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Cloning, Sequencing, and Expression of the Gene Encoding *Clostridium paraputrificum* Chitinase ChiB and Analysis of the Functions of Novel Cadherin-Like Domains and a Chitin-Binding Domain

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The *Clostridium paraputrificum chiB* gene, encoding chitinase B (ChiB), consists of an open reading frame of 2,493 nucleotides and encodes 831 amino acids with a deduced molecular weight of 90,020. The deduced ChiB is a modular enzyme composed of a family 18 catalytic domain responsible for chitinase activity, two reiterated domains of unknown function, and a chitin-binding domain (CBD). The reiterated domains are similar to the repeating units of cadherin proteins but not to fibronectin type III domains, and therefore they are referred to as cadherin-like domains. ChiB was purified from the periplasm fraction of *Escherichia coli* harboring the *chiB* gene. The molecular weight of the purified ChiB (87,000) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, was in good agreement with the value (86,578) calculated from the deduced amino acid sequence excluding the signal peptide. ChiB was active toward chitin from crab shells, colloidal chitin, glycol chitin, and 4-methylumbelliferyl β-D-*N*,*N*′-diacetylchitobioside [4-MU-$(GlcNAc)_2$]. The pH and temperature optima of the enzyme were 6.0 and 45°C, respectively. The K_m and V_{max} values for 4-MU-$(GlcNAc)_2$ were estimated to be 6.3 μM and 46 μmol/min/mg, respectively. SDS-PAGE, zymogram, and Western blot analyses using antiserum raised against purified ChiB suggested that ChiB was one of the major chitinase species in the culture supernatant of *C. paraputrificum*. Deletion analysis showed clearly that the CBD of ChiB plays an important role in hydrolysis of native chitin but not processed chitin such as colloidal chitin.

Chitinase (EC 3.2.1.14) is a glycosyl hydrolase that catalyzes the degradation of chitin, an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine. Chitinases are present in a wide range of organisms, including bacteria, insects, viruses, plants, and animals, and play important physiological and ecological roles. On the basis of amino acid sequence homology, chitinases are divided into two unrelated families, families 18 and 19 of glycosyl hydrolases (15). Family 18 includes chitinases from bacteria, fungi, viruses, and animals and chitinases classified in class III or V from plants. On the other hand, family 19 includes almost exclusively plant chitinases classified in classes I, II, and IV but also a bacterial chitinase, *Streptomyces griseus* HUT 6037 chitinase C (32). To date, various chitinases were isolated from aerobic microorganisms such as *Bacillus circulans* (1, 55–57), *Serratia marcescens* (6, 13, 16, 20), an *Aeromonas* sp. (41, 51–53), an *Alteromonas* sp. (48), *Streptomyces plicatus* (37), *Streptomyces olivaceoviridis* (3, 36, 38), and *Janthiobacterium lividum* (12). Several genes encoding chitinases were cloned in *Escherichia coli* and characterized in detail along with their translated products (9, 12, 20, 36–38, 41, 43, 49, 56, 57). From these studies, chitinases were found to comprise two or more discrete domains, while the function of each domain has not yet been elucidated. Watanabe et al. reported that *B. circulans* ChiA1 has a chitin-binding domain (CBD) and two fibronectin type III (Fn3) domains in addition to a catalytic domain classified in family 18 (59). Deletion analysis indicated that a CBD but not Fn3 domains were responsible for binding of ChiA1 to insoluble chitins and further that the presence of a CBD in ChiA1 increased its hydrolytic activity on colloidal chitin. However, the function of Fn3 domains remains unclear. Many bacterial chitinases other than ChiA1, e.g., *Serratia marcescens* ChiA and ChiB, *Alteromonas* sp. Chi85, and *Aeromonas* sp. ChiII, consist of several conserved domains, while functions of those domains except for the CBD have not been clarified. Chitinases from a yeast, *Saccharomyces cerevisiae*, and a plant, *Nicotiana tabacum* (tobacco), also have been shown to consist of multidomains (18, 23), i.e., they contain CBDs that have no significant similarity to CBDs from bacterial chitinases. Although knowledge of chitinases and their genes from plants, animals, and aerobic microorganisms has been accumulating, there are no reports describing purification of chitinases or cloning of chitinase genes from anaerobic bacteria.

Clostridium paraputrificum is a strictly anaerobic, sporogenic, rod-like, gram-positive bacterium. We found that *C. paraputrificum* M21 isolated in this laboratory secreted chitinases into the culture medium when it was grown in a medium containing chitin as the sole carbon source (21). In this paper, we describe the cloning and sequencing of the *chiB* gene encoding a major chitinase expressed in the culture fluid of *C. paraputrificum* M21 and the characterization of its translated product. We also deal with the function of respective domains constituting the modular chitinase ChiB.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *C. paraputrificum* M21, which was isolated from soil, was used as the source of chromosomal DNA (21). This bacterium was cultivated at 37°C overnight in 100 ml of modified GS medium (21) with 1% glucose, chitin, or ball-milled chitin as a carbon source. The headspace in anaerobic culture vials was replaced with N_2 gas. Plasmids

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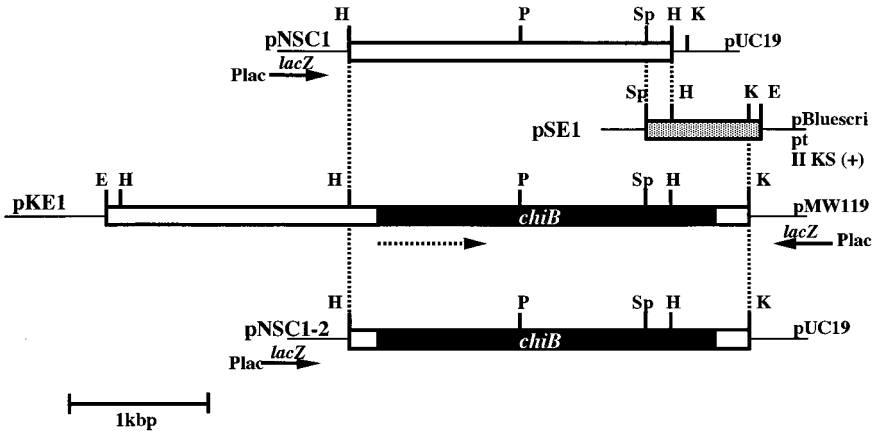

