Molecular Analysis of the Gene Encoding a Novel Cold-Adapted Chitinase (ChiB) from a Marine Bacterium, *Alteromonas* sp. Strain O-7

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The chitinase B (ChiB) secreted by *Alteromonas* sp. strain O-7 was purified, and the corresponding gene (*chiB*) was cloned and sequenced. The open reading frame of the *chiB* gene encodes a protein of 850 amino acids with a calculated molecular mass of 90,223 Da. ChiB is a modular enzyme consisting of two reiterated domains and a catalytic domain belonging to chitinase family 18. The reiterated domains are composed of chitin-binding domain (ChtBD) type 3 and two fibronectin type III (Fn3)-like domains. Expression plasmids coding for ChiB or deletion derivatives thereof were constructed in *Escherichia coli*. Deletion analysis showed that the ChtBD of ChiB plays an important role in efficient hydrolysis of insoluble chitin. The optimum pH and temperature of ChiB were 6.0 and 30°C, respectively. The enzyme showed relatively high catalysis, even at low temperatures close to 0°C, and remarkable thermal lability compared to ChiA and ChiC, which are the mesophilic chitinases of the same strain. The k_{cat}/K_m value for the ChiB reaction at 10°C was about 4.7 times higher than that of ChiC. These results suggest that ChiB is a cold-adapted enzyme. The RNA transcript of *chiB* was induced by 1% GlcNAc, and along with a rise in temperature, the RNA transcript showed a tendency to decrease. Thus, among the ChiA, ChiB, and ChiC chitinases, production of ChiB may be advantageous for the strain, allowing it to easily acquire nutrients from chitin and to survive in cold environments.

Chitin, an insoluble homopolymer of β -(1,4)-linked *N*-acetylglucosamine (GlcNAc), is an abundant organic compound in nature. This polysaccharide is found in the exoskeletons and endoskeletons of many marine organisms, including mollusks, coelenterates, protozoa, fungi, and crustaceans. Since carbon and nitrogen are generally limited in the marine environment, chitin is a particularly important nutrient source for marine organisms. Chitinolytic marine bacteria play a crucial role in the recycling of chitinous materials for maintenance of the ecosystem in the marine environment (13). The concerted action of two chitinolytic enzymes, chitinase (EC 3.2.1.14) and β -*N*-acetylglucosaminidase (EC 3.2.1.30), is considered to be essential for the complete hydrolysis of chitin to GlcNAc. Chitinases cleave glycosidic linkages of GlcNAc randomly to produce soluble oligosaccharides, mainly chitobiose, which are further hydrolyzed to GlcNAc by β -*N*-acetylglucosaminidases. Finally, the degradation products, mainly GlcNAc, are then taken up by the cells as a carbon and nitrogen source.

Alteromonas sp. strain O-7 is a gram-negative, flagellated, motile, and aerobic rod-shaped bacterium of marine origin and an efficient producer of chitinolytic enzymes (33). This strain produces at least three chitinases (ChiA, ChiB, and ChiC), a chitinase-like enzyme (ChiD), three β -*N*-acetylglucosaminidases (GlcNAcases A, B, and C), a transglycosylative enzyme (Hex99), a chitin-binding protein (Cbp1), and a chitin-binding protease (AprIV) in the presence of chitin. We have cloned

and sequenced all of the genes involved in the chitin degradation of the strain except the *chiB* gene (21, 34–39, 41). Furthermore, they have been expressed in *Escherichia coli* and the biochemical properties of each recombinant protein have been investigated. ChiA, ChiC, and ChiD showed sequence similarity to bacterial chitinases of family 18; however, ChiD was found to be a chitinase-like enzyme with high activity only toward chitin oligosaccharides of sizes ranging from trimers to hexamers but not toward chitin (34, 37, 41). Outer membrane-associated GlcNAcase A and periplasmic GlcNAcase B belong to glycosyl hydrolase family 20, and cytoplasmic GlcNAcase C belongs to glycosyl hydrolase family 3 (35, 36, 39). Hex99, belonging to glycosyl hydrolase family 20, synthesized β -(1,6)-(GlcNAc)₂ from β -(1,4)-(GlcNAc)₂, which is one of the smallest molecules that induce chitinase production in the microorganism (38).

