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Title: Isolation of Genes Coding for Chitin-Degrading Enzymes in the Novel Chitinolytic Bacterium, *Chitiniphilus shinanonensis,* and Characterization of a Gene Coding for a Family 19 Chitinase

Running title: GENE CLONING OF CHITINASES FROM C. SHINANONENSIS

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Abbreviations: ChBD, chitin-binding domain; GH, glycosyl hydrolase; GlcNAc, *N*-acetyl-$_D$-glucosamine; 4MU, 4-methylumberiferryl; NAG, β-*N*-acetylglucosaminidase; pNP, *p*-nitrophenyl

Abstract

Chitiniphilus shinanonensis type strain SAY3^T is a strongly chitinolytic bacterium, originally isolated from the moat water in Ueda, Japan. To elucidate the chitinolytic activity of this strain, 15 genes (*chiA-chiO*) coding for putative chitin-degrading enzymes were isolated from a genomic library. Sequence analysis revealed the genes comprised 12 family 18 chitinases, a family 19 chitinase, a family 20 β-*N*-acetylglucosaminidase, and a polypeptide with a chitin-binding domain but devoid of a catalytic domain. Two operons were detected among the sequences: *chiCDEFG* and *chiLM*. The gene coding for the polypeptide (*chiN*) showed sequence similarity to family 19 chitinases and was successfully expressed in *Escherichia coli*. ChiN demonstrated a multi-domain structure, composed of the N-terminal, two chitin-binding domains connected by a Pro- and Thr-rich linker, and a family 19 catalytic domain located at the C-terminus. The recombinant protein rChiN catalyzed an endo-type cleavage of *N*-acetyl-$_D$-glucosamine oligomers, and also degraded insoluble chitin and soluble chitosan (degree of deacetylation of 80%). rChiN exhibited an inhibitory effect on hyphal growth of the fungus *Trichoderma reesei*. The chitin-binding domains of ChiN likely play an important role in the degradation of insoluble chitin, and are responsible for a growth inhibitory effect on fungi.

Introduction

Chitin, a linear polysaccharide consisting of β-1, 4-linked *N*-acetyl-$_D$-glucosamine (GlcNAc), is widely distributed in nature, such as in the exoskeletons of crustaceans and insects, and in the cell walls of fungi (1, 2). More than $1x10^{11}$ tons of chitin is synthesized annually in its various forms, and it is the most abundant biomass next to cellulose. Chitin and its deacetylated derivative, chitosan, have attracted considerable

interest because of their biological properties, and are widely used in various fields including the health care, food, agriculture, chemical, and environmental engineering industries (3-5).

GlcNAc oligomers prepared from chitin have useful biological activities, such as immunostimulation and induction of plant defense responses (6). Additionally, GlcNAc can be utilized as a sweetener and nutritional supplement. Chemical hydrolytic reactions involving strong acids have been used for production of GlcNAc and its oligomers on an industrial scale, but costs associated with treatment of waste products to prevent environmental pollution are prohibitive. Hence, microbial chitinolytic enzymes have been investigated extensively for their potential use in the enzymatic production of GlcNAc and its oligomers in an eco-friendly manner.

A wide variety of bacteria produce chitinolytic enzymes to utilize chitin as a carbon and nitrogen source. In general, chitin degradation proceeds via two successive steps in which two different types of chitinolytic enzymes participate. First, the chitin polymer is hydrolyzed by an endo-type chitinase (E.C.3.2.1.14) into small oligosaccharides composed mainly of *N, N'*-diacetylchitobiose, $(GlcNAc)_2$. Second, these oligosaccharides are hydrolyzed into GlcNAc by the action of an exo-type β-*N*-acetylglucosaminidase (NAG) (E.C.3.2.1.52) (3). Although a large number of chitinolytic bacteria have been isolated and characterized, they represent only a small proportion of the types of bacteria that play major roles in degradation and recycling of chitin in natural environments. Most environmental bacteria are unculturable and remain uncharacterized (7). We examined the extent of diversity of chitinolytic bacteria in different natural environments, including a river, a moat, and soil, and found that a wide variety of bacteria are likely to be involved in the degradation and recycling of chitin (8). The mixed culture derived from the sampling sites enabled us to isolate

one chitinolytic bacterium, which was classified into a new genus (9). The strain was named and registered as the type strain SAY3^T under the scientific name of *Chitiniphilus shinanonensis* (10).

C. shinanonensis strain SAY3^T is strongly chitinolytic, as evidenced by its rapid growth on colloidal chitin plates and the formation of a clear halo around subsequent colonies. Additionally, it can degrade and utilize flake chitin (large particles of 1-2 mm in diameter) more efficiently than *Aeromonas hydrophila* strain SUWA-9, a representative aquatic chitinolytic bacterium (9, 11). These characteristics suggest that chitinolytic enzymes from this strain may be used for efficient production of GlcNAc and its oligomers from native chitin.

Endo-type chitinases are classified as belonging to either family 18 or family 19 of the glycosyl hydrolases (GH), based on the amino acid sequence similarity of their catalytic domains (12-14). Both families of chitinases differ in their reaction mechanisms: Family 18 chitinases release a β-anomer product by a retaining mechanism, while family 19 chitinases produce an α-anomer through an inverting mechanism. Family 18 chitinases are distributed widely in microorganisms, plants, and animals. In contrast, family 19 chitinases are mostly found in plants. Interestingly, family 19 chitinases have recently been characterized in a limited group of prokaryotes (15). In this paper, we describe the isolation and analysis of 15 genes coding for putative chitinolytic enzymes from strain SAY3^T. Of these, one gene (*chiN*) encodes a polypeptide with significant similarity to family 19 chitinase. We examined the enzymatic characteristics of the recombinant protein obtained by expressing *chiN* in *Escherichia coli,* and discuss the possible origin of the gene.

Materials and Methods

Strains and culture conditions

C. shinanonensis strain SAY3^T was grown in Luria-Bertani (LB) broth at 30ºC for the preparation of genomic DNA. *E. coli* JM109 was used for the construction of recombinant plasmids. *E. coli* BL21(DE3) was used to host the chitinase gene.