FIG. 1. Restriction maps of pNSC1, pSE1, pKE1, and pNSC1-2 containing the *chiB* gene. Thin lines and open bars correspond to vector plasmid DNAs and cloned DNA fragments, respectively. The coding region is represented by solid bars. A broken arrow indicates the direction of the transcription of *chiB*. H, *Hin*dIII; E, *Eco*RI; K, *Kpn*I; Hp, *Hpa*I; Hi, *Hin*cII; E22, *Eco*T22I; P, *Pst*I; E47, *Eco*47III; Sp, *Spe*I; S, *Sal*I.

pUC19 (TOYOBO, Tokyo, Japan), pBluescript II SK(−) and SK(+) (Stratagene), and pMW119 (Nippon Gene, Tokyo, Japan) were used as the cloning vectors. *E. coli* XL1-Blue {*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lac* [F′ *proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tet^r)]} was used as the cloning host for recombinant plasmids. *E. coli* JM109 {*supE44 hsdR17 recA1 endA1 gyrA96 thi relA1* Δ(*lac-proAB*) [F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]} was used as the host for pNSC1-2 to produce ChiB. *E. coli* M15(pREP4) (Qiagen) was used as the host for derivatives of plasmid pQE-30 (Qiagen), which provides proteins containing a six-His affinity tag at their N-termini. All *E. coli* strains were grown at 37°C on LB medium.

Construction of the gene library and screening of chitinase-producing recombinants. Chromosomal DNA of *C. paraputrificum* was isolated by the procedure of Silhavy et al. (42), partially digested with *Hin*dIII, and separated on a 0.7% agarose gel. DNA fragments with appropriate sizes were recovered from the agarose gel by the GeneClean (Bio 101, La Jolla, Calif.) procedure and ligated into the dephosphorylated *Hin*dIII site of pUC19. *E. coli* XL1-Blue was transformed with the ligation mixture by electroporation. Transformants were cultivated on LB agar plates containing ampicillin (80 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 50 μg/ml) at 37°C. White colonies carrying pUC19 with genomic DNA inserts were tested for chitin-degrading activity by the Congo red method (44) described originally for detection of endoglucanase activity. Carboxymethyl (CM) cellulose used in the original protocol was replaced with CM chitin (DAIICHI KOGYO, Kyoto, Japan) for detection of chitin-degrading activity. Colonies with clear zones against a red background were selected as chitinase-producing recombinants.

Cloning of the full-length *chiB* gene. Chromosomal DNA of *C. paraputrificum* was completely digested with *Eco*RI and *Kpn*I. To clone the full-length *chiB* gene, DNA fragments in the 4- to ~6-kb size range were recovered as described above and inserted between the *Eco*RI and *Kpn*I sites of pMW119 to generate pKE1 (Fig. 1). *E. coli* XL1-Blue was transformed with the ligation mixture and screened for the existence of the *chiB* gene by colony hybridization with a 2.4-kb *Hin*dIII fragment of pNSC1 as a probe.

DNA sequencing. Nucleotide sequencing was carried out on Applied Biosystems model 373A or Licor (Lincoln, Nebr.) model 4000L automated DNA sequencer, with appropriate dye primers and a series of subclones. The nucleotide sequence data was analyzed with GENETYX computer software (Software Development Co. Ltd., Tokyo, Japan). Homology searches in GenBank were carried out with a BLAST program.

Purification of ChiB. To place the full-length *chiB* gene under the control of the *lac* promoter, plasmid pNSC1-2 was constructed by replacing the *Pst*I-*Kpn*I fragment of pNSC1 with the *Pst*I-*Kpn*I fragment of pKE1 (Fig. 1). *E. coli* JM109 harboring pNSC1-2 was cultured in 1 liter of LB medium containing ampicillin (50 μg/ml) at 37°C for 12 h. Cells (1.7 g) were collected by centrifugation (8,000 × *g*, 5 min), and the periplasm fraction was prepared by the osmotic shock method (30). The periplasmic proteins were loaded onto a DEAE-Toyopearl 650 M column (1.0 by 20 cm; Tosoh, Tokyo, Japan) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) and eluted with a linear gradient of 0 to 1.0 M sodium chloride in the same buffer at flow rate of 2 ml/min. Active fractions were combined and applied to a MonoQ HR 5/5 column (0.5 by 5.0 cm; Pharmacia, Uppsala, Sweden) equilibrated with 10 mM potassium phosphate buffer (pH 7.0), and the enzyme was eluted with a linear gradient of 0 to 1.0 M sodium chloride at a flow rate of 0.5 ml/min. Active fractions were combined and used as the purified enzyme.

Enzyme and protein assays. 4-Methylumbelliferyl β-D-*N*,*N*′-diacetylchitobioside [4-MU-$(GlcNAc)_2$; Sigma] was dissolved in *N*,*N*′-dimethylformamide to a concentration of 10 mM and stored at −20°C. Chitinase activity was measured in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM 4-MU-$(GlcNAc)_2$ as a substrate by the modified procedure of O'Brien and Colwell (31). Reaction mixtures were incubated at 37°C for 10 min, and the amount of 4-methylumbelliferone released from the substrate was spectrofluorometrically measured with an excitation wavelength at 355 nm and an emission wavelength at 465 nm on a Hitachi (Tokyo, Japan) spectrofluorometer F-2000. Alternatively, chitinase activity was measured by the modified method of Schales (17) with glycol chitin (SEIKAGAKUKOGYO Co. Ltd., Tokyo, Japan), ball-milled chitin, colloidal chitin, or unprocessed chitin (crab shell; Nacalai tesque, Kyoto, Japan). Colloidal chitin was prepared by the method of Jeuniaux (19), and chitin purchased from Nacalai tesque was used as unprocessed chitin. One unit of chitinase activity was defined as the amount of enzyme which produces 1 μmol of 4-methylumbelliferone or 1 μmol of reducing sugar as an *N*-acetylglucosamine standard per min. The protein concentration was measured by the method of Bradford (5) with bovine serum albumin (Sigma) as a standard unless stated otherwise.

SDS-PAGE and zymogram analyses. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was done by the method of Laemmli (24). Zymogram analysis was performed as follows. Protein samples were added to SDS-PAGE sample buffer and heated at 100°C for 3 min. Proteins were separated with an SDS–8% polyacrylamide gel containing 0.1% glycol chitin, and the gel was immersed in refolding buffer (50 mM Tris-HCl buffer [pH 7.5], 5 mM 2-mercaptoethanol, 1 mM EDTA) at 4°C overnight. The gel was transferred to fresh refolding buffer, incubated at 37°C for 2 h to allow the refolded enzymes to hydrolyze glycol chitin, and then stained with 0.1% Congo red solution for 15 min; this was followed by washing with 1 M NaCl.

