The third chitinase gene (*chiC*) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases

Kazushi SUZUKI*, Mayumi TAIYOJI*, Noriko SUGAWARA*, Naoki NIKAIDOU*†, Bernard HENRISSAT‡ and Takeshi WATANABE*†[1]

*Department of Biosystem Science, Graduate School of Science and Technology, Niigata University, 8050 Ikarashi-2, Niigata 950-2181, Japan, †Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, 8050 Ikarashi-2, Niigata 950-2181, Japan, and ‡Architecture et Fonction des Macromolècules Biologiques, AFMB-CNRS IFR1, 31 Chemin Joseph Aiguier, F-13402 Marseille cedex 20, France

The third chitinase gene (*chiC*) of *Serratia marcescens* 2170, specifying chitinases C1 and C2, was identified. Chitinase C1 lacks a signal sequence and consists of a catalytic domain belonging to glycoside hydrolase family 18, a fibronectin type III-like domain (Fn3 domain) and a C-terminal chitin-binding domain (ChBD). Chitinase C2 corresponds to the catalytic domain of C1 and is probably generated by proteolytic removal of the Fn3 and ChBDs. The loss of the C-terminal portion reduced the hydrolytic activity towards powdered chitin and regenerated chitin, but not towards colloidal chitin and glycol chitin, illustrating the importance of the ChBD for the efficient hydrolysis of crystalline chitin. Phylogenetic analysis showed that bacterial family 18 chitinases can be clustered in three subfamilies which have diverged at an early stage of bacterial chitinase evolution. *Ser. marcescens* chitinase C1 is found in one subfamily, whereas chitinases A and B of the same bacterium belong to another subfamily. Chitinase C1 is the only *Ser. marcescens* chitinase that has an Fn3 domain. The presence of multiple, divergent, chitinases in a single chitinolytic bacterium is perhaps necessary for efficient synergistic degradation of chitin.

Keywords: chitin-binding domain, fibronectin type III, hydrophobic cluster analysis, phylogenetic analysis.

INTRODUCTION

Chitinases (EC 3.2.1.14) hydrolyse the β-1,4-linkages in chitin, the second most abundant biopolymer in nature next to cellulose. The chitinases so far sequenced are classified into two different families based on the amino acid sequence similarity of their catalytic domains. These form families 18 and 19 in the family classification system of glycoside hydrolases [1]. Family 18 contains chitinases from bacteria, fungi, viruses and animals, and some plant chitinases. On the other hand, family 19 contains only plant chitinases and the recently identified *Streptomyces griseus* chitinase C [2]. The chitinases of the two different families do not share amino acid sequence similarity, have completely different three-dimensional (3D) structures [3–5] and molecular mechanisms, and are therefore likely to have evolved from different ancestors.

Bacterial chitinases generally consist of multiple functional domains, such as chitin-binding domains (ChBDs) and fibronectin type III-like domains (Fn3 domains), linked to the catalytic domain. The importance of the ChBD in the degradation of insoluble chitin has been demonstrated for some bacterial chitinases [6–9]. Many bacteria, including *Bacillus circulans*, *Strep. lividans*, *Aeromonas* sp. and *Serratia marcescens* have been shown to produce multiple chitinases from different genes [10–14], and the efficient degradation of chitin is assumed to be achieved by the combined action of the multiple chitinases.

Ser. marcescens is an efficient biological degrader of chitin and one of the most extensively studied chitinolytic bacteria. The *chiA* and *chiB* genes, encoding chitinases A and B of four different strains of *Ser. marcescens*, QMB1466 [13,14], BJL200 [15,16], KCTC2172 [17] and 2170 [18], have been cloned and sequenced. Nucleotide and deduced amino acid sequences of the *chiA* and *chiB* genes of these strains are very similar to each

other. The 3D-structure of chitinase A from one of the strains, QMB1466, has been reported [3]. In addition to the *chiA* and *chiB* genes, the nucleotide sequence encoding the 52 kDa chitinase gene of *Ser. marcescens* KCTC2172 has been reported recently [19]. To initiate a genetic analysis of chitin degradation by bacteria we have chosen *Ser. marcescens* 2170 as a model organism, since this strain produces higher levels of chitinase activity and is more amenable to genetic analysis than the more extensively studied strain QMB1466. As shown in our previous report [18], strain 2170 releases a relatively limited number of proteins into the culture medium when grown in the presence of chitin. The proteins detected in the culture supernatant include four chitinases (A, B, C1 and C2) and a 21 kDa chitin-binding protein (CBP21) lacking chitinase activity [20]. Chitinases C1 and C2 probably correspond to the chitinolytic proteins of *Ser. marcescens* QMB1466 with molecular masses of 48 and 36 kDa [21], but it is unclear whether these proteins are derived from their own gene(s) or are proteolytic derivatives of chitinase(s) A and/or B.

In this report we describe the structure of the *chiC* gene for chitinases C1 and C2 and show that they are not proteolytic derivatives of chitinase(s) A and/or B. Chitinase C1 is the only *Ser. marcescens* chitinase that has an Fn3 domain, and chitinase C2 is a derivative of chitinase C1 generated by removal of the Fn3 domain and the C-terminal ChBD.

EXPERIMENTAL

Bacterial strains, plasmids and culture conditions

Ser. marcescens 2170 [22] was grown at 30 °C in a yeast extract-supplemented minimal (YEM) medium [23] containing 0.5% colloidal chitin for chitinase production and was grown in Luria–Bertani (LB) medium for chromosomal DNA extraction.

Escherichia coli JM109 carrying pUC119 and its derivatives were grown at 33 °C for plasmid preparation and at 30 °C for chitinase production in LB medium containing 100 μg/ml ampicillin.

SDS/PAGE

SDS/PAGE and detection of chitinase activity after renaturation of enzymes were performed as described previously [24].

CNBr cleavage of chitinases C1 and C2

Chitinases C1 and C2 in the culture supernatant of *Ser. marcescens* 2170 were collected by ammonium sulphate precipitation (80 % saturation) and partially purified by chitin affinity column chromatography as described previously [20]. The fractions containing both chitinase C1 and C2 were collected and the two chitinases were separated by SDS/PAGE (10 % slab). The strips of the polyacrylamide gel containing chitinase bands were cut out and chitinase proteins were extracted from the gel electrophoretically. Purified chitinase C1 and C2 proteins were dissolved in 75 μl of 70 % formic acid and subjected to CNBr cleavage by adding 10 μl of CNBr (100 mg in 75 μl of 70 % formic acid) and incubating at 37 °C for 24 h in the dark. After 24 h, CNBr was removed and trapped in NaOH pellets by repeated freeze-drying of the samples. CNBr-free samples were then treated with SDS sample buffer and analysed by SDS/PAGE (16.5 % slab) using the modification of the discontinuous procedure of Schagger and von Jagow [25].