Our ultimate goal is to clarify the chitinolytic system of *Alteromonas* sp. strain O-7 at the molecular level. In this report, we describe the molecular characterization and structural organization of ChiB, which is responsible for chitinolytic activity in *Alteromonas* sp. strain O-7. Furthermore, we have overexpressed ChiB in *E. coli* and investigated the biochemical properties of the enzyme and its role in the chitin degradation system of this bacterium.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Alteromonas* sp. strain O-7 was grown at 27°C in Bacto Marine Broth 2216 (Difco) and used as the source of chromosomal DNA. *E. coli* JM109 and BL21(DE3) were grown at 37°C in Luria-Bertani medium supplemented with appropriate antibiotics for selection of transformants and for production of recombinant proteins, respectively. For agar medium, Luria-Bertani medium was solidified with 1.5% (wt/vol) agar (Nacalai

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TABLE 1. Sequences of primers used in this study

Primer	Sequence ^a	Location ^b
P-1	5'-TGTTT(TTT(A/G)CCAAT(A/T)GGAAA(A/G)TC-3'	1763-1741
P-2	5'-TATTGGCATAA(C/T)TT(C/T)GT(A/T)AA(C/T)CC-3'	1777-1799
P-3	5'-AATTGAGTGAATTACCCAAGGTA-3'	1538-1515
P-4	5'-TCCGACATGCAGGCTCTACAAGCG-3'	1963-1986
P-5	5'-TCCCTAAGTCGAGGTTTCGTAGCC-3'	1186-1163
P-6	5'-GGTACTGTTTTGGTCAATGCGCCC-3'	1234-1257
P-7	5'-GGCCAAGCGAGCTCCTACGAC-3'	244-264
P-8	5'-CGTTAATTTGTCGACAATGGG-3'	2718-2698
P-9	5'-TCAAGGCTTGGTGAGCTCGGCAACCTTG-3'	951-979
P-10	5'-TCAGGTGTCCAGGAGCTCCGTGACAGTTC-3'	1704-1732
P-11	5'-CCACCACTATCTCTGTCAAGTGCTC-3'	1256-1279
P-12	5'-CTAAGTGTTCCTTGATTGGCTGTG-3'	1361-1338

^a The mutagenized restriction sites are underlined.

^b Nucleotide numbering of the *chiB* gene (accession no. AB086847).

Tesque, Kyoto, Japan). For production of chitinase, *Alteromonas* sp. strain O-7 was grown at 27°C in Bacto Marine Broth 2216 containing 0.1% powdered chitin from crab shells (Nacalai Tesque) or 1.0% GlcNAc.

Purification of ChiB. *Alteromonas* sp. strain O-7 was grown at 27°C with agitation at 200 rpm on a rotary shaker for 48 h. The culture supernatant was collected by centrifugation at 10,000 × *g* and 4°C and was used as a crude enzyme solution. All purification steps were carried out at 4°C. The crude enzyme solution was dialyzed against 50 mM Tris-HCl buffer (pH 7.5), and the dialyzed solution was applied to a DEAE-Toyopearl 650 M column (1.9 by 45 cm; Tosoh Co., Tokyo, Japan) equilibrated with the same buffer. The column was washed with the buffer (300 ml) and then with a linear gradient of NaCl (0 to 1.0 M) at a flow rate of 24 ml/h. ChiB activity was eluted at about 0.4 M NaCl. The active fractions were pooled and concentrated by ultrafiltration with a Q0100 membrane (Advantec, Tokyo, Japan). The concentrated sample was applied to a Sephadex G-100 column (1.9 by 50 cm; Amersham Bioscience) equilibrated with the same buffer containing 0.1 M NaCl. For further purification, the active fraction was chromatographed by using a fast protein liquid chromatography Resource Q (6 ml; Amersham Bioscience) column equilibrated with buffer and then the enzyme was eluted with a linear gradient of NaCl (0 to 0.6 M). Active fractions were eluted at a concentration of about 0.15 M and used as the purified enzyme solution.

General recombinant DNA techniques. *Alteromonas* chromosomal DNA was isolated as described previously (34). Plasmids pUC18 and -19 were used as cloning vectors. Plasmid pET-20b(+) (Novagen) was used as the expression vector. Agarose gel electrophoresis, plasmid DNA preparation, transformation of *E. coli*, and Southern hybridization were performed as described by Sambrook and Russell (30). Restriction enzymes and other modifying enzymes were purchased from Toyobo (Osaka, Japan).

Cloning of *chiB*. Chromosomal DNA of *Alteromonas* sp. strain O-7 was digested with various restriction enzymes and electrophoresed on a 0.6% agarose gel. The fragments from 1.0 to 3.0 kb were excised from the gel and purified with a GenElute gel extraction kit (Sigma). These fragments were self-ligated and used as template DNAs. Degenerate inverse primers P-1 and P-2 (Table 1) were synthesized on the basis of the N-terminal amino acid sequence of ChiB. PCR amplification was performed with KOD-Plus-DNA polymerase (Toyobo) for 30 cycles consisting of 94°C for 30 s, 55°C for 15 s, and 68°C for 5 min. The 2.3-kb fragment was amplified by using the *Hind*III-digested template and phosphorylated by T4 DNA polynucleotide kinase. The phosphorylated fragment was cloned into the dephosphorylated *Sma*I site of pUC18. Analysis of the entire nucleotide sequence of the inserted DNA indicated that the 5' upstream region of the *chiB* gene was missing. To clone the 5' upstream region of the *chiB* gene, a second inverse PCR was performed. Two primers, P-3 and P-4 (Table 1), were synthesized on the basis of the nucleotide sequence of the first inverse PCR product. The 1.1-kb fragment was amplified by using the *Hpa*I- and *Eco*RV-digested template. However, analysis of the nucleotide sequence indicated that the 5' upstream region of the *chiB* gene was still missing. Thus, a third inverse PCR was performed by using two primers, P-5 and P-6 (Table 1), synthesized on the basis of the nucleotide sequence of the second PCR product. The 2.2-kb fragment was amplified by using the *Hind*III-digested template.