Construction of genomic library and expression screening

SAY3^T genomic DNA was prepared using the Nexttec^{TM} Genomic DNA Isolation kit for Bacteria (Nexttec Biotechnologie GmbH, Hilgertshausen, Germany) according to the manufacturer's instructions. The resulting chromosomal DNA was fragmented to an average size of 40 kb by treating with a sonicator. The DNA fragments were ligated into fosmid vector pCC1FOS, packaged into lambda phage particles, and transfected into *E. coli* EPI300 using the CopyControl^{TM} Fosmid Library Production Kit (EPICENTRE Biotechnology, Madison, USA). Expression screening using selection plates of M9 synthetic medium containing fluorescent substrates, 8 mM of 4-methylumbelliferryl (4MU)-GlcNAc and 1 mM of 4MU-$(GlcNAc)_3$, was used to identify chitinolytic bacteria. Ten *E. coli* clones with chitinolytic activity were selected out of approximately 5,000 library clones.

Gene analysis

Recombinant fosmid DNA was prepared from the 10 positive *E. coli* clones using the QIAGEN Large-Construct Kit (QIAGEN, Tokyo, Japan). Next-generation sequencing of fosmid DNA was carried out using a Genome Sequencer FLX system (Roche Diagnostics, Tokyo, Japan). The nucleotide sequences of sub-cloned inserts were obtained using an ABI PRISM 3100 Genetic Analyzer and BigDye Terminator v3.1

Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan). ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) and BLAST (hppt://www.ddbj.nig.ac.jp/search/blast-j.html) were used to reveal a group of genes coding for chitin-degrading enzymes from the resultant nucleotide sequences. Putative sequences for signal peptides were deduced using SignalP (http://www.cbs.dtu.dk/services/SignalP). Domain structures and functions were estimated by Pfam (http://pfam.sanger.ac.uk) and Cazy (http://www.cazy.org/).

Expression of *chiN* in *E. coli*

The DNA fragment corresponding to the *chiN* ORF, minus the N-terminal signal peptide (31 amino acid residues), was amplified by PCR using the following primers: forward primer 5'- GCGAATTCGCCCAGCAATGGGCCGAA-3' (*Eco* RI cleavage site underlined) and reverse primer 5'- GCGTCGACTCAGCAACTGGATTGTTG-3' (*Sal* I cleavage site underlined). To produce a truncated polypeptide missing the two chitin-binding domains (ChBDs) at the N-terminus, the forward primer was replaced with 5'- GCGAATTCGCTGTCACCTGCAGTGG -3'. The amplified DNA fragments were ligated into expression vector pCold I (Takara, Kyoto, Japan). The resulting recombinant plasmids were sequenced to confirm the integrity of inserted sequences, and designated pCold I-*chiN* (for the full-length) and pCold I-*chiN*ΔChBD (for the truncated) and maintained in *E. coli* BL21(DE3).

E. coli BL21(DE3) strains containing either pCold I-*chiN* or pCold I-*chiN*ΔChBD were grown in 200 ml LB medium supplemented with ampicillin (50 μg/ml) at 37ºC with shaking (150 rpm). When the absorbance reached OD_{600}=0.4-0.6, cultures were cooled and incubated at 15ºC for 30 min. Isopropyl-β-thiogalactopyranoside was then added to a final concentration of 0.5 mM, and the culture was successively shaken at

15ºC for 24 h. Cells were harvested by centrifugation, suspended in 4 ml of 20 mM sodium phosphate buffer (pH7.4) (0.5 M NaCl, 20 mM imidazole), and disrupted by sonication (Astrason, Model 2020, Kyoto, Japan). Cell debris was removed by centrifugation (10,000 x g, 10 min) and recombinant ChiN (rChiN) and ChiNΔChBD (rChiNΔChBD) proteins were purified from the supernatant using His GraviTrap Columns (GE Healthcare, Tokyo, Japan)

Enzyme assay

Chitinase activity was assayed using the modified Schales method, with chitinous compounds used as the substrates (16). Activity is determined by measuring the amount of reducing sugar liberated during the reaction. The synthetic substrates *p*-nitrophenyl (pNP)-$(GlcNAc)_{1-3}$ were also used to assay chitinase by detecting a release of pNP. The products of the enzymatic hydrolysis of GlcNAc oligomers and pNP-$(GlcNAc)_{1-3}$ were analyzed by thin-layer chromatography with Merck HPTLC Silica Gel 60 (aluminum sheets), as described previously (17). The products in the plate were visualized using a diphenylamine reagent. The anomer formation ratios during enzymatic hydrolysis of GlcNAc hexamer were analyzed by Acquity UPLC system (Waters, Milford, MA) using an Acquity UPLC BEH Amide column (2.1 mm x 100 mm, 1.7 μm particle size). After the reaction, the mixture was added with twice volume of acetonitrile and kept on ice. Then the sample was analyzed immediately after filtration (0.2 μm pore size). GlcNAc oligomers were eluted with 70% acetonitrile at a flow rate of 0.25 mL/min and detected by absorbancy at 210 nm.

Chitin-binding assay

Flake chitin (2-3 mm in diameter) (a gift from Kyowa Technos, Tokyo, Japan) was

subjected to a binding test using the purified recombinant chitinases, rChiN and rChiNΔChBD. Ten mg of flake chitin was mixed thoroughly with 20 μg of each recombinant protein in 300 μl of 20 mM sodium acetate buffer (pH5.6). The mixture was kept on ice for 2 h with mixing every 15 minutes. After the flake chitin was removed by centrifugation, the amount of protein in the supernatant was determined with a protein assay kit (Bio-Rad, Tokyo, Japan) using bovine serum albumin as a standard. The amount of bound protein was calculated from the difference in the amount of protein present following 2 h of incubation compared with that at time zero.

Growth inhibition test

The fungus *Trichoderma reesei* QM9414 was used for hyphal extension-inhibition assays to examine antifungal activity of rChiN and rChiNΔChBD. A small plug of mycelial colony was inoculated onto the center of potato-dextrose agar plates (Difco, Franklin Lakes, USA). After incubation at 28°C for 24 h, wells (5 mm in diameter) were punched around the edge of the colony and inoculated with recombinant proteins. The plate was further incubated at 28°C for 18 h to detect growth inhibitory zones around the wells.

Nucleotide sequences

The nucleotide sequences determined in this work have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB649129-AB649134.