N-terminal amino acid sequence analysis

N-terminal amino acid sequence determination of the purified chitinases, obtained by chitin affinity chromatography, and CNBr fragments of chitinases were performed as described previously [10].

DNA extraction, PCR, library construction and screening

Chromosomal DNA of *Ser. marcescens* 2170 was extracted from the cells as described by Silhavy et al. [26].

To prepare ^{32}P-labelled probes for Southern hybridization and colony hybridization, a part of the *chiC* gene was amplified by PCR by using a Takara PCR Thermal Cycler MP (Takarashuzo, Osaka, Japan). The amplified fragments were ligated with *Hinc*II-cut pUC119, and the resulting plasmid, pTCCP12, was maintained in *E. coli* JM109. Inserted fragments were prepared from pTCCP12 when necessary and labelled with [α-^{32}P]dCTP using the Rediprime DNA labelling system (Amersham Pharmacia Biotech, U.K.) according to the manufacturer's instructions.

For Southern hybridization, restriction enzyme-digested chromosomal DNA of *Ser. marcescens* 2170 was fractionated in a 0.7 % agarose gel, transferred onto a nylon membrane (Hybond N, Amersham) by the capillary method, and hybridized with the ^{32}P-labelled probe described above.

For library construction, chromosomal DNA of *Ser. marcescens* 2170 was digested with *Sal*I and separated on a 0.7 % agarose gel. The gel segment corresponding to the size between 4 and 6 kb was cut out, and DNA fragments in the gel were recovered by using GENECLEAN II (BIO 101, Inc., Vista, CA, U.S.A.). The DNA was ligated to *Sal*I-digested pUC119 and used to transform *E. coli* JM109 cells. The library was screened by the colony hybridization technique using the ^{32}P-labelled probe as described previously [2], except that hybridization and washing were carried out at 42 °C.

Nucleotide sequence determination and sequence analysis

Various restriction fragments of pTCC1 were subcloned into pUC119, and the resulting plasmids were sequenced with an automated laser fluorescence sequencer (Model 4000L, LI-COR, Lincoln, NE, U.S.A.). Sequencing reactions were done by using the Thermosequenase fluorescence-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) with a double-stranded template according to the supplier's instructions. The deduced amino acid sequence was compared with those available in the translated GenBank, the SWISS-PROT protein sequence data bank, and the National Biomedical Research Foundation (NBRF) protein data bank by using the Lipman–Pearson algorithm [27].

Production and purification of chitinases C1 and C2

E. coli JM109 cells harbouring plasmid pTCC2 carrying the cloned *chiC* gene were grown in LB medium supplemented with 100 μg/ml ampicillin and 0.4 mM isopropyl β-D-thiogalactoside for 24 h. Cells were collected by centrifugation and disrupted by sonication. After removing unbroken cells and debris, proteins were collected by ammonium sulphate precipitation (60 % satd.). Precipitated proteins were dissolved in a small volume of 2 mM sodium phosphate buffer (pH 6.0) and subjected to chitin affinity column chromatography as described previously [20]. Fractions containing chitinases C1 and C2 were collected and further purified by Sephadex G-75 gel-filtration chromatography.

Enzyme and protein assay

Chitinase activity was measured by a modification of the Schales' procedure [28] with colloidal chitin as the assay substrate. One unit of chitinase activity was defined as the amount of enzyme that produces 1 μmol of reducing sugar per min. Protein concentration was measured by the method of Lowry et al. [29] using BSA as the standard. The chitin-binding assay was performed in a mixture containing 4 mg of binding-assay substrates, 1 M NaCl and various concentrations of chitinase in 20 mM sodium phosphate buffer (pH 6.0). After incubation for 1 h on ice with stirring every 15 min, the mixture was centrifuged and the amount of unadsorbed chitinase in the supernatant was determined. The amount of adsorbed protein was estimated by subtracting the amount of unadsorbed protein from the total protein added to the tube.

MS

The mass spectrometric analysis of chitinases was done using a Voyager Elite matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) mass spectrometer (PerSeptive Biosystems, Framingham, MA, U.S.A.).

Chemicals

Colloidal chitin and glycol chitin were prepared from powdered chitin purchased from Funakoshi Chemical Co. (Tokyo, Japan) following the methods described by Jeuniaux [30] and Yamada and Imoto [31] respectively. Chitin EX (powdered prawn-shell chitin) used in chitin affinity column chromatography and chitosan 8B (approx. 80 % deacetylated) were purchased from Funakoshi Chemical Co. (Tokyo, Japan). Regenerated chitin for the chitin-binding assay was prepared from chitosan 8B by the method of Molano et al. [32]. Restriction enzymes and modification enzymes were purchased from Takarashuzo (Osaka,

Japan), Toyobo Biochemicals (Osaka, Japan) and New England Biolabs (Beverly, MA, U.S.A.).

RESULTS AND DISCUSSION

N-terminal and internal amino acid sequences of chitinases C1 and C2

Chitinases C1 and C2, as well as chitinases A and B and the chitin-binding protein CBP21, were detected in the culture supernatant of *Ser. marcescens* 2170 grown in the presence of chitin as described previously [18]. The molecular masses of chitinases C1 and C2 were estimated to be 48 kDa and 36 kDa respectively from SDS/PAGE analysis. The two chitinases both exhibited multiple peaks in each cycle of N-terminal amino acid sequence determination, and the amino acid residues detected in each cycle were identical for the two chitinases [18]. Thus we inferred that chitinases C1 and C2 both contain two or more polypeptides, and that C2 was derived from C1 by loss of its C-terminal portion.

Owing to the complexity of the N-terminal amino acid sequences, internal amino acid sequences were determined by analysing N-terminal amino acid sequences of CNBr fragments of chitinases C1 and C2. The CNBr fragments, derived from either chitinase C1 or C2, were separated by SDS/PAGE and their N-terminal amino acid sequences were determined by automated Edman degradation after blotting the separated CNBr fragments onto a PVDF membrane. Three major polypeptides (4, 8 and 20 kDa, as estimated by SDS/PAGE analysis) were obtained by CNBr cleavage of purified chitinase C1, and two major fragments (5 and 8 kDa) were obtained from purified chitinase C2. The N-terminal amino acid sequence analysis of these polypeptides revealed that the 8 kDa fragments from the two chitinases had the same N-terminal amino acid sequence except for one unidentified amino acid residue (Figure 1), supporting the idea that chitinase C2 is a derivative of C1.