Construction of expression plasmids. Expression plasmid pET-ChiB, encoding ChiB residues 25 to 839, was constructed as follows. We synthesized two oligonucleotide primers, P-7 and P-8 (Table 1), that were modified to contain *Sac*I and *Sal*I restriction sites to facilitate cloning in frame into pET-20b(+). The

chiB gene was amplified by PCR with the primers and with chromosomal DNA digested with *Bam*HI as the template. PCR was performed for 30 cycles consisting of 94°C for 30 s, 50°C for 15 s, and 68°C for 2 min. The amplified DNA was digested with *Sac*I and *Sal*I, and the resulting fragment (2.0 kb) was inserted into the corresponding sites of pET-20b(+). The truncated ChiB protein (ChiBΔN1) lacking the N-terminal region (residues 1 to 261) and the truncated ChiB protein (ChiBΔN1ΔN2) lacking the region corresponding to residues 1 to 512 were also amplified by PCR in the same manner as pET-ChiB. The primers used to encode ChiBΔN1ΔN2 were P-8 and P-9 (Table 1), and those used to encode ChiBΔN1ΔN2 were P-8 and P-10 (Table 1). The PCR products were inserted in frame between the *Sal*I and *Sac*I sites. The resulting plasmids, encoding the regions including residues 262 to 839 and residues 513 to 839 of ChiB, were designated pET-ChiBΔN1 and pET-ChiBΔN1ΔN2, respectively. The nucleotide sequences of the junctions between vectors and inserts and the whole amplified DNA were confirmed with the DYEnamic ET terminator cycle sequencing premix kit (Amersham Bioscience) on a DNA sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems).

Purification of recombinant proteins. *E. coli* BL21(DE3) cells harboring pET-ChiB, pET-ChiBΔN1, or pET-ChiBΔN1ΔN2 were induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase and further incubated for 4 h at 37°C. Cells were harvested by centrifugation and washed and resuspended with phosphate-buffered saline. The cells were disrupted by sonication, and the lysate was centrifuged at 10,000 × *g* for 20 min. Each of the His-tagged proteins accumulated in the cells as inclusion bodies. The pellets were then solubilized in 6 M guanidine hydrochloride, and the recombinant proteins were purified with a HisTrap column (Amersham Bioscience). ChiB, ChiBΔN1, and ChiBΔN1ΔN2 were slowly dialyzed against 50 mM Tris-HCl buffer (pH 7.5) by several buffer changes. The supernatant was further chromatographed with a linear gradient of NaCl with a Resource Q (Amersham Bioscience) fast protein liquid chromatography column. Active fractions were combined and used as purified enzymes.

Enzyme assay and kinetic parameters. Chitinase activity was measured as described previously, with colloidal chitin, glycol chitin, or *p*-nitrophenyl-*N*,*N*'-diacetylchitobiose [pNP-(GlcNAc)₂] as the substrate (34, 37, 41). Kinetic parameters were determined for ChiA, ChiB, and ChiC. The recombinant ChiA and ChiC proteins were purified as described previously (41). Reaction mixtures containing 50 mM citrate buffer (pH 6.0), a range of concentrations of pNP-(GlcNAc)₂ (0 to 3.3 mM), and enzymes were incubated at 10, 20, or 30°C for 6 min. The reactions were terminated by the addition of 2.0 M Na₂CO₃, and the release of *p*-nitrophenol was monitored at 420 nm. Enzyme activity was calculated on the basis of an extinction coefficient for *p*-nitrophenol of 14,000 M⁻¹ cm⁻¹. The enzyme assays were done in triplicate. *K_m* and *k_{cat}* values were obtained from extrapolated intercepts of Lineweaver-Burk plots (1/[*s*] versus 1/[*v*]), where [*s*] and [*v*] are the pNP concentration and initial velocity, respectively) by using Microsoft Excel 2000. Protein was assayed with Coomassie blue dye by using a Bio-Rad protein assay kit (5).

N-terminal amino acid sequence. The N-terminal amino acid sequence was analyzed with an ABI Procise 491 HT protein sequencer (Applied Biosystems) connected to an online phenylthiohydantoin derivative analyzer.