Results

Isolation of 15 genes coding for putative chitinolytic enzymes

C. shinanonensis strain SAY3^T is a type strain for a novel genus, *Chitiniphilus*, and

1 was isolated from moat water at Ueda Castle, Nagano, Japan (10). It is characterized by
2 fast growth on chitin agar with a zone of clearing around the colony, and efficient
3 utilization of insoluble flake chitin (9). To elucidate the chitinolytic pathway of *C.*
4 *shinanonensis*, we attempted to purify chitinolytic enzymes from SAY3^T culture
5 medium. Cultures were grown in synthetic medium containing colloidal chitin as the
6 sole carbon source; however, this approach proved difficult because of a large number
7 of proteins with chitinolytic activity. We then attempted to isolate genes coding for
8 chitinolytic enzymes to characterize a repertoire of chitin-degrading enzymes in this
9 bacterium.
10 A SAY3^T genomic library was constructed using fosmid vector pCC1FOS, which
11 can incorporate DNA fragments of up to approximately 40 kb to be packaged into
12 lambda phage particles. The constructed library was composed of approximately
13 5,000 *E. coli* clones; which, assuming a genome size similar to that of *E. coli* (4.6 Mbp),
14 should provide coverage of the entire SAY3^T genome with more than 99% probability
15 (18).
16 We then screened clones to isolate those with chitinolytic activity. Screening was
17 carried out using selection plates containing a mixture of fluorescent substrates,
18 4MU-GlcNAc and 4MU-$(GlcNAc)_3$, in which the former is degraded by an exo-type
19 NAG, and the latter is preferentially digested by an endo-type chitinase. A negligible
20 background of chitinolytic activity in host *E. coli* enabled us to select 10 clones
21 exhibiting blue-white fluorescence under exposure of ultraviolet light. A mixture of
22 fosmids prepared from the 10 clones was sequenced using a next-generation sequencer.
23 As a result, six contigs (more than 10 kb in size) were constructed, encompassing a total
24 length of 197 kb (Table S1). The sequence contained a total of 102 putative ORFs, and
25 BLAST analyses revealed that 15 of these showed significant similarity to various

chitinolytic enzymes. These genes were designated *chiA-chiO* and are summarized in Table 1. Although their exact locations in the genome are yet to be determined, seven of the genes (*chiB-chiH*) form a cluster. Of these, five genes (*chiCDEFG*) are likely to constitute an operon, as indicated by short distances between adjacent genes (135-217 bp) and the absence of promoter consensus sequences. Promoter-like consensus sequences were also identified upstream of the first gene in the predicted operon (*chiC*). In addition, *chiL* and *chiM* constitute another putative operon, *chiLM*.

Table 1

Possible domain structures of 15 *chi* ORFs

Deduced domain structures of the 15 *chi* ORFs with homology to chitin degradation and utilization genes are summarized in Fig. 1. Twelve of the 15 ORFs have a putative catalytic domain that is similar to family 18 chitinases, and one ORF (ChiN) shows sequence similarity to family 19 chitinases. The ORF designated Chi I shows sequence similarity to an exo-type NAG belonging to the GH family 20. ChiF does not share sequence similarity with any previously reported GH catalytic domains. The location of *chiF* in the deduced operon (*chiCDEFG*), and the presence of two putative ChBDs at the N-terminus of the *chiF* ORF, suggest the possible participation of this gene product in degradation and utilization of chitin.

Fig. 1

Using SignalP, all ORFs, not including ChiI and ChiM, were found to have a signal peptide, indicating that their gene products are likely to be secreted. Other than ChiL and ChiM, all ORFs possess ChBDs that could be classified into carbohydrate binding module 5 (CBM5) (19). In most cases, two ChBDs are connected by Pro-, Thr-, and Val-rich linkers, while a similar linker region is not found in ChiI.

Expression of *chiN* in *E. coli*

chiN encodes a polypeptide consisting of 386 amino acid residues, with a molecular weight of 40.3 kDa. The *chiN* ORF contains a putative catalytic domain with significant sequence similarity to family 19 chitinases (Fig. 1). The domain structure of the *chiN* ORF is composed of an N-terminal signal domain (31 amino acid residues), two ChBDs joined by a Pro, Thr-rich linker, and a C-terminal family 19 chitinase catalytic domain. The amino acid sequence corresponding to the putative catalytic domain was compared with those of other representative family 19 chitinases (Fig. 2). Fig. 2 The *chiN* catalytic domain shows 94% sequence similarity to chitinase G of *Streptomyces coelicolor* (20), followed by *S. griseus* chitinase C (92%) (21), rice chitinase (77%) (22), papaya chitinase (74%) (23), and barley chitinase (74%) (24). Two Glu residues were determined to be indispensable for catalysis, where one acts as a proton donor and the other acts as a nucleophile (25, 26). These residues are highly conserved among family 19 chitinases, including the *chiN* ORF (corresponding to Glu240 and Glu249).

To analyze the function of the *chiN* gene product, the DNA fragment corresponding to the whole ORF minus the N-terminal putative signal peptide was PCR-amplified, inserted into the expression vector pCold I, and expressed in *E. coli* BL21 (DE3). Similarly, the DNA fragment corresponding to the catalytic domain without the two ChBDs was expressed. The two resulting recombinant proteins (designated rChiN and rChiNΔChBD) were successfully obtained in a soluble form and subsequently purified by one-step affinity chromatography targeting a polyhistidine tag fused to the recombinant proteins. The purified proteins were analyzed by SDS-PAGE, and their apparent molecular weights (41.4 kDa for rChiN, 28.3 kDa for rChiNΔChBD) were in agreement with the calculated values for the expected recombinant proteins (Figure S1).

These purified enzymes were used in subsequent experiments.

Characterization of recombinant protein rChiN

The ability of rChiN to degrade various chitinous substrates was examined. The recombinant protein hydrolyzed soluble chitosan with the greatest efficiency, with a deacetylation degree of 80%. Insoluble chitin (colloidal and powdered chitin) was also degraded to a lesser extent (Table 2). With colloidal chitin as the substrate, the optimal pH and temperature for the reaction were 5.6 and 50°C, respectively. The enzyme did not release any pNP from the chromogenic substrates, pNP-$(GlcNAc)_{1-3}$ even after a prolonged reaction period (data not shown). Table 2

Reaction products (GlcNAc oligomers and chromogenic substrates) were analyzed by thin layer chromatography after a prolonged reaction period. GlcNAc trimers, tetramers and pentamers were degraded to a mixture of GlcNAc monomers and dimers, whereas rChiN did not have any activity towards GlcNAc dimers (Fig. 3A). rChiN also did not react with pNP-GlcNAc or pNP-$(GlcNAc)_2$, but converted pNP-$(GlcNAc)_3$ into GlcNAc dimers and pNP-GlcNAc (Fig. 3B). Considering that pNP was not released from pNP-$(GlcNAc)_3$, the enzyme appears to preferentially cut the second bond from the non-reducing end. Reaction products from the reaction using GlcNAc hexamers as the substrate were analyzed at appropriate intervals (Fig. 3C). At the initial stage, GlcNAc hexamers were degraded into two trimers or a dimer plus a tetramer, indicating that ChiN catalyzes an endo-type cleavage reaction. The products at the initial stage of reaction were analyzed by UPLC to detect the ratios of α to β anomers. The products, GlcNAc dimers, trimers and tetramers exhibited a higher ratio of α anomers than those indicated by the standard oligomers that were in equilibrium (Fig. 4). This result indicates that rChiN hydrolyzes the substrate by an inverting Fig. 3 Fig. 4

mechanism to produce α anomers by hydrolyzing β glycosidic bonds.