Based on the N-terminal amino acid sequences of 8 kDa and 20 kDa polypeptides from chitinase C1, four degenerate PCR primers were designed. A pair of the primers allowed us to amplify a 0.5 kb DNA fragment when chromosomal DNA of *Ser. marcescens* 2170 was used as a template. The nucleotide sequence of the amplified fragment was determined and the deduced amino acid sequence was compared with those of the *chiA* and

Figure 2   Nucleotide sequence of the *chiC* gene of *Ser. marcescens* 2170 and deduced amino acid sequence of the gene product

A possible Shine–Dalgarno sequence and −35/−10 hexamers of the possible promoter are doubly underlined and underlined with a dotted line respectively. The amino acid sequence region matching the N-terminal amino acid sequence determined for chitinases C1 and C2 is boxed. The N-terminal amino acid sequences of the CNBr fragments of chitinase C1 are shaded and those of C2 are underlined. Arrows indicate the inverted repeat. The asterisk marks the stop codon, i.e. the end of the coding region.

Figure 1   N-terminal amino acid sequences of peptide fragments, obtained by CNBr cleavage of chitinases C1 and C2, and degenerate synthetic oligonucleotide primers

X represents unidentified amino acid residues.

Figure 3   Alignment of the C-terminal domain of chitinase C1 with other chitinases, endoglucanase of *Bacillus* sp. and protease C of *Strep. griseus*

A black background indicates amino acid residues identical with those of chitinase C1. Asterisks indicate conserved aromatic residues. Abbreviations of enzyme sources are given in Table 1.

Table 1   Abbreviations of enzyme sources with accession numbers for either GenBank, SWISS-PROT or NBRF

Abbreviation	Organism and enzyme	Accession number
Acav A	*Aeromonas caviae* chitinase A	U09139
Aero II	*Aeromonas* sp. No. 10S-24 chitinase II	D31818
Aero 1	*Aeromonas* sp. No. 10S-24 chitinase ORF1	D63139
Aero 2	*Aeromonas* sp. No. 10S-24 chitinase ORF2	D63139
Alte A	*Alteromonas* sp. O-7 chitinase A	D13762
Alte C	*Alteromonas* sp. O-7 chitinase C	AB004557
Bcir A1	*Bacillus circulans* WL-12 chitinase A1	M57601
Bcir C	*Bacillus circulans* WL-12 chitinase C	D89568
Bcir D	*Bacillus circulans* WL-12 chitinase D1	D10594
Blic	*Bacillus licheniformis* TP chitinase	U71214
Bsub	*Bacillus subtilis* chitinase	AF069131
Cpar B	*Clostridium paraputrificum* chitinase B	AB001874
Eagg	*Enterobacter agglomerans* chitinase (Chia-Entag)	U59304
Ente A	*Enterobacter* sp. G-1 chitinase A	U35121
Eame	*Ewingella americana* chitinase	X90562
Jliv 69	*Janthinobacterium lividum* chitinase 69	U07025
Kzop	*Kurthia zopfii* chitinase	D63702
Smar A	*Serratia marcescens* 2170 chitinase A	AB015996
Smar B	*Serratia marcescens* 2170 chitinase B	AB015997
Smar C	*Serratia marcescens* 2170 chitinase C1	This study
Smal A	*Stenotrophomonas maltophilia* chitinase A	AF014950
Sery	*Streptomyces erythraeus* chitinase	P14529
Sgri PC	*Streptomyces griseus* protease C	L29018
Sliv B	*Streptomyces lividans* chitinase B	D84193
Sliv C	*Streptomyces lividans* chitinase C	D12647
Sliv A	*Streptomyces lividans* 66 chitinase A	D13775
Soli 01	*Streptomyces olivaceoviridis* exo-chitinase 01	X71080
Spli 63	*Streptomyces plicatus* chtinase 63	M82804
Sthe	*Streptomyces thermoviolaceus* OPC-520 chitinase	D14536
Vhar A	*Vibrio harveyi* chitinase A	U81496
Afae PHB	*Alcaligenes faecalis* poly(3-hydroxybutyrate) depolymerase	J04223
Baci EA	*Bacillus* sp. strain N-4 endoglucanase A	P06566
Baci EB	*Bacillus* sp. strain N-4 endoglucanase B	P06565
CbhA	*Cellulomonas fimi* cellobiohydrolase A	L25809
CbhB	*Cellulomonas fimi* cellobiohydrolase B	L38827
CenD	*Cellulomonas fimi* endo-1,4-β-D-glucanase (CenD)	L02544
Ppic PHB	*Peudomonas pickettii* poly(3-hydroxybutyrate) depolymerase	D25315
AmyA180	Unclassified alkalophilic Gram-positive bacteria DSM 5853 exo-maltopentaohydrolase (amylase)	X53373

chiB genes of this bacterium. Although some local similarities were observed, no identical region was found with either chitinase A or B, clearly indicating that chitinase C1, and C2, are not derivatives of either chitinase A or B and, therefore, are derived from an unidentified chitinase gene.

Cloning and nucleotide sequence of the gene encoding chitinase C1

The 0.5 kb amplified fragment was radiolabelled and used as a hybridization probe for cloning of the gene specifying chitinase C1 (and C2). Southern hybridization experiments with chromosomal DNA of strain 2170 were carried out and a strong signal at the position around 5 kb was observed when chromosomal DNA was digested with *Sal*I. Thus, *Sal*I-digested fragments with sizes between 4 and 6 kb were prepared, ligated with *Sal*I-cut pUC119 and transformed into *E. coli* JM109 cells. Colony hybridization of the transformant colonies was carried out with the same radiolabelled probe, and one positive clone was detected among 800 transformant colonies. The positive clone contained a plasmid (designated pTCC1) with a 4.5 kb inserted fragment and the nucleotide sequence of a 2.9 kb region was determined.

As shown in Figure 2, one open reading frame of 1440

nucleotides, starting from an ATG initiation codon, was identified in the sequenced region. The translated polypeptide is 480 amino acids long with a calculated size of 51.7 kDa, slightly larger than the size of chitinase C1 (48 kDa) estimated by SDS/PAGE. The internal amino acid sequences determined using five CNBr fragments perfectly matched the sequences of the corresponding regions of the deduced polypeptide. Four out of five CNBr fragments were generated by cleavages at the C-terminus side of tryptophan but not at methionine residues. The reason for such frequent cleavage at tryptophan is unclear, although cleavage at tryptophan has been reported with other proteins [33]. Multiple peaks were observed in each cycle of the N-terminal amino acid sequence determination of chitinases C1 and C2 as described above. This can be explained by the presence of four polypeptides starting from Ala-9, Val-10, Ala-11 and Ala-12 in each chitinase preparation. The N-terminal region of the deduced polypeptide, which is absent from chitinases C1 and C2, does not have the typical features of a signal sequence.