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described before (41). Proteins on the gel was transferred to Sequi-Blot polyvinylidene difluoride membrane. The membrane was incubated for 1 h at room temperature with anti-ChiB polyclonal rabbit anti-

TABLE 2. Purification of ChiB from *Alteromonas* sp. strain O-7

Fraction	Total protein amt (mg)	Total enzyme activity (U)	Sp act (U/mg of protein)	Purification factor (fold)	Yield (%)
Culture supernatant	37.20	52.9	1.4	1.0	100.0
DEAE-Toyopearl 650M	4.15	11.9	2.9	2.1	23.0
Sephadex G-100	1.43	9.4	6.6	4.7	18.0
Resource Q	0.06	1.9	30.8	22.0	3.5

serum diluted to 1:1,000 in phosphate-buffered saline containing 2.0% skim milk (Difco). Bound antibody was detected as described previously (21).

Real-time quantitative PCR analysis of the *chiB* transcript. *Alteromonas* sp. strain O-7 was cultured in Bacto Marine Broth 2216 or the same medium containing 1.0% GlcNAc at 17, 27, or 37°C until the optical density at 600 nm reached 1.2. Total RNA was extracted from 1.0-ml suspensions of *Alteromonas* sp. strain O-7 cells with an SV total RNA isolation system (Promega) in accordance with the manufacturer's instructions. Total RNA (5.0 µg) and primer P-12 (Table 1) were used to reverse the *chiB* transcripts. The reaction was carried out at 55°C for 60 min with Moloney murine leukemia virus reverse transcriptase (RNase H minus; Promega) and terminated by heating at 70°C for 15 min. The amount of the reverse transcript was measured by real-time quantitative PCR. P-11 and P-12 (Table 1) were the primers used. PCR amplification was monitored with a QuantiTect SYBR Green PCR kit (Qiagen) in a LightCycler (Roche Diagnostics). For each sample, a log-linear line was fitted automatically by selecting two points above the threshold band to determine the fractional cycle number of the crossing point. The data were calculated automatically by the LightCycler software (version 3.53; Roche Diagnostics).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank databases under accession no. AB086847.

RESULTS

Purification of ChiB. When *Alteromonas* sp. strain O-7 was grown in medium supplemented with chitin, zymogram analysis (48) showed four activity bands corresponding to proteins of 85 (ChiA), 65 (truncated form of ChiA), 45 (ChiC), and 35 (ChiB) kDa in the culture supernatant after 36 h (data not shown). To characterize ChiB, the 35-kDa protein was purified from the culture supernatant of the strain by successive column chromatographies on DEAE-Toyopearl 650 M, Sephadex G-100, and Resource Q. The purification procedure is summarized in Table 2. By the procedure described, the enzyme was purified about 22.0-fold with a yield of 3.5%. The molecular mass of the enzyme estimated by SDS-PAGE was 35 kDa (data not shown), and the N-terminal amino acid sequence was DKPIGKKHKLIGYWHNFVNP. The optimum pH and temperature of purified ChiB were 6.0 and 30°C, respectively, when pNP-(GlcNAc)₂ was used as the substrate.

Cloning and nucleotide sequence of the *chiB* gene. The nucleotide sequences of three amplified DNAs were determined and connected by overlapping the sequences to analyze the full-length *chiB* gene. The *chiB* gene consists of 2,550 nucleotides encoding a protein of 850 amino acids with a predicted molecular mass of 90,223 Da (Fig. 1). The open reading frame has an ATG start codon at position 187 that is preceded by a possible ribosome-binding site (AGGGA) at a distance of 11 nucleotides. An inverted repeat composed of a 6-bp stem and a loop of seven bases was located 11 bp downstream of the *chiB*

FIG. 1. (A) Deduced amino acid sequence encoded by the *chiB* gene. The signal peptide sequence is underlined. The type 3 ChtBD is boxed, and the Fn3-like domain is shaded. The regions that are homologous to the consensus sequence of the type 3 ChtBD are indicated by white letters on a black background. The N-terminal amino acid sequence of ChiB purified from the culture supernatant is shown by bold letters. Solid circles above amino acid residues represent the consensus sequences of family18 chitinases. (B) Diagram of the domain structure of ChiB: signal peptide, ■; ChtBD, ▨; Fn3-like domain, ▩; catalytic domain, □.