N-terminal amino acid sequencing. ChiB purified from a recombinant *E. coli* was fractionated by SDS-PAGE (24) and transferred onto an Immobilon-P^{SQ} transfer membrane (Millipore Corp., Bedford, Mass.) by electroblotting. The blotted protein was cut out from the membrane and subjected to N-terminal amino acid sequence analysis on an Applied Biosystem model 476A protein sequencer.

Preparation of antiserum and immunoblotting. Antiserum against the purified ChiB was raised in a BALB/c mouse. ChiB purified from *E. coli* and proteins in the culture supernatant of *C. paraputrificum* grown on ball-milled chitin as a carbon source were separated by SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane by use of an electroblotting apparatus, Sartoblot II (Sartrius, Gottingen, Germany) (47). Immunoreactive proteins were detected on a Western blot by an enzyme immunoassay using peroxidase-conjugated goat anti-mouse immunoglobulins (Tago Inc., Burlingame, Calif.) and 3,3′-diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan).

Construction of six-His-tagged ChiB and its truncated derivatives. The primers listed in Fig. 2A were used for the PCR-aided construction of plasmids that encode the full-length ChiB and its truncated derivatives containing a six-His tag in their N termini. The *Bam*HI and *Sal*I sites indicated in Fig. 2A were used for insertion of the PCR products from the primer combinations 1+2, 3+4, 3+5, and 3+6 between the *Bam*HI and *Sal*I sites of pQE-30, thus yielding pChiB-Δ2RC, pR1, pR1R2, and pR1R2C, respectively. Plasmids pChiB-Δ2RC, pR1, pR1R2, and pR1R2C encode the catalytic domain, domain R1, the tandem repeat R1-R2, and a stretch of the tandem repeat R1-R2 and CBD, respectively (Fig. 2B). Fidelity of all of the cloned DNA fragments was confirmed by DNA sequencing. The DNA fragments cloned in pR1, pR1R2, and pR1R2C were

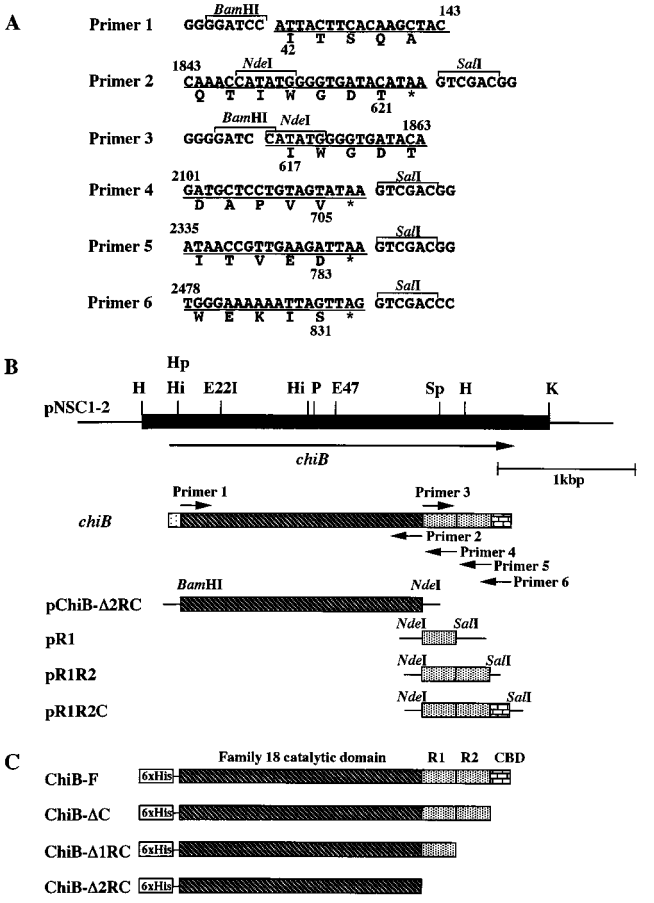

FIG. 2. (A) PCR primers used for amplification of DNA fragments encoding respective domains; (B) plasmids containing the amplified fragments; (C) domains of ChiB protein and its derivatives. Primer segments complementary to *chiB* sequences at the domain borders are underlined, and the translation of these parts of the primers is printed below the nucleotide sequence. Numbers beneath amino acid positions indicate the locations within the ChiB sequence shown in Fig. 3. Asterisks indicate stop codons in panel A. Thin lines correspond to vector plasmid DNA. The size and transcriptional orientation of the *chiB* gene are indicated by a horizontal arrow in panel B.

recovered after digestion of these plasmids with *Nde*I and *Sal*I and inserted between the same recognition sites of pChiB-Δ2RC, yielding pChiB-Δ1RC, pChiB-ΔC, and pChiB-F. The *Nde*I recognition sites were introduced into the DNA fragments carrying parts of *chiB* to connect two DNA fragments, resulting in no amino acid displacement. Plasmid pChiB-Δ1RC encodes the truncated enzyme lacking R2 and CBD, and pChiB-ΔC encodes the enzyme lacking CBD. Plasmid pChiB-F encodes the full-length ChiB with a six-His tag. These proteins produced by *E. coli* M15 recombinants were purified by a Ni-NTA spin kit (Qiagen).

Chitin-binding assay. The standard binding assay mixture (total volume, 800 μl) contained 50 μg of protein and 10 mg of insoluble polysaccharides, such as chitin, colloidal chitin, chitosan, Avicel, or phosphoric acid-swollen cellulose in 10 mM potassium phosphate buffer (pH 6.0). After the mixture was incubated for 1 h on ice with occasional stirring, the supernatant containing unadsorbed protein was separated from insoluble polysaccharide by centrifugation and the protein concentration in the supernatant was estimated by the method of Lowry et al. (26). The amount of adsorbed protein was estimated from the difference between the amount of protein added to the mixture and that in the supernatant.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB001874.