The results described above indicate that both chitinases C1 and C2 are products of the cloned gene, which was designated *chiC*. Recently, Gal et al. [19] reported the nucleotide sequence of the gene encoding 52 kDa chitinase from *Ser. marcescens* KCTC2172. The 52 kDa chitinase gene of strain KCTC2172 is apparently the counterpart of the *chiC* gene of strain 2170, since

```
Smar  C    332  TPPPGKPNAPTALTVAELGATSLKLSWAAATG---ALPIASYTVYRNGNPIGQTAGLSLTDSGLTPATQYSYFVTATDSQGNTSLPSSALAVKTANDG  426
CbhA       556  VEDLVAPTVPTGLTAGTTTASVPLSWTASTDN--VAVTGYDVYRGTTLVGTTAATSYTVIGLTPATAYSFTVRAKDAAGNVSAASAAAAATTQSGT  650
AmyA180   1392  DPDHTSPSKPTDLTIA-IATAHTVSLSWTASADD---VEVAGYKIYRDGVEIGVTESTTYTDSGLTAETTYSYAVQAYDTSNNFSALSDELTIETAEKT 1485
Ppic PHB   441  GPVQ-SAGTPTGLTVTGTTTTSVSLSWNAVTN------ATSYNVYRNGSKVGSSTSTTYTDTGLIAGTTYSYTVIEIDPTAGESAQSSAVSAKTQSSF  531
CbhB       855  TPDTTAPTVPTGLQAGVVTSTEATISWTASTDD---TRVTGYDVYRGATKVGTATTTSFTDTGLTASTAYAYTVRAFDAAGNVSAPSAALTVTTKATP  949
Sliv  C    136  VPGDAAPSAPGTPTASNITDTSVKLSWSAATD---DKGVKNYDVLRDGAKVATVTGTTYTDNGLTKGTAYSYSVKARDTADQTGPASGAVKVTTTGGG  230
Afae PHB   371  GTGQ-AGSAPTGLAVTATTSTSVSLSWNAVAN------ASSYGVYRNGSKVGSATATAYTDSGLIAGTTYSYTVTAVDPTAGESQPSAAVSATTKSAF  461
Smal  A    195  SNDTTPPSVPGGLASPSKTATTVNLVWSAATDNSGGSGVAGYDVYRNGSLVGPSATQYTDGGLTASTAYTYTVRARDNAGNASAQSGSISVTTAAGG  292
CenD       553  TPDTTAPTAPTGLRAGTPTASTVPLTWSASTDTGG-SGVAGYEVYRGTTLVGTTTATSYTVTGLAADSANTFSVRAKDGAGNTSAASAAVTARTAAGG  649
Bcir A1    554  GGDTQAPTAPTNLASTAQTTSSITLSWTASTDN---VGVTGYDVYNGTALATTVTGTTATISGLAADTSYTFTVKAKDAAGNVSAASNAVSVKTAAET  648
Kzop       554  GGDTQAPTAPTNLASTAQTTSSITLSWAASTDN---VGVTGYDVYNGTALATSVTGTTATISGLAADTSYTFTVKAKDAAGNSSVASNALTVKTAAGT  648
Spli 63    136  VPGDEAPSAPGTPTASNITDTVKLSWSAATD---DKGVKNYKLRDGATVATVTGTTYTDNGLTKGIDYSYSVKARDTGDQTGPASGSVKVTTTGGD  230
Bcir  D     87  TPDTTPPTVPAGLTSSLVTDTSVNLTWNASTDNVG---VTGYEVYRNGTLVANTSTTTAVVTGLIAGTTYVFTVKAKDAAGNLSAASTSLSVTTSTGS  181
Sliv  A    180  NPDPTVPSAPAGLSVSGTTPNSASLSWNTVSG------ATGYNVYRDGTKVTAVTGTSATVTGLAASTSYSFQVTATN-AAGESVKSAAVTARTTAPD  270
```

Figure 4   Alignment of the Fn3 domain of chitinase C1 with those of other bacterial enzymes

A black background indicates amino acid residues identical with those of chitinase C1. Abbreviations of enzyme sources are given in Table 1.

the coding regions of the two chitinase genes match 99.2 % in nucleotide sequence and 95 % in amino acid sequence.

Multidomain structure of chitinase C1

Comparing the deduced amino acid sequence with entries in the protein data banks readily identified the domains constituting chitinase C1. A large N-terminal region exhibited extensive similarity to the catalytic domains of several bacterial chitinases such as *Vibrio harveyi* chitinase A, *Strep. lividans* chitinases A and B and *B. circulans* chitinase D1. Thus, this region was considered to be the catalytic domain. The catalytic domain of chitinase C1 did not show apparent similarity with those of the other chitinases found in this bacterium (chitinases A and B), except for the conserved segments which are observed in all family 18 chitinases [18]. One of the conserved segments has been shown to contain the residue directly involved in the catalytic mechanism [3,34], which corresponds to Glu-141 of the deduced chitinase C1 polypeptide.

As shown in Figure 3, the C-terminal region of the deduced polypeptide exhibited significant similarity to the ChBDs and putative ChBDs of various bacterial chitinases. (Abbreviations of enzyme sources with accession numbers are listed in Table 1.) Similarities were found not only with chitinases but also with the C-terminal regions of *Bacillus* sp. endoglucanases and *S. griseus* proteinase C. The C-terminal region of chitinases A1 of *B. circulans* WL-12 was shown to be the ChBD and was also important for efficient hydrolysis of insoluble chitin [9]. Therefore it is highly probable that the C-terminal region is the ChBD of chitinase C1. Tyr and Trp residues are well conserved in the alignment shown in Figure 3, suggesting the involvement of the side-chains of these aromatic residues in chitin binding by analogy with the situation in the cellulose-binding domains of cellulases [35].

The region between the catalytic domain and the ChBD was found to display sequence similarity to Fn3 domains, as shown in Figure 4. The bacterial Fn3 domain was first identified in chitinase A1 of *B. circulans* WL-12 [36] and was later found in many bacterial enzymes such as chitinases, cellulases, amylases and poly(3-hydroxybutyrate) depolymerases [37]. Among bacterial enzymes degrading insoluble substrates, the Fn3 domains have been most frequently found in chitinases. The function of Fn3 domains is not clear yet, although a structural role to maintain an optimal distance and orientation between the catalytic domain and the ChBD for maximum chitinase activity has been suggested [9]. The frequent occurrence of this domain in chitinases may have a special importance for the degradation of the insoluble and crystalline polysaccharide chitin.
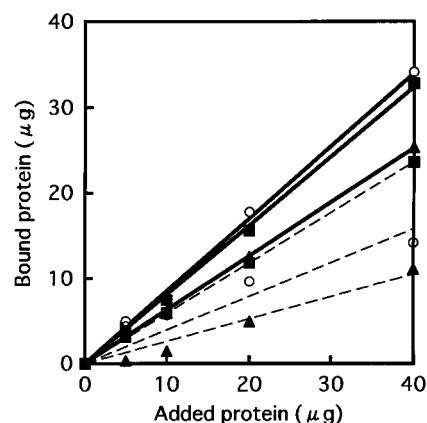
Figure 5   Binding activity of chitinases C1 and C2 to various chitin samples

Solid lines, chitinase C1; broken lines, chitinase C2; (○), regenerated chitin; (▲), powdered chitin; (■), colloidal chitin.