FIG. 2. Western blot analysis of ChiB. *Alteromonas* sp. strain O-7 was cultured at 27°C for 24 h. Culture supernatant samples were taken at 3, 5, 7, 9, and 24 h. Each of the culture supernatant samples (180 μ l) was added to 20 μ l of 20% trichloroacetic acid and centrifuged. The pellets were dissolved with 20 μ l of SDS-PAGE sample buffer and subjected to SDS-12% PAGE. Lane M, prestained molecular weight marker.

gene terminal codon (TAA). The deduced N-terminal 24-amino-acid sequence showed the typical features of signal peptides, which are composed of a positively charged region, a hydrophobic region, and a signal sequence cleavage site (27). From the characterization of the cleavage sites, it is presumed that the putative site of cleavage might be between alanine 24 and tyrosine 25, which is compatible with the $-3, -1$ rule of von Heijne (43). The molecular mass of the translated protein was much larger than that of the enzyme purified from the culture supernatant of *Alteromonas* sp. strain O-7 (35 kDa). The N-terminal amino acid sequence of the enzyme purified from the culture supernatant coincided precisely with the sequence starting from Asp519 of the deduced amino acid sequence (Fig. 1). These results suggest that ChiB purified from the culture supernatant appears to be generated by the action of a protease(s) present in the culture supernatant after the initial gene product is secreted into the culture medium. In order to detect the initial gene product in the culture supernatant, Western blot analysis with a polyclonal rabbit antibody to ChiB was performed (Fig. 2). When the strain was grown at 27°C in Bacto Marine Broth 2216 containing 1% GlcNAc, a band corresponding to a protein of 90 kDa was detected after 5 h. On the other hand, a band corresponding to a protein of 35 kDa appeared after 7 h of cultivation and as the amount of the 35-kDa protein increased, the amount of the 90-kDa protein decreased. These results demonstrate that the 35-kDa protein is a truncated form of the 90-kDa protein that is the initial product of the *chiB* gene.

Domain structure of ChiB. The BLAST analysis program was used to compare ChiB with proteins in databases. Most of the structural elements could be readily identified by sequence comparison. Analysis of the amino acid sequence of ChiB derived from the nucleotide sequence revealed that the enzyme consists of a multidomain structure (Fig. 1). An N-terminal signal peptide was followed by a fragment of 42 amino acids (Pro32 to Trp73) that showed significant homology to chitin-binding domain (ChtBD) type 3, as classified by the

National Center for Biotechnology conserved domain database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). The domain showed sequence identities of 54% to the ChtBD of *Alteromonas* sp. ChiC (37), 44% to the ChtBD of *Alteromonas* sp. ChiD (41), 43% to the ChtBD of *Pseudoalteromonas* sp. ChiA (31), and 38% to the ChtBD of *Vibrio cholerae* ChiA (8). Sequence alignment of the ChtBD of ChiB with other type 3 ChtBDs revealed a consensus sequence (AKWWTQ) that is well conserved in bacterial ChtBDs (6). The duplicated domains located between Pro87 and Gly164 and between Pro182 and Ser260 were adjacent to the ChtBD and showed similarity to a fibronectin type III (Fn3)-like domain that is quite prevalent in bacterial sequences (26). Furthermore, the similar domain organization, consisting of ChtBD and two Fn3-like domains, was reiterated in a 228-amino-acid-residue region corresponding to residues 281 to 508 of ChiB (Fig. 1). Although a sequence identity of 31% between the first and second ChtBDs was found, the consensus sequence of the type 3 ChtBDs was not perfectly conserved in the second ChtBD (Fig. 1). However, residue Trp308, which seems to be essential for chitin binding, was identified.

The catalytic domain of ChiB was next to the reiterated sequences. The amino acid sequence from Asp519 to Asn850 was homologous to a number of chitinases belonging to glycosyl hydrolase family 18 (14, 15). This region showed sequence identities of 79% to *Vibrio cholerae* ChiA (8), 52% to *Xanthomonas* sp. strain AK ChiA (29), 42% to *Bacillus circulans* ChiD (46), 39% to *Streptomyces lividans* ChiB (22), and 33% to *Streptomyces thermoviolaceus* Chi30 (40). Family 18 bacterial chitinases contain the consensus sequences SXGG and DXX DXDXE, as shown in Fig. 1. The sequences SXGG and DXX DXDXE are substrate-binding and active sites, respectively. These consensus sequences were also perfectly conserved in the catalytic domain of ChiB, indicating that Glu648 of ChiB is involved as the proton donor in the catalytic double-displacement mechanism during hydrolysis (15, 42). Among the seven domains, there were small Gly-, Ala-, Pro-, Ser-, and Thr-rich sequences that typically resemble domain linker regions (32).

Expression and purification of recombinant proteins. To elucidate the function of the region consisting of ChtBD and two Fn3-like domains, we constructed the recombinant ChiB protein and deletion derivatives thereof (ChiB Δ N1 and ChiB Δ N1 Δ N2). ChiB, ChiB Δ N1, and ChiB Δ N1 Δ N2 were purified by a combination of HisTrap affinity chromatography and Resource Q ion-exchange chromatography. The molecular masses of these proteins calculated from the deduced amino acid sequence with no signal peptide (ChiB Δ N1, 67 kDa; ChiB Δ N1 Δ N2, 41 kDa) are in reasonable agreement with those estimated by SDS-PAGE (ChiB Δ N1, 70 kDa; ChiB Δ N1 Δ N2, 43 kDa) (Fig. 3). However, the apparent molecular mass of the purified ChiB protein determined by SDS-PAGE was higher than that calculated on the basis of the deduced amino acid sequence of ChiB (92 kDa). These purified proteins were used for further enzymatic characterization.

