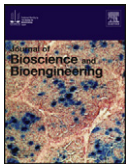

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Isolation of genes coding for chitin-degrading enzymes in the novel chitinolytic bacterium, *Chitiniphilus shinanonensis*, and characterization of a gene coding for a family 19 chitinase

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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.**

[**Key words:** *Chitiniphilus shinanonensis*; Family 19 chitinase; Chitin-binding domain; Fungal growth inhibition]

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 1×10^{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).

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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.

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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 30°C 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™ 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™ Fosmid Library Production Kit (EPICENTRE Biotechnology, Madison, WI, 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 5000 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′-GCGTCGACTCAGCAACTG-GATTGTTG-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 37°C with shaking (150 rpm). When the absorbance reached $OD_{600} = 0.4–0.6$, cultures were cooled and incubated at 15°C for 30 min. Isopropyl-β-thiogalactopyranoside was then added to a final concentration of 0.5 mM, and the culture was successively shaken at 15°C 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 (Model 2020, Astrason, Kyoto, Japan). Cell debris was removed by centrifugation (10,000 × *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, USA) using an Acquity UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm particle size). After the reaction, the mixture was added with twice the 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 milligrams of flake chitin was mixed thoroughly with 20 μg of each recombinant protein in 300 μl of 20 mM sodium acetate buffer (pH 5.6). The mixture was kept on ice for 2 h with mixing every 15 min. 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, NJ, 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 was isolated from moat water at Ueda Castle, Nagano, Japan (10). It is characterized by fast growth on chitin agar with a zone of clearing around the colony, and efficient utilization of insoluble flake chitin (9). To elucidate the chitinolytic pathway of *C. shinanonensis*, we attempted to purify chitinolytic enzymes from SAY3^T culture medium. Cultures were grown in synthetic medium containing colloidal chitin as the sole carbon source; however, this approach proved difficult because of a large number of proteins with chitinolytic activity. We then attempted to isolate genes coding for chitinolytic enzymes to characterize a repertoire of chitin-degrading enzymes in this bacterium.

A SAY3^T genomic library was constructed using fosmid vector pCC1FOS, which can incorporate DNA fragments of up to approximately 40 kb to be packaged into lambda phage particles. The constructed library was composed of approximately 5000 *E. coli* clones; which, assuming a genome size similar to that of *E. coli* (4.6 Mbp), should provide coverage of the entire SAY3^T genome with more than 99% probability (18).

We then screened clones to isolate those with chitinolytic activity. Screening was carried out using selection plates containing a mixture of fluorescent substrates, 4MU-GlcNAc and 4MU-$(GlcNAc)_3$, in which the former is degraded by an exo-type NAG, and the latter is preferentially digested by an endo-type chitinase. A negligible background of chitinolytic activity in host *E. coli* enabled us to select 10 clones exhibiting blue-white fluorescence under exposure of ultraviolet light. A mixture of fosmids prepared from the 10 clones was sequenced using a next-generation sequencer. As a result, six contigs (more than 10 kb in size) were constructed, encompassing a total length of 197 kb (Table S1). The sequence contained a total of