Table 2   Hydrolytic activity of chitinases C1 and C2 toward colloidal chitin, glycol chitin, regenerated chitin and powdered chitin

Substrate	Chitinase activity (units/μmol)	
	Chitinase C1	Chitinase C2
Colloidal chitin	153	160
Glycol chitin	289	264
Regenerated chitin	64.8	24.7
Powdered chitin	6.83	2.69

Chitinases C1 and C2 produced in *E. coli*

To verify the presence, the extent and the role of the putative ChBD in chitinase C1, chitinases C1 and C2 were produced in *E. coli* carrying the cloned *chiC* gene and purified. Since only a small amount of chitinase C1 was produced in *E. coli* JM109 harbouring the originally isolated plasmid pTCC1, we constructed plasmid pTCC2, which lacks the upstream region containing the probable ribosome-binding site and places the gene expression under the control of the *lac* promoter. Chitinase activity was mainly recovered in the cytoplasmic fraction (approx.

```
Smar A   159  VVGSYFVEWGVYGRNF----------TVDKIP----AQNLTHLLYGFIPICGGNG--INDSLK--EIEGSFQALQRSCQGREDFKVSIHDPFAAL-QKAQKGVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTL-------SDPFFF-
Ente A   159  VVGSYFVEWGVYGRNF----------TVDKIP----AQNLTHLLYGFIPICGGNG--INDSLK--EIEGSFQALQRSCQGREDFKVSIHDPFAAL-QKAQKGVTAWDDPYKGNFGQLMALKQARPDLKILPSIGGWTL-------SDPFFF-
Eagg A   159  VVGSYFVEWGVYGRNF----------TVDKIP----AQNLTHLLYGFIPICGGDG--INDSLK--GVEGSFQALQRSCQGREDFKVSIHDPFAAV-QKGQKGVTAWDDPYKGNFGQLMALKQARPDLKILPSIGGWTL-------SDPFFF-
Acav A   159  VVGAYYVEWGVYGRKF----------TVDKIP----AQNLTHILYGFTPICGGDG--INDSLK--GVEGSFEALQRSCAGREDFKVSIHDPWAAI-QMSQGNLSAWDEPYKGNFGNLMALKQAHPDLKILPSVGGWTL-------SDPFYF-
Alte A   159  VTGAYFVEWGIYGRNY----------DVTKIP----AHNLSHILYGFIPICGP-----NESLKSIEIGNSWRALQTACADSQDYEVVIHDPWAAV-QKSMPGVDAKD-PIRGVYSQLMALKQRYPDLKILPSVGGWTL-------SDPFHG-
Bcir A1   45  KIVGYYPSWAAYGRNY----------NVADID----PTKVTHINYAFADICWNGIHGNPDPSGPNPVTWTCQNEKSQTINVPNGTIVLGDPW-ID-TGKTFAGDTWDQPIAGNINQLNKLKQTNPNLKTIISVGGWTW------SNRFSDV
Kzop      45  KIVGYYPSWAAYGRNY----------NVTDID----PTKVTHINYAFADICWNGVHGNPDPSGPNPVTWTCQNEKSQTINVPNGTIVLGDPW-ID-TGKTFAGDTWDQPIAGNINQLNKLKQINPNLKTIISVGGWTW------SNRFSDV
Blic      42  KIIGYYPSWGAYGRNF----------QVWDMD----VSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENG-VIDAPNGTIVMGDPW-ID-AQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTW------SNRFSDV
Bsub      40  KIIGYYPSWGAYGRDF----------QVWDMD----ASKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENG-VIDVPNGSIVMGDPW-ID-VQKSNAGDTWDEPIRGNFKQLLKLKKNHPHLKTFISVGGWSW------SNRFSDV
Bcir C    47  KIIAYVAGWANWTAN-------------DIK----VEQLSHINYSFALIS----------------------NGK-------ATITNSD-------------------RTKLQMMVGLKSRNPDLKVLLSVGGWG--------ANGFSDA
Sliv C   241  VKMGYFTNWGVYGRNY----------HVKNLVTSGSADKITHINYAFGNVQG----------GKCTI-------------GDSYADYDKAYTAD-QSVDGVADTWDQPLRGNFNQLRKLKAKYPNIKILYSFGGWTW------SGGFPDA
Spli 63  242  VKMGYFTNWGVYGRNY----------HVKNLVTSGSAEKITHINLRFGNVQG----------GKCTI-------------GDAYADYDKAYTAD-QSVDGVADTWDQPLRANFNQLRNLKAEYPHIKILYSFGGWTW------SGGFPDA
Sthe      49  VKLGYFTEWGTYDRNF----------NVKNLDTSGTAAKITHINYAFGNVTG----------GKCAI-------------GDSYADYDKAFTAD-QSVSGQADTWDQPLRANFNQLRQLKAKYPHIKVLWSFGGWTW------SGGFADA
Smal A   299  RVIGYFTQWGIYGRNY----------RVKNIDTSGSAAKLTHINYAFGNVRN---------NRCEVGVTQASDPNTGVG-GDAFADYTKAFGAG-ESVSGSADTWDQPLRGNWNQLKQLKAKHPNVKVLISLGGWTW------SRGFSS-
Jliv 69  247  EVGSYFAQWGVYGRDY----------EVANVHTSGVADKLTFINYAFGNIYPKNGGYECGMINKAEPGATNPNSPDTGTG-GDAEADYIRTP--K-RTVDGQVIPWDAPLSGNFLQLKKLKAAHPNLKLFISLGGWSW------SKNFSVG
Smar B     6  AVIGYYFIPTNQINNYTETDTSVVPFPVSNITP-AKAKQLTHINFSFLDINS----------------------NLECAWDPATN-----------DAKARDVVNRLTALKAHNPSLRIMFSIGGWYYSNDLGVSHANYVNA
Cpar B    51  RNVMYYGDWSIWGGQG-------NFYPKDIP----ADKLTHLNFAFMDFN----------------------------ASGELIYCDKDAATGHPLGNAGVTYGDVNGGILNAFQVLKAENPNLKIGVSLGGWSK------SGDFSTI
Alte C   184  KIVGYFPSWQGAVS---------------DIQ----FDKLTHINYSFLLPHA------------------------DGTLRPLEN-----------------LSKMTALVNSAHA-NNVKVGIAIGGWNGG-----DDSAFETF
Smar C    26  ILMGFWHNWAAGASDGYQ------QGQFANMNLTDIPAEYNVVAVAFMKGQ--------------------------------GIPTFKP-------------YNLSDAEFRRQVGVLNS-QGRAVLISLGGADA-------HIELK--
Vhar A    36  VVVGYWQNWCDGG--GYQ------GGNAPCVTLDEVNPMYNIVNVSFMKVYD-------------VAD------------GRIPTFKLDP-----------TVGLSEEQFIDQVSELNK-QGRSVLLALGGADA-------HVELE--
Sliv B   278  ALVGYLHASFANG--------------SGYTRMADVPDSWDVIDLAFGEPTS------------VT----------SGDIRFERCP--------VAECPNVESDAEFKAAIKAKQA-AGKKVLISIGGQNG------QVQLT--
Soli 01  265  ALVGYLHASFANG--------------SGYTRLADVPDSWDVIDLAFGEPTS------------VT----------SGDIRFDRCP--------ATECPNVESDAEFKAAIKAKQA-AGKKVLISIGGQNG------QVQLT--
Aero 1   230  QLVGYLHARLCQR-------------FGLYPHGDVSDDWDVINLSFAEPDS------------PT----------NALLHFNRCS-------VSECPNVESDADFLAAIKAKQA-RGKKVLISIGGONG------EIQLT--
Bcir D   191  WLIGYWHN-FDNG-------------STNIKLRNVSTAYDVINVSFAEPIS------------PG----------SGTLAFT---------------PYNATVEEFKSDIAYLQS-QGKKVLISMGGANG------RIELTD-
Sliv A   282  AVTGYMQNFNNGAT-------------VQKIS--DVPSAYDIIAVAFADATT----------------------TPGAVTFNLDS-------AG----LGGYTVDQFKADVRAKQA-AGKKVIISVGGEKG-------TVSVN--