Characterization of recombinant proteins. The pH and temperature profiles of ChiB, ChiB Δ N1, and ChiB Δ N1 Δ N2 were examined by using pNP-(GlcNAc)₂, a chromogenic substrate for chitinase. The pH and temperature profiles of these enzymes were almost identical, and the optimal pH and temperature of each enzyme were 6.0 and 30°C, respectively (data not

FIG. 3. SDS-PAGE of recombinant ChiB, ChiBΔN1, and ChiBΔN1ΔN2. Lanes: M, molecular size standards; 1, ChiB; 2, ChiBΔN1; 3, ChiBΔN1ΔN2.

shown). The optimum pH of ChiB (pH 6.0) was identical to those of ChiA and ChiC, whereas the optimum temperature of ChiB was much lower than those of ChiA and ChiC from the same strain, and the respective activities of ChiB at 0, 10, and 20°C were 28, 41, and 92% of that at the optimal temperature (Fig. 4). Figure 4 shows the effect of temperature on the stability of ChiA, ChiB, and ChiC. The denaturation curves of these enzymes clearly illustrated the low thermal stability of ChiB. The half-time of inactivation of ChiB at 40°C was 7 min, and the enzyme retained only 15% of its original activity after incubation at 40°C for 60 min. On the other hand, after pre-incubation for 60 min at 40°C, ChiA and ChiC retained 100 and 80% of their original activities, respectively. These results indicate that ChiB has features typical of a cold-adapted enzyme: relatively high catalytic activity at low temperature and remarkable thermosensitivity (9, 16).

The kinetic properties of ChiA, ChiB, and ChiC are shown in Table 3. The k_{cat}/K_m value for the ChiB reaction at 20°C was 22.8- and 4.2-fold higher than those for the ChiA and ChiC

TABLE 3. Kinetic parameters and Arg, Pro, and Tyr contents of ChiA, ChiB, and ChiC

Protein and temp (°C)	Kinetic parameters			Full-length enzyme			Catalytic domain		
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	% Arg	% Pro	% Tyr	% Arg	% Pro	% Tyr
ChiB									
10	43.00	3.49	12.32						
20	98.11	4.62	21.24	1.5	5.5	3.4	2.1	6.9	2.7
30	173.90	4.81	36.15						
ChiA									
10	ND ^a	ND	ND						
20	2.10	2.26	0.93	2.3	5.8	4.6	2.9	5.5	6.0
30	3.10	1.89	1.64						
ChiC									
10	1.87	0.71	2.63						
20	3.12	0.61	5.11	3.4	4.7	4.7	4.4	4.0	4.8
30	5.50	0.68	8.09						

^a ND, not determined.

reactions, respectively. The value for the ChiB reaction at 10°C was 4.7-fold higher than that for the ChiC reaction. The activity of ChiA at 10°C was too low for measurement of the kinetic constant of ChiA.

Function of reiterated sequences. To clarify the function of the reiterated sequences found in ChiB, the hydrolytic activities of ChiB, ChiBΔN1, and ChiBΔN1ΔN2 were measured at various temperatures (10, 20, and 30°C) (Table 4). When colloidal chitin was used as the substrate, the respective activities of ChiBΔN1 at 10, 20, and 30°C were 70, 75, and 76% of those of ChiB. The respective activities of ChiBΔN1ΔN2 at 10, 20, and 30°C were 32, 24, and 22% of those of ChiB. On the other hand, when glycol chitin was used as the substrate, ChiBΔN1 and ChiBΔN1ΔN2 showed slightly higher activity than ChiB at the assay temperatures tested. These results indicate that both reiterated sequences in the N-terminal region (residues 25 to 508) of ChiB are essential for efficient hydrolysis of insoluble chitin but not soluble chitin. However, the reiterated domains do not contribute to the cold adaptation of ChiB.

FIG. 4. (A) Temperature dependence of ChiA, ChiB, and ChiC. Reactions were carried out at various temperatures for 30 min with 50 mM citrate buffer, pH 6.0. The ChiA, ChiB, and ChiC protein concentrations were 1.7, 1.1, and 1.9 ng/μl, respectively. Symbols: □, ChiA; ●, ChiB; △, ChiC. (B) Thermal stabilities of ChiA, ChiB, and ChiC. The enzymes were incubated at 40°C in 50 mM citrate buffer (pH 6.0), and residual activities of timed aliquots were measured by using pNP-(GlcNAc)₂ as the substrate. The ChiA, ChiB, and ChiC protein concentrations were 1.7, 1.1, and 1.9 ng/μl, respectively. Symbols: □, ChiA; ●, ChiB; △, ChiC.