RESULTS

Cloning of the *chiB* gene from *C. paraputrificum*. Approximately 5,700 transformants were obtained from the *C. paraputrificum* gene library and screened for chitinase productivity by the Congo red method. As a result, seven clones were selected as chitinase-productive recombinants. The plasmids isolated from these clones appeared to contain at least two different chitinase genes judging from their restriction maps (data not shown). Among them, pNSC1, which had the smallest insert (2.4 kb) and conferred the highest chitinase activity on *E. coli*, was chosen for further investigation (Fig. 1). Although the chitinase gene *chiB* contained in pNSC1 produced an active enzyme in *E. coli*, DNA sequencing of this gene revealed that the open reading frame (ORF) of *chiB* extended beyond the 3′ end of the genomic DNA insert. Since no recombinant plasmid which contained the complete *chiB* gene was found in our *C. paraputrificum* genomic DNA library, gene walking PCR was carried out to isolate a DNA fragment downstream of pNSC1 (data not shown) and resulting plasmid pSE1 was constructed from a 0.9-kb PCR product and pBluescript II KS(+) (Fig. 1). Analysis of the DNA sequence of pSE1 indicated that the insert DNA contained the region encoding the C terminus of ChiB. It is well-known that PCR products often experience unexpected mutations. Therefore, we attempted again to clone the full-length *chiB* gene from chromosomal DNA of *C. paraputrificum* M21. Since the DNA sequence of pSE1 and Southern hybridization analysis suggested that a 5.1-kb *Eco*RI-*Kpn*I fragment carried the complete ORF of *chiB*, we cloned this fragment into pMW119 to yield pKE1 (Fig. 1).

Nucleotide sequence of the *chiB* gene. Figure 3 shows the complete nucleotide sequence of the *chiB* structural gene along with its flanking region. The ORF of *chiB* consists of 2,493 nucleotides encoding a protein of 831 amino acids with a predicted molecular weight of 90,020. The putative initiation codon ATG was preceded at a spacing of 9 bp by a potential ribosome-binding sequence (5′-AGGAGG-3′) which was homologous to the consensus Shine-Dalgarno sequence (40). The sequences ATAACG and TATAAT, with 17-bp spacing, showing certain homology to the −35 and −10 *E. coli* promoter consensus sequences, TTGACA and TATAAT, respectively, were identified upstream of the coding region (39). A possible transcription terminator that consists of an 11-bp palindrome, corresponding to an mRNA hairpin loop with a ΔG of −23 kcal/mol (95.0 kJ/mol) (7), followed by four T's was found downstream of the TAA termination codon. This structure is similar to the rho factor-independent terminator of *E. coli.*

Amino acid sequence of ChiB. The deduced protein sequence of ChiB was compared with entries in the GenBank and SWISS-PROT databases. The N-terminal moiety of ChiB showed sequence homology with enzymes classified into family 18 of glycosyl hydrolases such as *Alteromonas* sp. Chi85 (sequence identity, 30.1%), *B. circulans* ChiA1 (24.7%), *B. circulans* ChiC (32.4%), *J. lividum* Chi69 (27.7%), *Serratia marcescens* ChiA (28.3%) and ChiB (25.4%), *Streptomyces plicatus* Chi63 (20.0%), and *Streptomyces lividans* ChiC (21.4%). Alignment of family 18 catalytic domains is shown in Fig. 4.

As shown in Fig. 5, the C-terminal portion of ChiB had significant homology with CBDs identified in some bacterial chitinases (12, 13, 32, 41, 43, 49, 51, 56, 57) and C-terminal domains of the *Bacillus* sp. cellulases CelA and CelB (10). Among these sequences, aromatic amino acid residues Trp and Tyr are fully conserved in positions 7 (789 in ChiB), 13, and 56 of the *C. paraputrificum* sequence and are highly conserved in positions 27, 32, and 33 except for some replacements by various amino acid residues.

Sequence analysis by dot matrix plot showed the presence of reiterated sequences of 78 amino acids with a spacing of 6 amino acids between the catalytic domain and the CBD. These sequences that showed 61.8% identity were referred to as

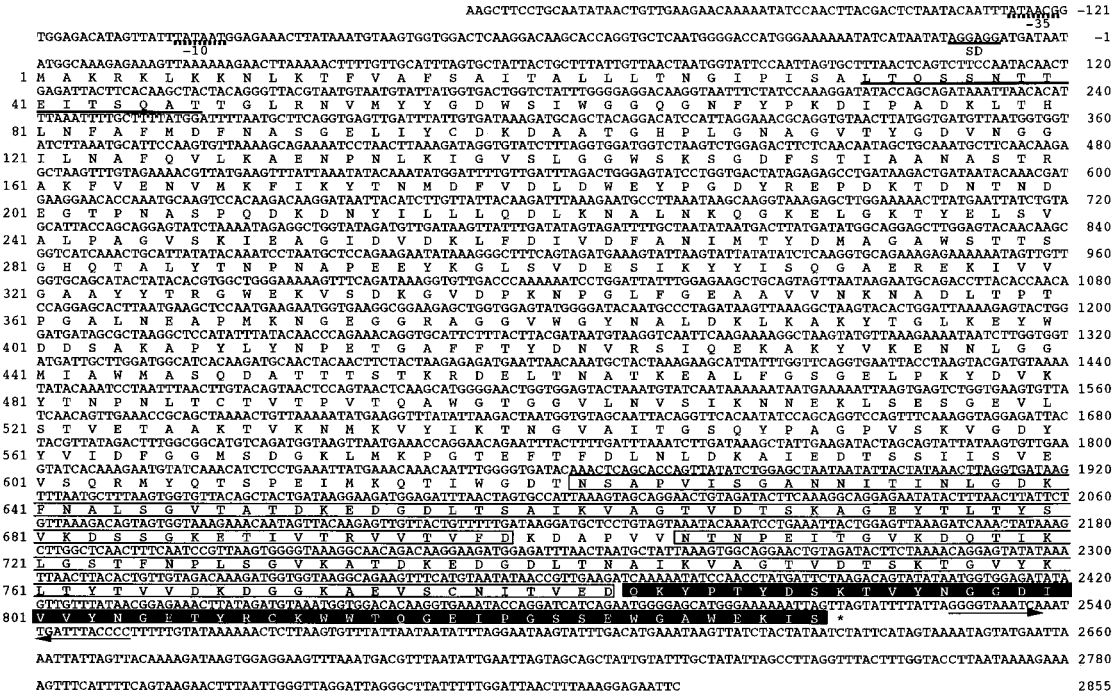

FIG. 3. Nucleotide sequence of the *chiB* gene and deduced amino acid sequence of the gene product. The −35 and −10 regions of a possible promoter sequence are indicated by broken underlines. The possible Shine-Dalgarno (SD) sequence is underlined. The N-terminal amino acids determined by protein sequencer 476A are indicated by underlines. Horizontal arrows indicate inverted sequences. Reiterated segments of ChiB are boxed, and the CBD is shaded.

segment R1 (Asn-622 through Asp-699) and segment R2 (Asn-706 through Asn-783). Comparing the amino acid sequences of R1 and R2 with entries in reiterated sequences (77 amino acids each) significantly homologous with R1 and R2 were found in *Vibrio harveyi* ChiA (accession no. U81496) as shown in Fig. 6A. Two 77-amino-acid sequences (Gly-353 through Ser-429 and Gln-430 through Asp-506) of ChiA showed 42.1 to 50.0% identity with the sequences of segments R1 and R2. Interestingly, these reiterated sequences showed some homology with sequences of cadherin domains conserved in cadherins, especially those of cadherin-related protein encoded by the *fat* gene of *Drosophila melanogaster* (27). Segments R1 and R2 and reiterated segments of *V. harveyi* ChiA are aligned with some cadherin domains from the *fat* gene product in Fig. 6B.

