchit1* belongs to chitinase family 18, and it has similar substrate binding and catalytic domains (SXGG

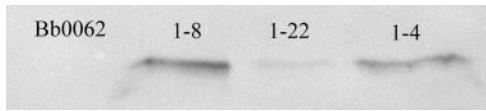

FIG. 9. Overproduction of Bbchit1 in *B. bassiana*. Western blotting was performed by probing lyophilized crude extracts of *B. bassiana* wild-type strain and transformant cultures grown in basal salt medium containing 2% (wt/vol) glucose (to repress the expression of the native *Bbchit1* gene) with rabbit antibody raised against Bbchit1. Each lane contained about 20 μg of protein. Signals were detected in transformants 1–22, 1–8, and 1–4, showing that there was Bbchit1 overproduction.

TABLE 2. Bioassay results for *B. bassiana* wild-type strain Bb0062 and transformants overproducing chitinase Bbchit1[a]

Strain	Time (h)	Slope	LC_{50}, 10^7 conidia/ml (95% fiducial limits)	r^2	Dose (10^7 conidia/ml)	Slope	LT_{50}, h (95% fiducial limits)	r^2
Bb0062	84	0.32	51.31 (10.79–91.81)	0.66	1.0	7.64	120.8 (118.5–123.1)	0.94
	96	0.40	9.60 (4.10–15.11)	0.74	5.0	9.50	99.8 (98.1–101.4)	0.98
1–4	84	0.61	7.10 (4.61–9.60)	0.86	1.0	8.40	96.9 (95.2–98.7)	0.98
	96	0.74	1.76 (1.39–2.13)	0.90	5.0	8.70	85.2 (83.1–87.4)	0.97
1–8	84	0.58	4.07 (2.80–5.33)	0.60	1.0	7.23	99.6 (97.3–101.9)	0.99
	96	0.70	1.07 (0.84–1.29)	0.74	5.0	5.54	78.6 (76.6–80.8)	0.51

[a] The insect used for bioassays was *M. persicae.* For each treatment, there were three replicates, each containing 30 aphids. The experiment was repeated three times. A χ^2 test showed that the variances of the three repeats of the experiments were homologous ($P < 0.05$). The values were calculated based on the data from all repeats. The SPSS program showed that the LC_{50}s and LT_{50}s for transformants 1–4 and 1–8 were significantly lower than the values for wild-type strain Bb0062.

and DXXDXDXE). However, the similarity at the amino acid level between the two chitinases encoded by *CHIT1* and *Bbchit1* is very low, and they are phylogenetically distant. Moreover, the SWISS-MODEL program, a knowledge-based protein model tool, was able to predict the three-dimensional (3D) structure of the *CHIT1* product based on the tertiary structures of chitinases in the database; however, it was unable to predict the 3D structure of the *Bbchit1* product, suggesting that the 3D structures of the *Bbchit1* and *CHIT1* products are different. The differences between the *Bbchit1* and *CHIT1* products in amino acid sequences and 3D structures, which determine the chitin binding and manner of cleavage, result in the different functions that they have. To date, only the *CHIT1* and *Bbchit1* chitinase genes, from *M. anisopliae* and *B. bassiana*, respectively, have been investigated in an insect fungus pathogenic context. The CHIT1 chitinase from *M. anisopliae* was shown to have a little or no effect on virulence (27), while overproduction of Bbchit1 did increase the virulence of *B. bassiana* for aphids. In order to elucidate the relationships between chitinases and virulence in insect-pathogenic fungi, overexpression and gene knockout experiments with other chitinase genes should be conducted.

ACKNOWLEDGMENTS

This work was supported by Major State Basic Research Development Program grant 2003CB114203 from the Ministry of Science and Technology of China, by Natural Science Foundation of China grant 30200183, and by Hi-tech Research and Development Program of China grant 2002AA245021 from the Ministry of Science and Technology of China.

We are grateful to Siyuan Wu for his assistance with the statistic analysis. We also thank M. J. Bidochka and L. Scully for critically reading the manuscript.

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