Role of ChBDs in ChiN

To investigate the function of two ChBDs located at the N-terminus of rChiN, chitin-binding assays were performed using flake chitin as substrate for both rChiN and rChiNΔChBD. Under the experimental conditions described in Materials and Methods, 61% of the initial rChiN protein had bound flake chitin after 2 h incubation, while only 12% of rChiNΔChBD was bound. This result indicates that ChBDs have the ability to bind insoluble chitin. The degradation activity of rChiNΔChBD was also examined using various chitinous substrates and compared with results obtained for rChiN (Table 2). rChiNΔChBD had a similar reaction velocity to rChiN for soluble chitosan, while the reaction rate decreased by 75 and 85% for colloidal chitin and powdered chitin, respectively. This result suggests that ChBDs assist ChiN catalytic domain-driven degradation of insoluble chitin by anchoring the catalytic domain on the surface of substrates. This mechanism has been proposed for most endo-type chitinases (19).

Growth inhibition effect of ChiN on the fungus *Trichoderma reesei*

Plant family 19 chitinases are known to inhibit the growth of plant pathogenic fungi as a mechanism against disease (27). The inhibitory effect of rChiN and rChiNΔChBD on hyphal growth was examined using *T. reesei* as a test strain. rChiN significantly inhibited the hyphal extension of *T. reesei*, with inhibition observed at dosage levels of 0.15 pmol and above (Fig. 5A). rChiNΔChBD also inhibited hyphal extension, but to a lesser extent (Fig. 5B). This result indicates that ChiN has the potential to inhibit fungal growth, and that ChBDs are important for antifungal activity, as well as hydrolytic activity toward insoluble chitin.

Fig. 5

Discussion

The novel bacterium *C. shinanonensis* SAY3^T was isolated from a stable microbial community capable of degrading chitin. The community was constructed by mixing samples of bacteria found tightly bound to the surface of chitin flakes that were collected from soil, river water and moat water (9). The strain SAY3^T is characterized by its rapid growth in synthetic medium containing chitin as the sole carbon source, and efficient degradation and utilization of flake chitin. To elucidate the chitinolytic pathway(s) of SAY3^T, we isolated genes encoding chitinolytic enzymes.

We isolated and sequenced 15 genes likely to be related to chitin degradation (Table 1 and S1). However, we cannot rule out the possibility that other chitin-degrading genes may exist in the genome, because the selection of fosmid clones used for sequencing was dependent on heterologous expression screening in *E. coli*. Bacteria can utilize chitin as a carbon source by degrading it into GlcNAc using an endo-type chitinase and exo-type NAG. GlcNAc can then be converted into fructose-6-phosphate by successive phosphorylation, deacetylation and deamination, and is finally introduced into the glycolytic pathway (28). Thus, the presence of these two enzymes is likely to be a minimum requirement for chitin utilization. Contrary to this, the representative chitinolytic soil-borne actinomycete*, Streptomyces coelicolor* A3(2), possesses 18 genes related to chitin utilization (11 family 18 chitinases, 2 family 19 chitinases and 5 exo-NAGs) (29). Although some of these genes may be redundant, acquisition of multiple copies of various chitinolytic genes would enable bacterial cells to utilize chitin efficiently. We have not yet determined which of the 15 genes are expressed in medium containing chitin. However, the presence of many species of chitinolytic proteins in the culture medium suggests that most, if not all, of the genes can be expressed in chitin medium.

Although there are 13 genes coding for putative endo-type chitinases, strain SAY3^T possesses only one gene encoding an exo-type NAG (*chiI*) (Fig. 1). Studies of the chitin utilization system of the marine bacterium *Vibrio furnissi* revealed that it possesses two different NAGs, namely cytoplasmic and periplasmic types (30). The limitations of expression screening mean that other genes encoding NAGs may have been overlooked in the current study. Preliminary experiments revealed the existence of a single species of protein with NAG activity in the periplasm and culture fluids, but not in the cytoplasm. Characterization of this enzyme is now underway.

C. shinanonensis SAY3^T has one gene coding for a family 19 chitinase (*chiN*), which are mainly distributed in plants. Animals and microorganisms predominantly contain family 18 chitinases. The presence of family 19 chitinases in microorganisms was first reported in the actinomycete *Streptomyces griseus* (21). Their prevalence in a wide range of actinobacteria has since been reported (31). However, family 19 chitinases have only been identified in a limited number of bacteria through whole genome sequencing.

We successfully expressed *chiN* in *E. coli* and used the recombinant rChiN to characterize its enzymatic function. rChiN exhibited greater degrading activity towards partially deacetylated soluble chitosan (approximately 80%) compared with insoluble colloidal and powdered chitin (Table 2). This tendency was also reported for other family 19 chitinases of prokaryotic origin (27, 32). TLC analysis of reaction products from GlcNAc hexamer degradation indicated that rChiN catalyzes an endo-type cleavage of GlcNAc oligomers (Fig. 3C). Moreover, rChiN hydrolyzed GlcNAc hexamers by an inverting mechanism, in which α anomers of dimers, trimers and tetramers were produced (Fig. 4). This result strongly supports our presumption that ChiN is a member of family 19 chitinases. Chromogenic compounds,

pNP-$(GlcNAc)_3$ but not pNP-$(GlcNAc)_1$ or $_2$, could generally be used as a substrate for endo-type chitinase, as shown by the release of pNP. However, rChiN did not release any pNP from chromogenic substrates, pNP-$(GlcNAc)_{1-3}$, although it cleaved pNP-$(GlcNAc)_3$ into pNP-GlcNAc and GlcNAc dimer (Fig. 3B). Hence, ChiN preferentially cuts the second bond from the non-reducing end of pNP-$(GlcNAc)_3$, but not a bond between GlcNAc and pNP.