Purification and characterization of ChiB. The gene product of *chiB* was purified 17-fold from the periplasm fraction of *E. coli* JM109(pNSC1-2) with a recovery of 10% by DEAE-Toyopearl 650 M and MonoQ HR5/5 column chromatographies. The final preparation gave a single band on SDS-PAGE, and the molecular weight of the enzyme was estimated to be around 87,000 (Fig. 7A and B, lanes 2), which is good agreement with the value (86,578) estimated from the deduced amino acid sequence of ChiB excluding the signal peptide. The N-terminal amino acid sequence of the purified ChiB was identified as Leu-Thr-Gln-Ser-Ser-Asn-Thr-Thr-Glu-Ile-Thr-Ser-Gln-Ala-Thr, which was found in the deduced amino acid sequence of ChiB at amino acid positions 33 to 47 (Fig. 3). Therefore, 32 N-terminal amino acids of the premature ChiB, which has a typical signal peptide characteristic of bacterial extracellular enzymes (54), appear to function in secretion of ChiB into the periplasmic space of *E. coli.*

We observed that ChiB expressed in *E. coli* sometimes underwent proteolysis by an *E. coli* protease(s) during cultivation of cells and purification of the enzyme and it turned into the smaller form (ChiB-S). The N-terminal amino acid sequence of ChiB-S, determined to be Ile-Thr-Ser-Gln-Ala-Thr, indicated that proteolysis occurred between amino acid positions 41 and 42 shown in Fig. 3. The chitinase activity of ChiB-S was as same as that of the mature ChiB (data not shown). For general characterization of the enzymatic properties, the mature form of ChiB was used.

ChiB has high activity toward 4-MU-$(GlcNAc)_2$ (42.1 U/mg) and relatively low activities toward chitin (0.75 U/mg), glycol chitin (9.75 U/mg), and colloidal chitin (6.74 U/mg). Paper chromatography analysis indicated that major hydrolysis products of chitin and colloidal chitin by ChiB were a monomer and a dimer of *N*-acetylglucosamine (data not shown). The initial rates of reaction were measured at 37°C in various concentrations of 4-MU-$(GlcNAc)_2$. From a Lineweaver-Burk plot, the K_m and V_{max} values were estimated to be 6.3 μM and 46 μmol/min/mg of enzyme, respectively. The enzyme activity was completely inhibited by 1 mM $HgCl_2$ and was partly inhibited by a 1 mM concentration of $CuCl_2$, $FeCl_3$, or $AlCl_3$ but not affected by the 1 mM $MgCl_2$, $CaCl_2$, or EDTA tested. The optimum pH for activity was found to be 6.0 when the enzyme activity was assayed in Britton-Robinson's universal buffer solution (40 mM phosphoric acid, 40 mM boric acid, 40 mM acetic acid; pH adjusted with NaOH) at various pHs. The enzyme was stable in the range of pH 6 to 9 when incubated in the same buffer solution at various pHs at 25°C for 24 h. The enzyme was optimally active at 45°C and stable under heat treatment at 40°C for 10 min.

Immunological detection of ChiB. As shown in Fig. 7A and B, SDS-PAGE and zymogram analyses indicated that some proteins with chitinase activity were produced in the culture supernatant of *C. paraputrificum* grown on ball-milled chitin as a carbon source and further that among these proteins a chitinase with a molecular weight of 87,000 was a major protein in

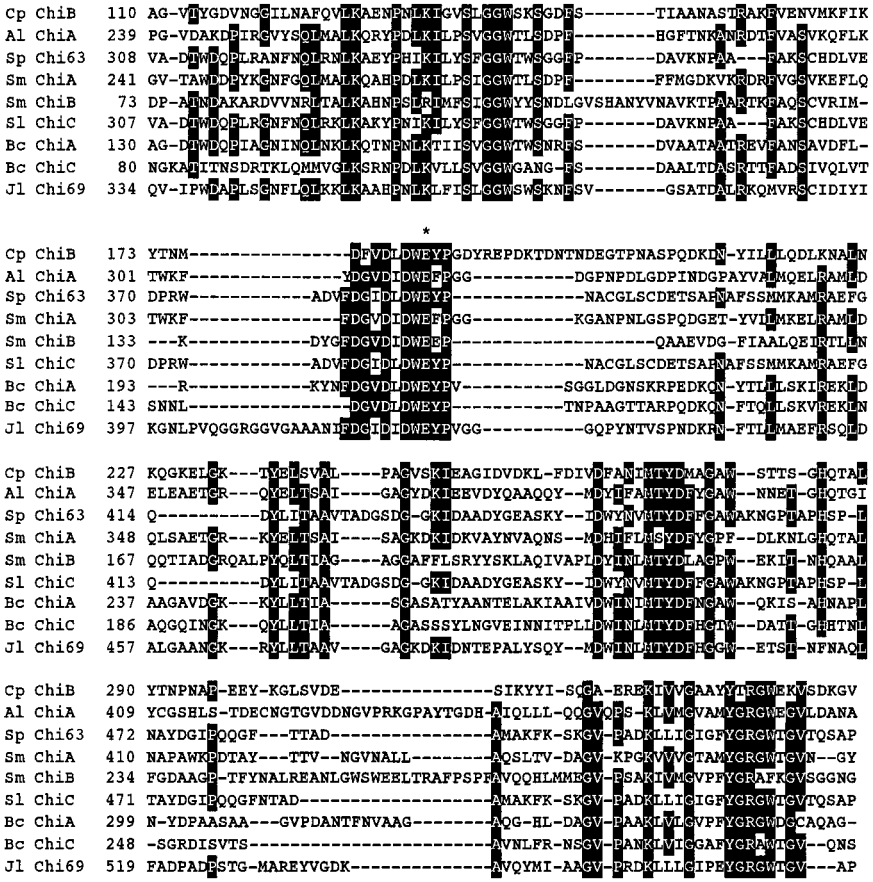

FIG. 4. Alignment of family 18 catalytic domains of *C. paraputrificum* (Cp) ChiB, *Alteromonas* (Al) Chi85, *Streptomyces plicatus* (Sp) Chi63, *Serratia marcescens* (Sm) ChiA and ChiB, *Streptomyces lividans* (Sl) ChiC, *B. circulans* (Bc) ChiA1 and ChiD, and *J. lividum* (Jl) Chi69. A Glu residue identified as a proton donor is marked with an asterisk. Amino acids which are conserved in at least six of the nine sequences are shaded. Dashes indicate gaps left to improve alignment. Numbers refer to amino acid residues at the start of the respective lines; all sequences are numbered from Met-1 of the peptide.