TABLE 4. Hydrolytic activities of ChiB and truncated derivatives thereof

Temp (°C) and sample	Mean chitinase activity (U/mmol) ^a ± SD	
	Glycol chitin	Colloidal chitin
10		
ChiB	8.0 ± 0.4 (100)	6.3 ± 0.4 (100)
ChiBΔN1	8.2 ± 0.6 (103)	4.4 ± 0.7 (70)
ChiBΔN1ΔN2	8.8 ± 0.5 (110)	2.0 ± 0.3 (32)
20		
ChiB	14.5 ± 0.6 (100)	14.2 ± 0.2 (100)
ChiBΔN1	14.8 ± 0.4 (102)	10.6 ± 0.7 (75)
ChiBΔN1ΔN2	15.8 ± 0.9 (109)	3.4 ± 0.7 (24)
30		
ChiB	23.4 ± 0.9 (100)	19.0 ± 0.6 (100)
ChiBΔN1	24.5 ± 0.3 (105)	14.4 ± 0.9 (76)
ChiBΔN1ΔN2	26.0 ± 3.2 (111)	4.2 ± 0.8 (22)

^a Means of three independent experiments. The data in parentheses are relative activities.

FIG. 5. Real-time quantitative PCR analysis of the *chiB* transcript. *Alteromonas* sp. strain O-7 was cultured in Bacto Marine Broth 2216 (open bar) or the same medium containing 1.0% GlcNAc (solid bar) at 17, 27, or 37°C. The data shown are the means $\pm$ the standard deviations of three independent experiments.

Gene expression. *Alteromonas* sp. strain O-7 is a marine bacterium that grows in the range of 10 to 37°C (33). To examine whether expression of the *chiB* gene is regulated by growth temperatures, real-time quantitative PCR analysis was performed. The strain was cultured in Bacto Marine Broth 2216 with or without 1.0% GlcNAc at various temperatures (17, 27, and 37°C). The RNA transcript of *chiB* was induced by 1% GlcNAc at the cultivation temperatures examined, and along with a rise in temperature, the RNA transcript of *chiB* showed a tendency to decrease (Fig. 5). The respective amounts of RNA transcript of *chiB* at 27 and 37°C were 70 and 41% of that at 17°C. On the other hand, when the strain was cultured with no inducer, production of the RNA transcript of *chiB* did not depend on the temperature. These results suggest that the *chiB* gene is regulated by the growth temperature in the presence of 1% GlcNAc.

DISCUSSION

Alteromonas sp. strain O-7 is an efficient degrader of chitin in the marine environment (33). We have already shown that the chitinolytic system of the strain consists of three chitinases (ChiA, ChiB, and ChiC), three β -N-acetylglucosaminidases (GlcNAcase A, GlcNAcase B, and GlcNAcase C), a chitinase-like enzyme (ChiD), a transglycosylative enzyme (Hex99), a chitin-binding protein (Cbp1), and a chitin-binding protease (AprIV) (21, 34–39, 41). In this paper, we report that ChiB is a modular enzyme with a high level of catalytic activity at low temperatures compared with ChiA and ChiC. The *chiB* gene encodes a protein of 890 amino acids that has a novel domain architecture. The region consisting of one type 3 ChtBD and two Fn3-like domains was reiterated in the amino-terminal half of the protein. To clarify the function of the ChtBD and the Fn3-like domain, truncated forms of the enzyme (ChiB Δ N1 and ChiB Δ N1 Δ N2) were constructed and the enzyme activities toward soluble and insoluble chitin were examined. When insoluble chitin was used as the substrate, the activities of the truncated enzymes decreased remarkably in comparison with that of full-length ChiB. On the other hand,

when soluble chitin was used as the substrate, the truncated enzymes showed slightly higher activities than ChiB. The type 3 ChtBD was found in ChiA, ChiC, ChiD, Cbp1, and AprIV of *Alteromonas* sp. strain O-7 as a common structural unit. We have already demonstrated that the type 3 ChtBD strongly binds α - and β -chitin and is essential for efficient hydrolysis of insoluble chitin (21, 37, 41). These results indicate that the type 3 ChtBD of ChiB directly participates in the binding to chitin molecules and plays an important role in the hydrolysis of the insoluble substrate. On the other hand, the reiterated Fn3-like domains of ChiB were located between the first and second type 3 ChtBDs and between the second type 3 ChtBD and a catalytic domain. Fn3-like domains are structurally characterized as β -sheets containing an immunoglobulin-like fold (7). Fn3-like domains are found in various prokaryotic chitinases in widely different arrangements (11, 28, 37, 45). Watanabe et al. reported that removal of the Fn3-like domains within chitinase A1 from *Bacillus circulans* WL-12 resulted in lower chitin-hydrolyzing activity, although the domain had no effect on chitin binding (47). Therefore, Fn3-like domains of ChiB may function as linkers to maintain the optimal distance between and orientation of the domains, although the true function of these Fn3-like domains remains to be elucidated (23).