ChiN has two ChBDs at its N-terminus, which belongs to carbohydrate-binding module (CBM) family 5 (Fig. 1). This family is mainly comprised of ChBDs, and helps catalytic domains to digest insoluble chitin efficiently by anchoring the domains on the surface of chitin (19). Some family 19 chitinases found in prokaryotes lack ChBDs and are not effective in degrading crystalline chitin. Family 19 chitinases that do contain ChBDs mainly only have a single ChBD. Family 19 chitinases possessing two ChBDs, like ChiN, are not common. Full-length rChiN and truncated rChiNΔChBD, devoid of the two ChBDs, were obtained to examine the function of the ChBDs in ChiN. As a result, it was confirmed that the two ChBDs act to bind insoluble flake chitin, and thereby accelerate degradation velocity of the catalytic domain toward insoluble chitin but not soluble chitosan (Table 2).

Plant family 19 chitinases are known to be involved in defense systems against plant pathogenic fungi (14). The chitinases act by inhibiting fungal growth through the degradation of chitin, which is a major component of fungal cell walls. Family 19 chitinases found in actinobacteria also have the ability to inhibit hyphal extension of the fungus *T. reesei* (27). We determined that ChiN inhibited hyphal extension of *T. reesei* to some extent (Fig. 5). ChBDs are likely to play a stimulatory role in this inhibition, as shown by the finding that rChiNΔChBD exhibited a lesser inhibition than rChiN. It may be postulated that family 19 chitinases in microorganisms have

antifungal properties similar to those of plant family 19 chitinaes.

The presence of genes coding for family 19 chitinases in a limited member of actinobacteria and bacteria has led to the assumption that the genes were horizontally transferred from plants. Kawase *et al*. proposed that a family 19 chitinase gene was first acquired by an ancestor of the *Streptomycineae* and spread among the actinobacteria through horizontal transfer (31). Recently, another study supported this horizontal gene transfer theory based on the precise analysis of phylogenetic distribution of family 19 chitinase genes in plants and other organisms (33). This recent study proposed a model of two independent transfers: a transfer from plants to purple bacteria in the distant past, and a more recent transfer from plant to actinobacteria. However, the scenario where these ancestral genes were spread to such a limited number of species of bacteria and actionobacteria is yet to be elucidated. The high amino acid sequence similarity of ChiN to actinobacteria family 19 chitinases suggests that *chiN* was procured by some actinobacteria. The roles of family 19 chitinases (ChiN) in the antifungal activity and the utilization of chitin as a nutrient by C. *shinanonensis* SAY3^{T} remain unclear. Kawase *et al*. postulated that actinobacteria have acquired some advantages in the interaction with fungi by obtaining the family 19 chitinase genes (31).

In this paper, we identified 15 genes with potential roles in chitin degradation and utilization. We further analyzed one of these genes, *chiN*, which encodes a family 19 chitinase. We are now undertaking functional analyses of recombinant proteins obtained from the remaining *chi* genes, in addition to analyses of expression of *chi* genes in different culture conditions. These studies will assist in elucidating the strong chitinolytic system in this bacterium.

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Figure legends

FIG. 1. Deduced domain structures of 15 *chi* ORFs. Black and gray boxes indicate signal peptides and chitin-binding domains, respectively. White boxes indicate catalytic domains of glycosyl hydrolases (GH) with family numbers. Vertical lined bars indicate Pro-, Thr-, Val-rich linker. The dashed box indicates chitobiase/β-hexosaminidase C-terminal domain.

FIG. 2. Alignment of amino acid sequences of the catalytic domains of family 19 chitinases. Residues conserved in all sequences are enclosed in a black box, and those conserved in >66% of the sequences are enclosed in a gray box. The two catalytic Glu residues conserved among family 19 chitinases are indicated by asterisks. Numerals represent the number of residues starting from the initial codon or the N-terminus of catalytic domain. ChiN, *C. shinanonensis* ChiN in this work; *S. coelicolor*, strain A3(2) ChiG (accession number, Q8CK55); *S. griseus*, strain HUT 6037 ChiC (O50152); rice, *Oryza sativa* chitinase 2 (Q7DNA1); papaya, *Carica papaya* endochitinase (P85084); barley, *Hordeum vulgare* endochitinase 2 (P23951).

FIG. 3. Analysis of reaction products from various GlcNAc oligomers and chromogenic substrates. (A) Final products after a prolonged reaction using GlcNAc dimers (lane 1), trimers (lane 2), tetramers (lane 3), and pentamers (lane 4). (B) Final products after a prolonged reaction using pNP-$(GlcNAc)_3$ (lane 1), pNP-$(GlcNAc)_2$ (lane 2) and pNP-GlcNAc (lane 3). (C) Reaction products were analyzed from reaction mixtures at various times using GlcNAc hexamer as substrate. The reaction times are: 0 min (lane 1), 1 min (lane 2), 5 min (lane 3), 10 min (lane 4), 20 min (lane 5), 40 min

(lane 6) and 60 min (lane 7). Lanes M denote standard compounds; G1, GlcNAc; G2, GlcNAc dimer; G3, GlcNAc trimer; G4, GlcNAc tetramer; G5, GlcNAc pentamer; G6, GlcNAc hexamer; PG1, pNP-GlcNAc; PG2, pNP-$(GlcNAc)_2$; PG3, pNP-$(GlcNAc)_3$.

FIG. 4. UPLC analysis of hydrolysates from GlcNAc hexamers. (A) GlcNAc and its oligomers from dimer to hexamer were analyzed as standards. (B) Reaction products from GlcNAc hexamers were analyzed at the initial stage of reaction. The elution of α and β anomer of each oligonucleotide is indicated with the ratio of α to β.

FIG. 5. Antifungal activity of rChiN and rChiNΔChBD. Different concentrations of protein samples were put in the wells around the *T. reesei* hyphal colonies, and the inhibitory effect on hyphal extension was examined. (A) Inhibition of hyphal extension by different concentrations of rChiN. Sterile water (well 1); 1.5 pmol (well 2), 0.73 pmol (well 3), 0.29 pmol (well 4), 0.15 pmol (well 5) and 0.06 pmol (well 6) of rChiN. (B) Comparison of antifungal activities of rChiN and rChiNΔChBD. Sterile water (well 1); 1.3 pmol of rChiN (well 2), 0.26 pmol of rChiN (well 3), 0.052 pmol of rChiN (well 4), 1.3 pmol of rChiNΔChBD (well 5) and 0.26 pmol of rChiNΔChBD (well 6).