the supernatant. In Western blotting analysis (Fig. 7C), the antiserum directed against ChiB reacted with a protein with a molecular weight of 87,000 in the culture supernatant of *C. paraputrificum*. These results suggest that ChiB is a major chitinase in *C. paraputrificum.*

Function of R1, R2, and CBD. We constructed the truncated derivatives of ChiB as fusion proteins with a six-His tag to facilitate purification of the enzymes (Fig. 2). To avoid proteolytic removal of the six-His tag from these proteins, a portion of ChiB-S was fused to the six-His tag. Molecular weights of

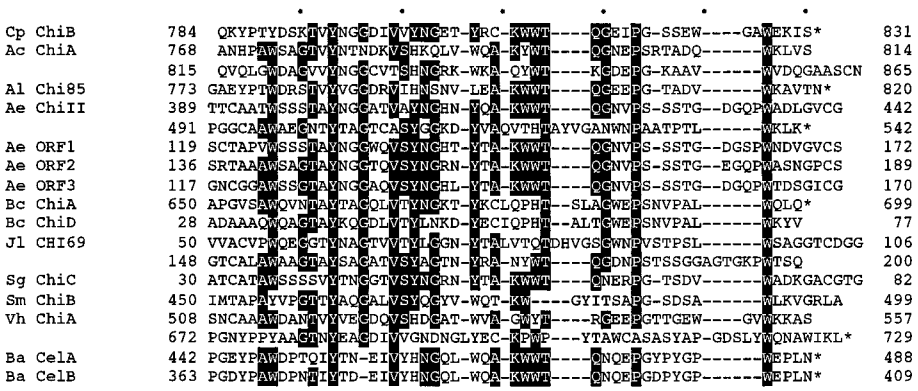

FIG. 5. Alignment of CBDs of *C. paraputrificum* (Cp) ChiB, *Aeromonas caviae* (Ac) ChiA, *Aeromonas* sp. (Ae) ChiII, ORF1, ORF2, and ORF3, *Alteromonas* sp. (Al) Chi85, *B. circulans* (Bc) ChiA1 and ChiD, *J. lividum* (Jl) Chi69, *Streptomyces griseus* HUT 6037 (Sg) ChiC, *Serratia marcescens* (Sm) ChiB, *V. harveyi* (Vh) ChiA, and *Bacillus* sp. strain N-4 (Ba) cellulases CelA and CelB. Amino acids which are conserved in at least 10 of the 19 sequences are shaded. Dashes indicate gaps left to improve alignment. Numbers refer to amino acid residues at the start or the end of the respective lines; all sequences are numbered from Met-1 of the peptide.

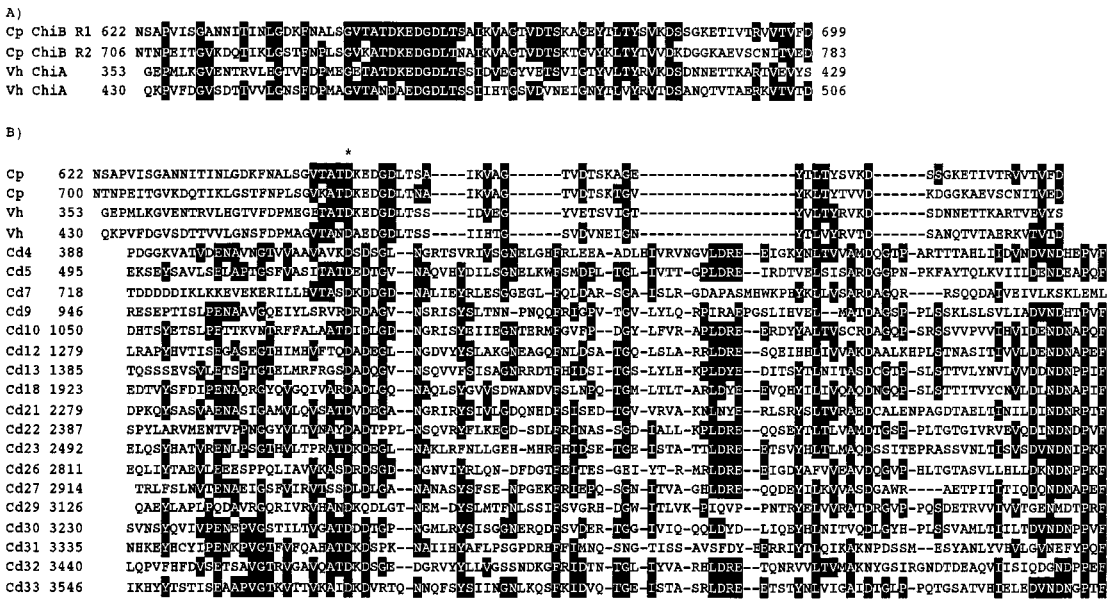

FIG. 6. (A) Alignment of reiterated segments found in *C. paraputrificum* (Cp) ChiB and *V. harveyi* (Vh) ChiA; (B) alignment of the sequences shown in panel A with cadherin domains (Cd) of tumor suppressor (Fat protein) of *D. melanogaster*. Of 34 tandem cadherin domains in Fat protein, 18 were aligned with the R1 and R2 segments of ChiB and reiterated sequences of *V. harveyi* ChiA. Amino acids which are conserved in at least 3 of the 4 sequences in panel A and at least 8 of the 22 sequences in panel B are shaded. Dashes indicate gaps left to improve alignment. Numbers refer to amino acid residues at the start of the respective lines; all sequences are numbered from Met-1 of the peptide. An Asp residue identified as Ca^{2+}-binding site of uvomorulin is marked with an asterisk.