Sery       7  SASPYLYNGWGNPPS-----------PTEVMN----ASGIKNFTLAFILADG----------------------TCNPAWDG----------------NRPLDGQDKATIDAIRG-AGGDVIPSIGGYSG--------SKLG--
Eame       4  LYTPYIDVSLNALWSDWQNYPKGR--PNPIYSQQAISYGVDGLVLDFLTLSP---------------------SNKACWAAS---------------DAMPLEWALPLANELNAANRQVIVSFGGASN--------ADIS--
Aero II   33  EAAPYFFTWGYGESSY----------KFTSLVDAKQKAGLDAATLAFIITGS-----------------------SGCSLSN------------------DVDNMLSDVLAFQQQ--GGRLIISFGGAAVP--------MWKP-
Aero 2   222  PVSGYRFASVYRRERQ----------CGLAP---AGRKQPAAALSRWPSST-----------------------RQAVAMAAG---------------RRSESNLISQAASLKA-LGGGVIISSGGWNA--------SDIVR-
```

```
MGDKVKRDRFVGSVKEFLQT--------------WKFFDGVDIDWEFPGGKGANPNLG----------SP-QDGETYVLLMKELRAMLDQLSVETGRKYELTSAISA-GKD------------------KIDKVAYNVAQNSMDHIFLMSY  390
MGDKVKRDRFVGSVKEFLQT--------------WKFFDGVDIDWEFPGGKGANPNLG----------SP-QDGETYVLLMKELRAMLDQLSAETGRKYELTSAISA-GKD------------------KIDKVAYNVAQNSMDHIFLMSY  390
MGDKVKRDRFVGSVKEFLQT--------------WKFFDGVDIDWEFPGGGGANPKLG----------NA-QDGATYVQLMKDLRAMLDQLSAETGRKYELTSAISA-GKD------------------KIDKVDYNTAQNSMDHIFLMSY  390
LGDKTKRDTFVASVKEFLQT--------------WKFFDGVDIDWEFPGGQGANPSLG----------GP-NDGATYVVLMKELRAMLDELEAETGRQYELTSAISA-GGD------------------KIAKVDYQAAQQYMDHIFLMSY  390
FTNKANRDTFVASVKQFLKT--------------WKFYDGVDIDWEFPGGDGNPDLG----------DPINDGPAYVALMQELRAMLDELEAETGRQYELTSAIGA-GYD------------------KIEDVDYQAAQQYMDYIFAMTY  389
AATAATREVFANSAVDFLRK--------------YNFDGVDLDWEYPVSGGLDGNSK----------RP-EDKQNYTLLLSKIREKLDAAGAVDGKKYLTIASGA-SAT------------------YAANTELAKIAAIVDWINIMTY  279
AATAATREVFANSAVDFLRK--------------YNFDGVDLDWEYPVSGGLDGNSK----------RS-GDKQNYTLLLSKIREKLDAAGAVDGKKYLLIASGA-STT------------------YAANTELANIASIVDWINIMTY  279
AADPVARGNFAASAVEFLRK--------------YGFDGVDLDWEYPVSGGLPGNST----------RP-EDKRNYTLLLQEVRKKLDAAEAKDGKEYLLIASGA-SDR------------------YVSNTELDKIAQTVDWINIMTY  275
AADPAARENFAASAVNFLRK--------------YGFDGVDLDWEYPVSGGLPGNST----------RP-EDKRNYTLLLSKVREKLNAQGGQINGKQYLLTVSGA-SPE------------------YVSNTELDKIAETVDWINIMTY  273
ALTDASRTTFADSIVQLVTS--------------NNLDGVDLDWEYPTN-PAAGTTA----------RP-QDKQNFTQLLSKVREKLNAQGQINGKQYLLIAAGA-SSS------------------YLNGVEINNITPLLDWINLMTY  228
VKNPAA---FAKSCHDLVED---PR---------WADVFDGIDLDWEYPNACG--LSCD----------ET-SAPNAFSSMMK-------AMRAEFGQDYLITAAVTADGSDGG----------------KIDAADYGEASKYIDWYNVMTY  451
VKNPAA---FAKSCHDLVED---PR---------WADVFDGIDLDWEYPNACG--LSCD----------ET-SAPNAFSSMMK-------AMRAEFGQDYLITAAVTADGSDGG----------------KIDAADYGEASKYIDWYNVMTY  452
AKRPAA---FAQSCVNLVHD---PR---------WADVFDGIDLDWEYPNACG--LTCD----------S--SGPDAFRNLMA-------AVRSTFG-DELVIAAVTADGTPGG----------------KIEATDYAGAAQYVDWYNVMTY  255
AAQPANRQAFVASCVDAYIKGNLPVTDGAGGVGAAAGVFDGIDIDWEYPVACG--LSCG----------TP-ADNANYTALLAEFRRQLDAVRPG-----LLTIAVGA-GID------------------KIRVTDPAAYHPYLDFINVMTY  536
SATDALRKQMVRSCIDIYIKGNLPVQGGRGGVGAAANIFDGIDIDWEYPVSGGGQPYNTV----------SP-NDKRNFTLLMAEFRSQLDALGAANGKRYLLIAAVGA-GKD------------------KIDNTEPALYSQYMDWINLMTY  499
VKTPAARTKFAQSCVRIMKD--------------YGFDGVDIDWEYPQAAEVDGFIA------------ALQEIRTLLNQQT----IADGRQALP-YQLTIA-GAGGAFFLS----------------RYYSKLAQIVAPLDYINLMTY  211
AANASTRAKFVENVMKFIKY--------------TNMDFVDLDYEPDKTDNTNDEGTPNASP-QDKDNYLLLQDLKNALNKQGKELGKTYELSVALPA-GVS------------------KIEAGIDVDKLFDIVDFANIMTY  270
ASSQQGRARFVQEVIAFVEQ--------------HNLDGVDMDVEYPDP-G--DSAN----------------NYALLMKELS--VELRAR-GK--FLTAAVVALGYT----------------GGGVLESVFQDIDFLNLMAY  351
---TGDEDKLKDEIIRLVEV--------------YGFDGLDIDLEQAAIGAANNKT-----------------VLPAALKKVK----DHYAAQGKNFIISMAPEFPYLRT----------N------GTYLDYINALEGYYDFIAPQY  208
---TGDERAFADEIIRLTER--------------YGFDGLDIDLEQAAVTAANNQT-----------------VIPDALKLVK----DHYRAEGKNFLITMAPEFPYLTT----------G------GKYVPYIDNLEGYYDWINPQFY  224
--STAARDKFVSSVSAIIDE--------------YGLDGLDIDFEGHSLSLNADDTD----------FKNPKTPVIVNLVSAVK----TLKAKYGDGFVLTMALETFVQLGYGQYQYTGKWGGQDPRAGAYLPVIHALRDDLTLLHVQDY  488
--TTAARDTFVSSVSKIIDE--------------YGLDGLDIDFEGHSLSLNADDTD----------FKNPKTPVIVNLIQALK----TLKAKYGDDFVLTMAPETFFVQLGYQYYGTGKWGGQDPRAGAYLPVIHALRDDLTLLHVQDY  475
--TTAARDNFVNSISAIIDK--------------WSLDGLDIDFEGHSLQLNAGDNN---------VAAPTTPVIVNLISALK----TLKAKYGSKFMLTMAPETFFVQLGYQFYG-GNCSCGCDTRAGAYLPVIYAMKDDLSWLQVQDY  439
--ATKKRQQFEDSLKSIIST--------------YGFNGLDIDLEGSSLSLNAGDTD----------FRSPTTPKIVNLINGVK----ALKSHFGANFVLTAAPETAYVQGGYLNYG-GPWG-------AYLPVIHALRNDLTLLHVQHY  386
--SSASATNFANSVYSVMRE--------------YGFDGVDIDLENGLN-----PT-------------YMTQALR-------ALSAKAGPDMILTMAPQTIDMQST--------------QGGYFQTALNVKDILTVVNMQYY  440
-EVCQDSQSLAGAYQKVIDA--------------YGLKAIDVDIEATEFENDASETR----------------VLEALKIVK----EANPGLRTVVTFPTLVNGPNDVG-------------KRMIDKAARIGSDVDVWTQMPF  182
--TKFTVDQLVQTYTDVVQK--------------FKAKQLDFDLENGQY-----DYN----------------KISSALAIFQ----KSNPSVRISFTLPTMPAGLVTEG----------------LNIIQSAKNAGVKFSVNGMAMDY  189
---AVPAPRWPRWWMPCCNA--------------PACVPLDFDIEGSQLSQTALNS----------------VRNEAIKTLQ----AKYPDLYISFTLPTNPTGLTSEGLN--------------LLRGAQAAGVRTDVVNVMTMDY  210
--TCTDARSIATVYENVLER--------------FGADHLDSDPEHGDQEQSGSGPL-----------LIAAMAALKILQDRFKAKGKTAYVSFTLGVIRSAGFDVKTS------------TCLQSGQGPRCGCERSVNPMITDY  409
```