The recombinant ChiB protein was purified to homogeneity by employing both affinity and conventional chromatographies. ChiB belongs to chitinase family 18 and shares most of its properties with other bacterial chitinases (14, 15). However, this enzyme showed a relatively high catalysis level, even at low temperatures close to 0°C, and remarkable thermal lability compared to ChiA and ChiC, which are the mesophilic chitinases of the same strain. ChiB showed properties similar to those of ArChiA and ArChiB, which are produced by a psychrophilic marine bacterium, *Arthrobacter* sp. strain TAD20 (17), which is a solitary instance of cold-adapted chitinase regardless of the increase in the number of bacterial chitinases. However, ChiB differs from ArChiA and ArChiB in the following points. (i) ChiB is an enzyme produced by the mesophilic marine bacterium *Alteromonas* sp. strain O-7 together with mesophilic chitinases ChiA and ChiC. (ii) The domain structure of ChiB differs from those of ArChiA and ArChiB. (iii) ChiB retained only 15% of the original activity after incubation at 40°C for 60 min, whereas ArChiA and ArChiB retained 18 and 30% of their original activity after incubation at 50°C for 60 min. In general, cold-adapted enzymes display an apparent optimal activity shifted toward low temperatures and manifest pronounced heat lability (9, 16, 18). The temperature-activity and temperature-stability relationships of ChiB were clearly different from those of ChiA and ChiC (Fig. 4). Furthermore, the kinetic parameters were determined for ChiA, ChiB, and ChiC at three different temperatures. The k_{cat}/K_m ratio of ChiB was 12.32 at 10°C, which is approximately 4.7-fold higher than that of ChiC. However, the activity of ChiA at 10°C was too low for measurement of the kinetic constant of the enzyme. These findings suggest that ChiB has properties in common with other cold-adapted enzymes. There are usually multiple chitinase genes in chitinolytic bacteria; however, the contributions of individual enzymes to chitin degradation have not been elucidated in detail. *Alteromonas* sp. strain O-7 is a marine bacterium that grows at temperatures ranging from 10 to 37°C (33). The amounts of *chiB* RNA transcript showed a

tendency to decrease with a rise in the temperature, although the mode of regulation has not been clarified. Thus, among chitinases ChiA, ChiB, and ChiC, production of ChiB may be advantageous for the strain, allowing it to easily acquire nutrients from chitin and to survive in cold environments.

It has been proposed that increased flexibility is the most important factor for the catalytic efficiency of cold-adapted enzymes at low temperature (9). Analyses of the crystal structure and primary structure of cold-adapted enzymes have suggested that these enzymes have lower proline and arginine contents than their mesophilic counterparts (1–3, 10, 24, 25, 44). A structural role for arginine in multiple hydrogen bonds to backbone carbonyl and side chain oxygens has been proposed (4, 20, 24). On the other hand, proline residues are thought to modulate the entropy of protein unfolding by affecting backbone flexibility (19). The model structure of class C β -lactamase from the Antarctic psychrophile *Psychrobacter immobilis* A5 indicated that the enzyme possesses the lowest arginine and proline contents of any class C enzyme (10). Furthermore, Galkin et al. have reported a clear relationship between arginine residue content and thermostability in the three NAD⁺-dependent alanine dehydrogenases from psychrotrophic and mesophilic bacteria (12). Thus, we compared the arginine and proline contents of ChiA, ChiB, and ChiC as shown in Table 3. The ratio of arginine residues to the total number of amino acid residues of ChiB or its catalytic domain was lower than those of ChiA and ChiC. However, there was no clear relationship between Arg content and adaptation to cold because ChiA, which had unmeasurable activity at 10°C, had the next lowest Arg content and nearly the same Arg content in its catalytic domain as ChiB. Therefore, the Arg content of ChiB may not contribute to the thermal lability of the enzyme. Moreover, like that of NAD⁺-dependent alanine dehydrogenases (12), the thermal flexibility of ChiB could not be explained by the proportion of proline residues. Interestingly, ChiB also has a low proportion of tyrosine residues, which could provide polar interactions between aromatic rings (9). Unlike the Arg content, there was a clear relationship between the tyrosine contents in the catalytic domains of ChiA, ChiB, and ChiC and their thermostabilities. These results suggest that the increased flexibility related to the increased heat lability of ChiB could be explained by its tyrosine residue content. To clarify the strategy that ChiB uses to adapt to low temperatures, crystallization of ChiB is now being performed in our laboratory.

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