TABLE 1. Analysis of 15 ORFs related to chitin-degrading enzymes

ORF	Number of amono acids	Molecular weight (kDa)	The closest relative sequence[a]	Accession number	Similarity (%)	GH family[b]
ChiA	677	71.7	*Janthinobacterium lividum* chitinase 69	U07025	62	18
ChiB	692	74.0	*Doohwaniella chitinasigens* chitinase 67	U81007	68	18
ChiC	644	68.2	*Janthinobacterium lividum* chitinase 69	U07025	75	18
ChiD	844	89.7	*Hahella chejuensis* chitinase	CP000155	74	18
ChiE	557	58.7	*Aeromonas* sp. Chitinase (ORF1)	D63139	62	18
ChiF	528	58.1	*Stigmatella aurantiaca* hypothetical protein	CP002271	65	–
ChiG	508	53.7	*Aeromonas* sp. Chitinase (ORF2)	D63139	52	18
ChiH	552	58.5	*Chromobacterium violaceum* chitinase	AE016825	81	18
ChiI	834	92.0	*Aeromonas* sp. β-N-acetylglucosaminidase	AB031320	83	20
ChiJ	729	77.7	*Janthinobacterium lividum* chitinase 69	U07025	70	18
ChiK	631	67.4	*Janthinobacterium lividum* chitinase 69	U07025	58	18
ChiL	410	45.3	*Collimonas fungivorans* chitinase	EU599185	67	18
ChiM	666	72.8	*Lysobacter enzymogenes* chitinase A	Y667480	60	18
ChiN	386	40.3	*Streptomyces* sp. chitinsase IS	GG657750	69	19
ChiO	512	53.7	*Aeromonas* sp. chitinase II	D31818	66	18

[a]Results were obtained by blastp analysis.

[b]Glycosyl hydrolase family

TABLE 2. Degradation activities of rChiN and rChiNΔChBD toward chitinous substrates

Substrates	Relative activity (%)[a]	
	rChiN	rChiNΔChBD
Soluble chitosan (DD 80%)[b]	100	102
Colloidal chitin	5.3	0.85
Powder chitin	3.6	0.97

[a]The relative amount of reducing sugars liberated from each substrate at the initial stage of reacton is shown using the value of rChiN toward chitosan (DD 80%) as a standard (8.62 μmole of GlcNAc equivalent/min/mg-protein). Average values from three independent experiments are shown.
[b]DD: degree of deacetylation

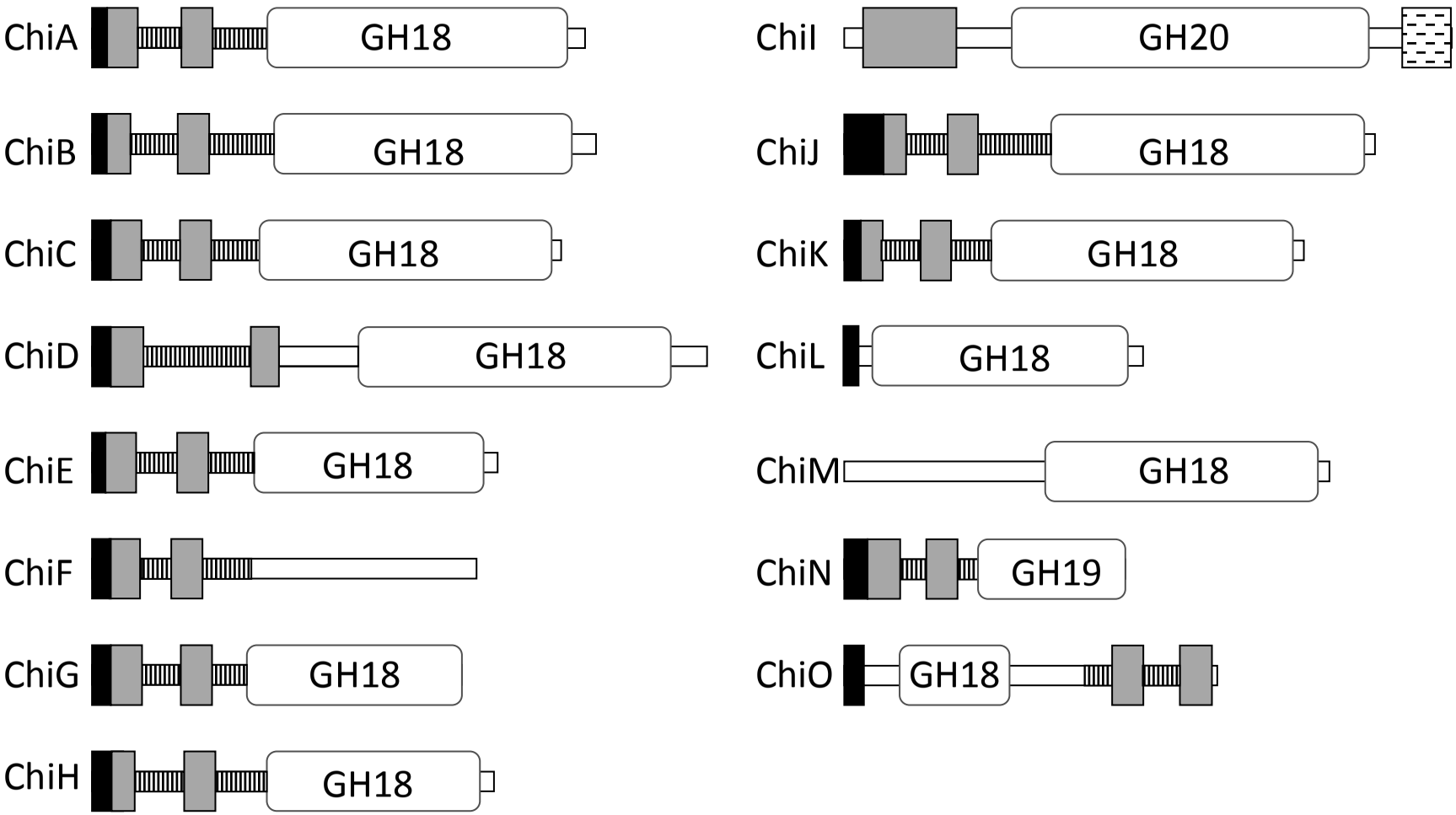

FIG. 1. Huang *et al*.