the truncated chitinases purified from *E. coli* recombinants were consistent with the values calculated from their sequences, i.e., 63,263 for ChiB-Δ2RC, 71,895 for ChiB-Δ1RC, 80,055 for ChiB-ΔC, and 85,616 for ChiB-F. To confirm that the C-terminal domain of ChiB homologous with bacterial CBDs has chitin-binding activity, the truncated chitinases were subjected to a chitin-binding assay. As shown in Table 1, ChiB-F, which contains the C-terminal domain (i.e., the CBD), bound to chitin to a great extent. Deletion of the CBD (ChiB-ΔC) markedly reduced chitin-binding activity of ChiB-F. However, further truncation, i.e., removal of R1 and R2 (ChiB-Δ2RC and ChiB-Δ1RC) did not decrease its chitin-binding activity any further. These results clearly indicate that the C-terminal domain, which is homologous to the CBD of bacterial chitinases, plays a major role in binding of ChiB to chitin but that the catalytic domain itself has some affinity for the substrate. Binding of ChiB derivatives devoid of CBD is not caused by nonspecific adsorption, because bovine serum albumin adsorbed negligibly onto the same substrates. In addition to chitin, ChiB shows a significant affinity for cellulosic materials such as Avicel and phosphoric acid-swollen cellulose (Table 1). The reproducibility of the binding assay with these polysaccharides was high; i.e., values of the amount of adsorbed proteins were reproducible to about 5 to 11% for the respective polysaccharides on repeated runs.

The effects of removal of the CBD and segments R1 and R2 from ChiB on hydrolytic activity toward insoluble substrates were examined with chitin and colloidal chitin as substrates. As shown in Fig. 8A, when unprocessed chitin was used as a substrate, all truncated enzymes devoid of the C-terminal CBD (i.e., ChiB-Δ2RC, ChiB-Δ1RC, and ChiB-ΔC) exhibited only half the activity exhibited by the parental enzyme ChiB-F. By contrast, the truncated enzymes fully retained their activity toward colloidal chitin as compared to that of ChiB-F (Fig. 8B). Furthermore, the truncation of R1, R2, and the CBD enhanced their enzyme activity on 4-MU-$(GlcNAc)_2$ by 30 to 40% as compared to the activity of ChiB-F (data not shown).

DISCUSSION

Many chitinases and their genes from aerobic microorganisms, plants, viruses, and animals have been investigated. On the other hand, there have not been any detailed studies on chitinases from anaerobic bacteria although it was known that some anaerobic bacteria produced a chitinase(s) and utilized chitin as a carbon and energy source (34). Therefore, this is the first report of the characterization of a chitinase gene along with its translated product from an anaerobic chitinolytic bacterium, *C. paraputrificum* M21. The *chiB* gene encodes a chitinase composed of an N-terminal signal peptide, a catalytic domain of family 18, reiterated segments R1 and R2, and a

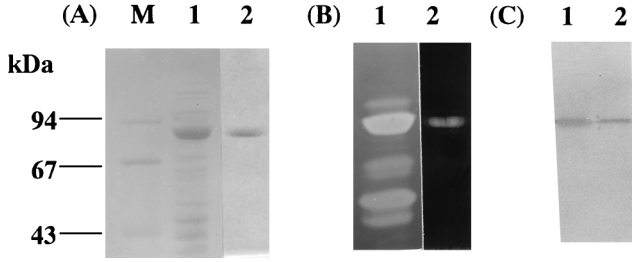

FIG. 7. SDS-PAGE, zymogram, and Western blot analyses of proteins in the culture supernatant of *C. paraputrificum* and ChiB purified from *E. coli*(pNSC1-2). (A) Gel stained with Coomassie brilliant blue R-250; (B) chitinase activity detected on a gel containing 1% glycol chitin; (C) ChiB proteins detected by Western blot analysis with a polyclonal mouse antiserum raised against the purified ChiB. Lanes: M, size markers; 1, culture supernatant of *C. paraputrificum* grown with ball-milled chitin as a carbon source; 2, ChiB purified from *E. coli*.

TABLE 1. Binding specificity of ChiB-F and its truncated ChiB derivatives

Substrate	Amt of adsorbed protein (μg)[a]			
	ChiB-F	ChiB-Δ2RC	ChiB-Δ1RC	ChiB-ΔC
Chitin	41.1	22.0	16.3	16.1
Colloidal chitin	47.1	32.6	32.0	27.6
Chitosan	14.8	ND[b]	ND	ND
Avicel	36.9	ND	ND	ND
Phosphoric acid-swollen cellulose	39.5	ND	ND	ND

[a] Fifty micrograms of a protein was used in each assay.
[b] ND, not determined.

CBD. Immunological analysis suggests that ChiB is a major chitinase in the culture supernatant of *C. paraputrificum* grown with chitin.

All bacterial chitinases reported to date except *Streptomyces griseus* ChiC belong to family 18 of glycosyl hydrolase (15). Among them, the crystal structure of *Serratia marcescens* ChiA has been determined (35), and based on the results of site-directed mutagenesis experiments (50, 58), Glu-315 has been identified as the proton-donating residue involved in catalysis. This residue is conserved in all catalytic domains in family 18. In *C. paraputrificum* ChiB, a Glu residue was conserved as Glu-184.

Removal of the CBD from ChiB significantly decreased the enzyme activity of ChiB toward unprocessed chitin irrespective of the presence or absence of reiterated segments R1 and R2; this was accompanied by a drastic decrease in the chitin-binding activity. These results indicate that this CBD plays an important role in the hydrolysis of the insoluble substrate (Fig. 8A). By contrast, ChiB-ΔC, which is devoid of CBD, retained the original activity of ChiB toward colloidal chitin or inversely exhibited an increased activity toward 4-MU-$(GlcNAc)_2$, a small synthetic substrate. Similar phenomena were reported for many cellulases (2); e.g., when a cellulose-binding domain was removed from a *Bacillus subtilis* endoglucanase, the truncated enzyme was fourfold more active on CM cellulose but fivefold less active on insoluble cellulose (14). One of the functions of the cellulose-binding domain is thought to be the disruption of noncovalent interactions between adjacent molecules of crystalline cellulose; i.e., a cellulose-binding domain supplies a catalytic domain with amorphous regions of cellulose, which is an easily digestible substrate for cellulases. Although the precise structure of native chitin is not exactly known, it is assumed that crystallinity of colloidal chitin is lower than that of unprocessed chitin. Therefore, CBDs in various chitinases might convert tightly packed chitin into a more amorphous structure in a manner similar to that of a cellulose-binding domain.