Figure 6 Alignment of the sequences corresponding to the major part of the catalytic domains of family 18 bacterial chitinases

The sequences shown here correspond to the regions extending from the first to sixth β-strands in the (α/β)8-barrel fold of the catalytic domain of each chitinase. Overbars indicate sequence segments corresponding to the β-strands predicted by HCA. The alignment was made by using the CLUSTAL W program [45] and manually edited by using HCA. Identical amino acids around the β-strands are indicated by a black background. The abbreviations of enzyme sources are given in Table 1.

Figure 7   Relationships among family 18 bacterial chitinases

The unrooted phylogenetic tree was calculated for 29 chitinases based on the alignment shown in Figure 6 using the neighbour-joining method [43] implemented in the program CLUSTAL W [45] and drawn using the program TreeView. Numbers at branch points indicate bootstrap analysis values obtained using 1000 resampled data sets. The horizontal scale bar corresponds to 0.1 amino acid change per position. The abbreviations of enzyme sources are given in Table 1.

90 %), as expected from the absence of a typical signal sequence at the N-terminus. The cytoplasmic fraction contained two chitinase molecules with sizes corresponding to chitinases C1 and C2. The N-terminal amino acid sequences of the chitinases corresponding to chitinases C1 and C2 were identical (STNNIINAVA) and matched the sequence starting from Ser-2 of the deduced polypeptide (see Figure 2). Therefore we considered the chitinases produced in *E. coli* to be essentially identical with chitinases C1 and C2, although they have a few additional amino acids at the N-terminus and may have a few amino acid differences at the C-terminus. Chitinases C1 and C2 extracted from *E. coli* cells were purified by chitin affinity column chromatography followed by gel-filtration chromatography. A total of 6.77 mg of purified chitinase C1 and 0.98 mg of purified chitinase C2 were obtained from a 1 litre culture of *E. coli* JM109 harbouring pTCC2. The molecular mass of chitinase C2 was determined to be 36 048 Da, as measured by MALDI–TOF-MS. This value coincided with the calculated molecular mass of the deduced polypeptide from Ser-2 to Gln-328, which corresponds to the catalytic domain of chitinase C1. Therefore it was confirmed that chitinase C2 is a derivative of C1, generated by loss of the Fn3 domain and the ChBD, and therefore corresponds to the catalytic domain of chitinase C1.

Chitinase C1 does not have a signal sequence and was recovered in the cytoplasmic fraction when expressed in *E. coli*. Similar results were obtained with chitinase B of this bacterium, which also lacks a signal sequence at its N-terminus [18]. On the other hand, chitinase A has a signal sequence and therefore the excretion paths of chitinase C1 and B must be different from that of chitinase A, although the production of these chitinases seems to be regulated in a similar manner [20]. The *sec*-independent secretion of extracellular enzymes of *Ser. marcescens* has been

shown for a metalloprotease [38] and a lipase [39], which are secreted by the ATP-binding cassette (ABC) protein-mediated exporter. The proteins excreted through the ABC pathway are known to contain a Gly-rich sequence that is repeated 4–36 times at the C-terminus [39,40]. However, chitinases C1 and B do not possess this type of secretion signal at their C-termini.