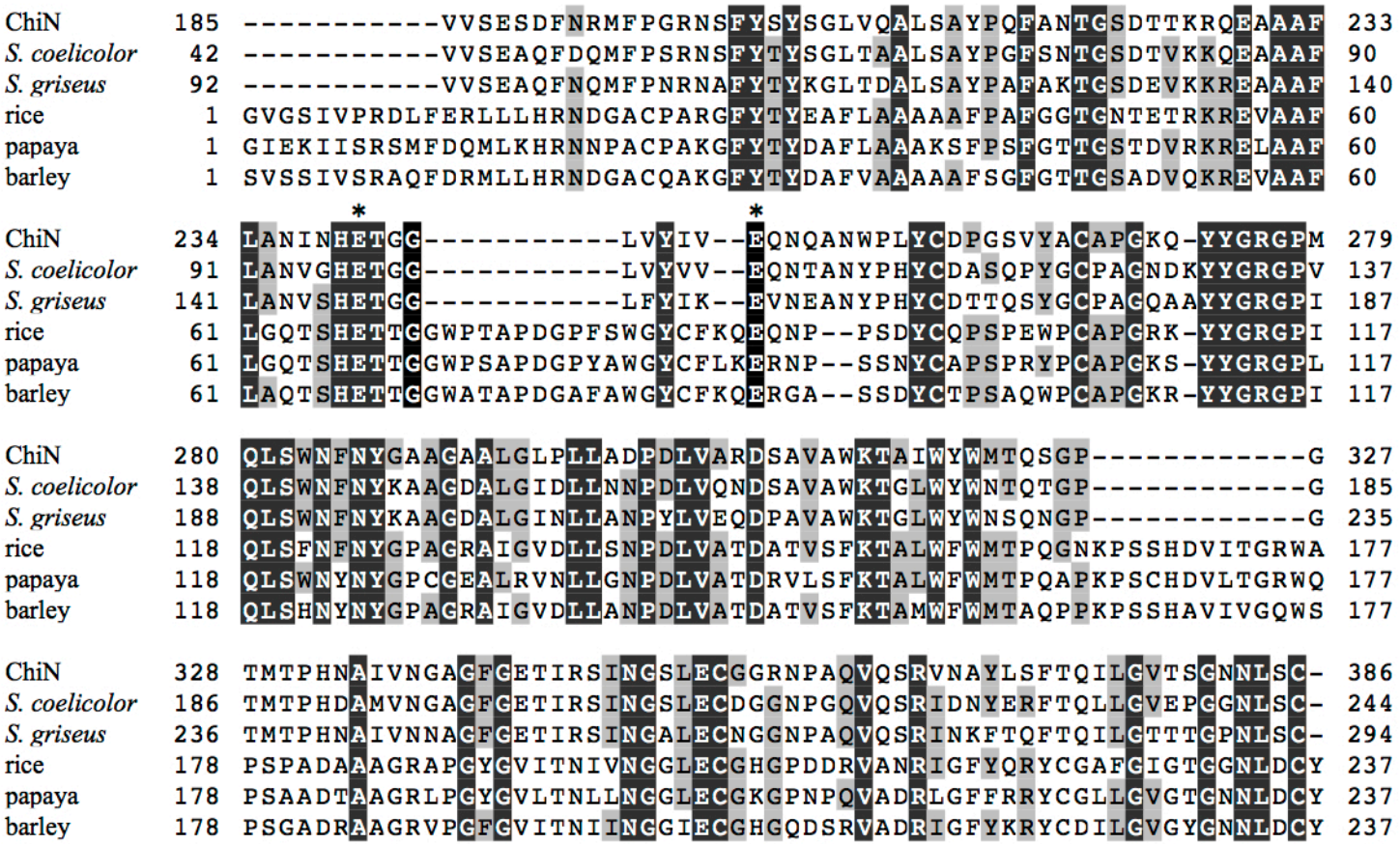

FIG. 2. Huang *et al.*

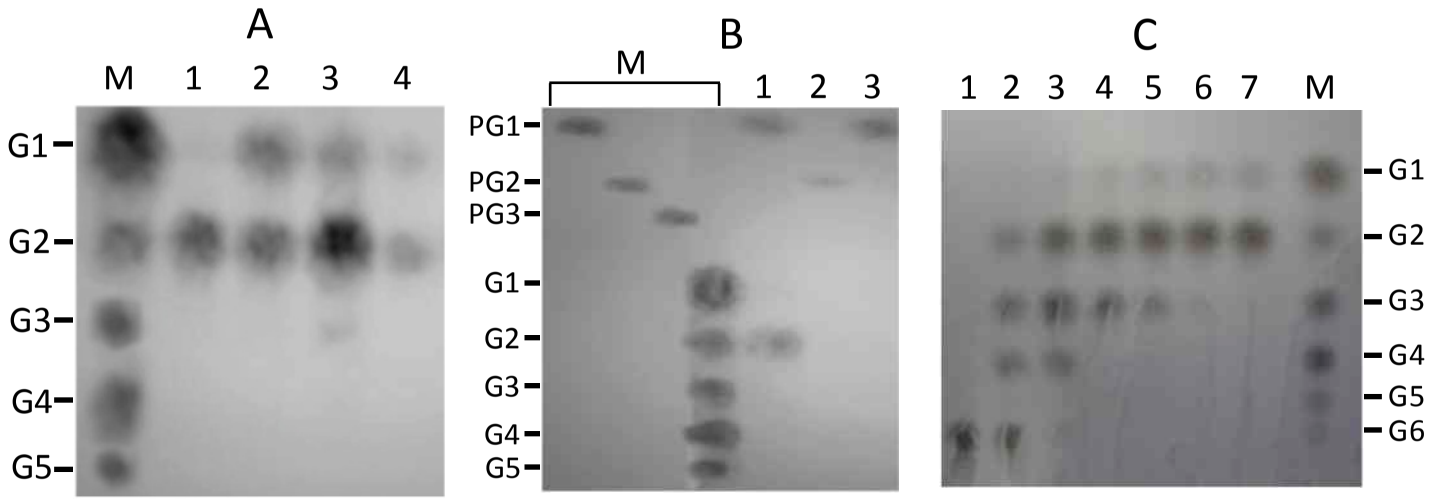

FIG. 3. Huang *et al*.