The tertiary structure of CBD has not yet been determined, and the amino acid sequence of the CBD of ChiB is not related to those of cellulose-binding domains reported to date. However, since the CBD has a certain affinity for cellulosic materials and homologous sequences are found in cellulases (Fig. 5), CBDs and cellulose-binding domains are expected to both have the mechanism of binding to their substrates and the overall feature of tertiary structures. Cellulose-binding domains can be grouped into 10 families on the basis of amino acid sequence similarity (45). Among them, tertiary structures of three different cellulose-binding domains with high affinity for crystalline cellulase were solved by X-ray analysis or nuclear magnetic resonance spectroscopy, i.e., cellulose-binding domains from *Trichoderma reesei* CBHI (family I) (22), *Cellulomonas fimi* Cex (family II) (60), and *Clostridium thermocellum* CipB (family III) (46). Based on a comparison of the structures of these cellulose-binding domains, a general model for the binding of cellulose-binding domains to crystalline cellulose was presented (46). This model included the following observations: specific types and combinations of amino acids appear to interact selectively with glucose moieties positioned on three adjacent chains of the cellulose surface; the major interaction is characterized by the planar strip of aromatic residues, which aligns along one of the cellulose chains; in addition, polar amino acid residues anchor the cellulose-binding domain molecule to two other adjacent chains of crystalline cellulose. In all cellulose-binding domains belonging to each family, Tyr and Trp residues and several polar hydrogen-bonding groups are well conserved (45). In CBDs of ChiB and other chitinases, Tyr and Trp residues are highly conserved (Fig. 5), suggesting that these aromatic side chains are involved in stacking against the pyranosyl rings of *N*-acetylglucosamine residues in chitin. The fact that the family III cellulose-binding domain of *Clostridium cellulovorans* CbpA has a high affinity for chitin may support that the mechanisms of binding to the substrates are common for CBDs and cellulose-binding domains.

Deletion of the CBD from ChiB did not affect the enzyme activity toward colloidal chitin. By contrast, Watanabe et al. observed that intact ChiA1 from *B. circulans* had a twofold-stronger activity toward colloidal chitin than the truncated derivatives of ChiA1 devoid of a CBD (59). Although the binding abilities of ChiB-F and intact ChiA1 toward colloidal chitin are on a similar level, ChiB derivatives devoid of a CBD have a higher binding ability for colloidal chitin than ChiA1 derivatives devoid of a CBD do (59). These observations suggest that the catalytic domain of ChiB itself has a strong affinity toward colloidal chitin, and therefore, ChiB without a CBD may retain hydrolytic activity on colloidal chitin (Fig. 8B).

C. paraputrificum ChiB contains reiterated segments R1 and R2 between the N-terminal catalytic domain and the C-terminal CBD. This domain organization is similar to that of *B. circulans* WL-12 ChiA1, although R1 and R2 are not homologous to the repeated segments of 95 amino acid residues in ChiA1. Two 95-residue tandem repeats in ChiA1 were named the fibronectin type III-like domain (Fn3) because of their similarity to fibronectin type III domains in the fibronectin of animal cells, and Fn3 modules are now known to be conserved

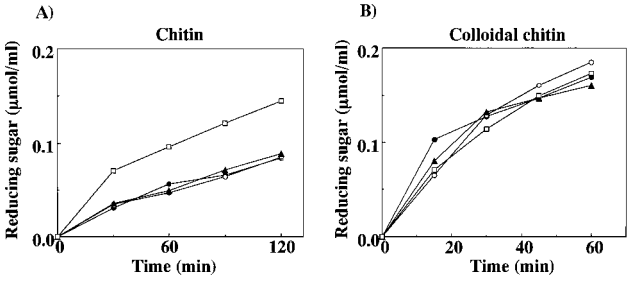

FIG. 8. Hydrolytic activity of ChiB and its truncated derivatives toward unprocessed chitin (A) and colloidal chitin (B). (A) Reaction mixtures contained 10 mg of chitin and 0.25 nmol of each chitinase in 400 μl of 10 mM potassium phosphate buffer (pH 6.0). Reactions were done at 37°C for 2 h, and the amount of reducing sugar generated was measured by the modified Schales procedure. (B) Reaction mixtures contained 1 mg of colloidal chitin and 0.05 nmol of each chitinase in 400 μl of 10 mM potassium phosphate buffer (pH 6.0). Reactions were done at 37°C for 1 h, and the amount of reducing sugar generated was measured by the same procedure. Symbols: ●, ChiB-Δ2RC; ○, ChiB-Δ1RC; ▲, ChiB-ΔC; □, ChiB-F.

in several glycosyl hydrolases such as chitinases (3, 9, 37, 56, 57), cellulases (11, 28), and amylases/pullulanase (29) from prokaryotes. Fibronectin is a multifunctional extracellular matrix and plasma protein and plays a central role in cell adhesion (4, 8, 25). On the basis of a systematic screen of a protein database, Bork and Doolittle concluded that bacterial Fn3 modules were initially acquired from an animal source and are being spread further by horizontal transfers between distantly related bacteria (4, 8, 25). On the other hand, R1 and R2, composed of 78 amino acids in *C. paraputrificum* ChiB, have no sequence homology with Fn3 but some homology with cadherin domains of the cadherin-related tumor suppressor protein (Fat) from *D. melanogaster*. Cadherins, all of which contain conserved cadherin domains of ca. 130 amino acids, are cell membrane proteins of animal cells and play an essential role in Ca^{2+}-dependent cell adhesion in contrast to Fn3, which is involved in Ca^{2+}-independent cell adhesion. A Ca^{2+}-binding site identified in uvomorulin (33), a member of the cadherin superfamily, and some other residues highly conserved in cadherins are also conserved in R1 and R2 and reiterated sequences of *V. harveyi* ChiA (Fig. 6B). Therefore, we propose to refer to the reiterated segments found in ChiB and *V. harveyi* ChiA as the cadherin-like domain. Although careful examination is necessary to conclude that the bacterial occurrences of cadherin-like domains are the result of acquisition by a bacterium of an animal gene but not of convergent evolution, it must be that cadherin-like domains are spread by horizontal transfers among many bacterial enzymes in the same way as Fn3 domains are.

To investigate the function of cadherin-like domains and the CBD, the truncated enzymes were constructed and examined for chitin-binding activity and enzyme activity. Although the importance of CBD was clearly demonstrated for binding of the enzyme to chitin and hydrolysis of the substrate with higher crystallinity, the function of cadherin-like domains remained unclear. Cadherin-like domains might act as a linker to maintain an optimal distance and orientation between the catalytic domain and CBD since cadherin-like domains by themselves did not have chitin-binding activity. Further studies are necessary to clarify the true function of the cadherin-like domains in ChiB.

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