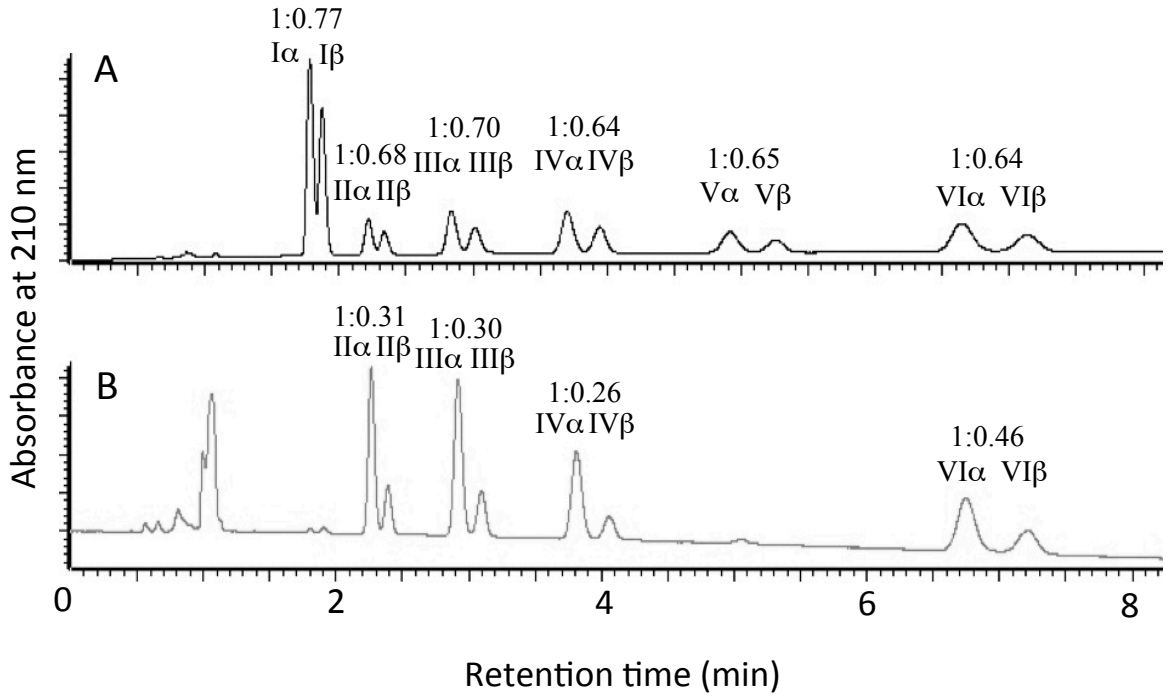

FIG. 4. Huang *et al*.

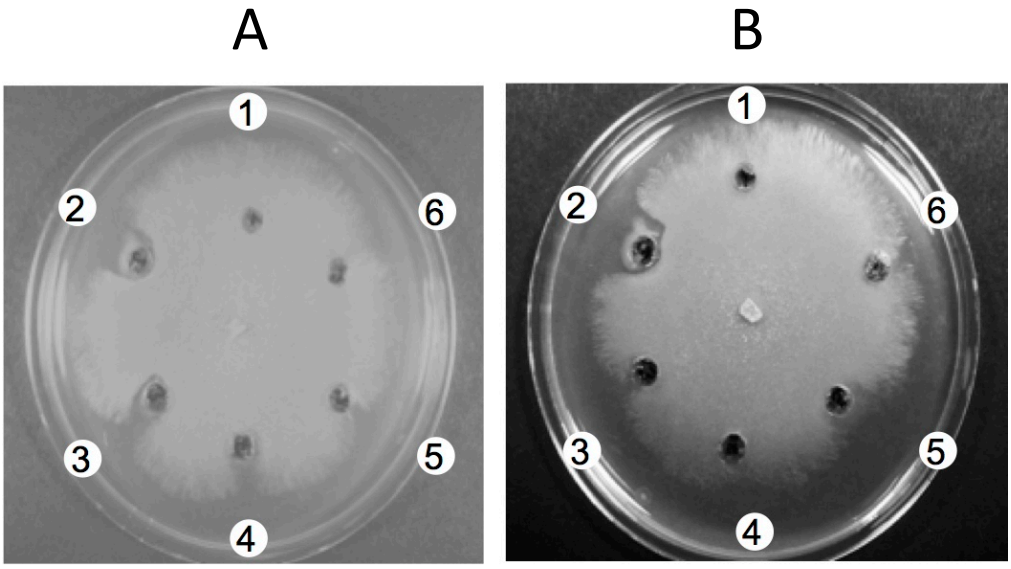

Fig. 5 Huang *et al.*

Table S1. ORF analysis of nucleotide sequences of fosmid clones selected from *C. shinanonensis* genome library

contig number[a]	length (bp)	accession number	number of putative ORFs	putative chitinolytic enzymes	gene symbols
contig 2	63,786	AB649129	31	8	*chiA, chiB, chiCDEFG, chiH*
contig 4	34,037	AB649130	14	1	*chiI*
contig 8	39,135	AB649131	20	2	*chiJ, chiK*
contig 18	22,150	AB649132	16	0	
contig 19	16,924	AB649133	12	2	*chiLM*
contig 22	20,737	AB649134	9	2	*chiN, chiO*
total	196,769		102	15	

[a]Contigs more than 10 kbp in length are shown

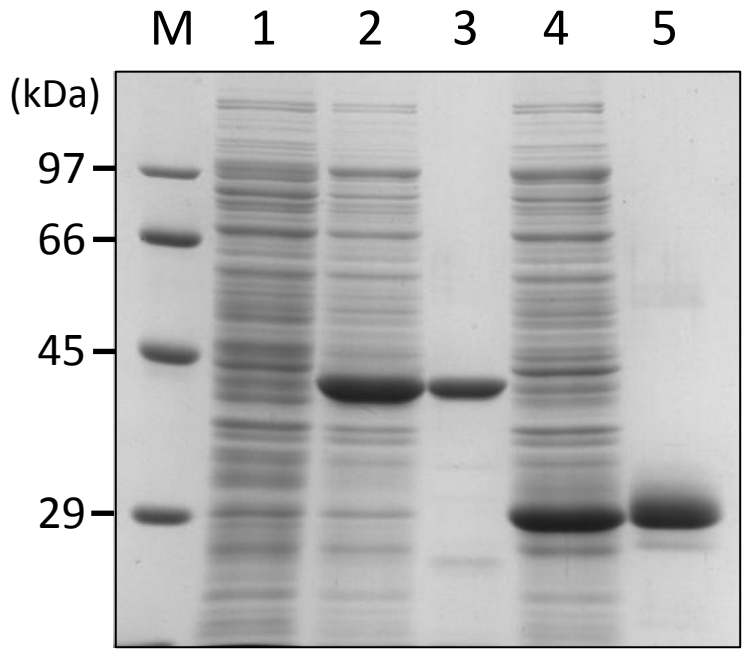

Figure S1. SDS-PAGE analysis of recombinant proteins, rChiN and rChiNDChBD.
Soluble crude proteins from *E. coli* BL21 (DE3) possessing pCold I alone (lane 1), pCold I-*chiN* (lane 2) and pCold I-*chiNDChBD* (lane 4) after isopropyl-β-thiogalactopyranoside induction. Recombinant proteins purified for rChiN (lane 3) and rChiNDChBD (lane 5). Lane M: molecular weight markers.