The role of the C-terminal portion of chitinase C1

To examine whether the C-terminal domain of chitinase C1 really acts as a ChBD, the binding activity of chitinase C1 to different types of chitin was measured and compared with that of chitinase C2, the catalytic domain of chitinase C1 (Figure 5). Chitinase C1 bound to regenerated chitin and colloidal chitin better than to powdered chitin (chitin EX). Chitinase C1 exhibited much higher binding activity than chitinase C2 with respect to all substrates tested. The level of binding of chitinase C2 to regenerated chitin and powdered chitin was approx. 40 % of that of chitinase C1, and was 70 % of the level of binding to colloidal chitin. Therefore it is clear that the C-terminal portion of chitinase C1 is important for the chitin-binding activity of chitinase C1, although the participation of the Fn3 domain in binding could not be evaluated in this experiment. It is notable that chitinase C2 itself has some binding activity with respect to chitinous substrates, especially colloidal chitin, although the activity is not high. Differences in binding activities between chitinases C1 and C2 were more marked for regenerated chitin and powdered chitin than for colloidal chitin.

To clarify whether the differences in chitin-binding activity affect the hydrolytic activity of the two chitinases, the activity of chitinases C1 and C2 against various chitinous substrates was determined. Chitinases C1 and C2 were incubated with various substrates at 37 °C for 10 min and the amount of reducing sugar generated was measured. As shown in Table 2, no differences were observed between the activities of chitinases C1 and C2 against glycol chitin and colloidal chitin. 4-Methylumbelliferyl-$(GlcNAc)_2$ and 4-methylumbelliferyl-$(GlcNAc)_3$ were also hydrolysed with similar efficiency (results not shown). On the other hand, the hydrolytic activity of chitinase C2 against powdered chitin and regenerated chitin was less than half of that of chitinase C1. These results indicate that the C-terminal portion of chitinase C1 does not affect the hydrolysis of soluble and amorphous substrates but is important for efficient hydrolysis of crystalline substrates.

We previously demonstrated that the C-terminal ChBD of chitinase A1 of *B. circulans* WL-12 was important for efficient hydrolysis of insoluble chitin, including not only regenerated and powdered chitin but also colloidal chitin. In this case, the catalytic domain did not show significant binding activity with respect to colloidal chitin. Therefore, the equal efficiencies of hydrolysis of colloidal chitin by chitinases C1 and C2 of *Ser. marcescens* 2170 may reflect strong affinity to colloidal chitin of the catalytic domain itself. Similar results have been reported by Morimoto et al. [7] with chitinase B of *Clostridium paraputrificum*. Deletion of the ChBD from chitinase B of *C. paraputrificum* did not affect the enzyme activity toward colloidal chitin, and the catalytic domain itself exhibited strong affinity for colloidal chitin.

Relation of chitinase C1 to other bacterial chitinases

The catalytic domain of chitinase C1 exhibited extensive sequence similarity with several bacterial chitinases, but only limited similarity with chitinases A and B of *Ser. marcescens* 2170. Based on the amino acid sequence similarity, we previously
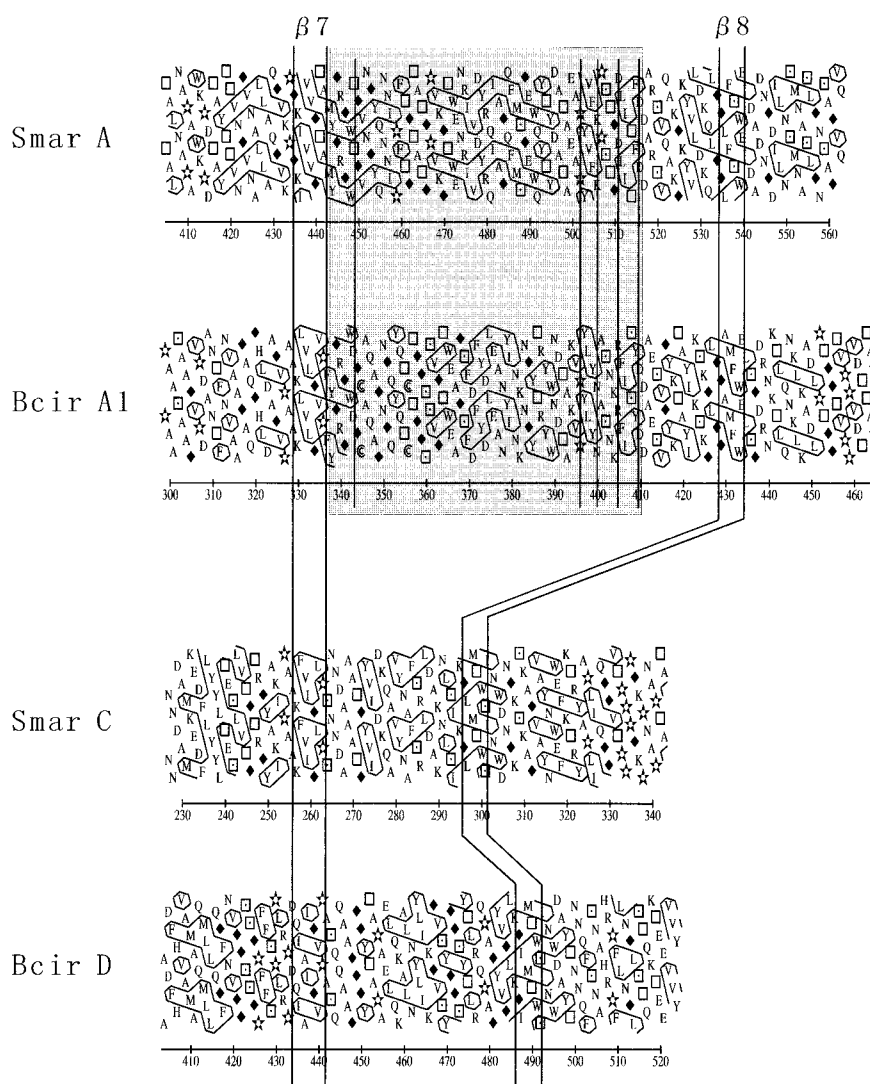
Figure 8   HCA plot of the regions between the seventh and eighth β-strands of the catalytic (β/α)8-barrel domain of subfamily A and subfamily B chitinases

Ser. marcescens chitinase A and *B. circulans* chitinase A1 are shown as examples of subfamily A, and *Ser. marcescens* chitinase C1 and *B. circulans* chitinase D1 are shown as examples of subfamily B. Vertical lines indicate the proposed correspondences between sequences. The grey area indicates the regions corresponding to the inserted domain. Proline, glycine, serine and threonine are represented by ☆, ◆, ⊡ and □ respectively.

proposed that the catalytic domains of bacterial chitinases could be divided into three groups, i.e. groups A, B and C [34]. This subdivision was made several years ago with the limited number of chitinase sequences available at that time by a pairwise comparison of sequences. For a better understanding of the relation of chitinase C1 to chitinases A and B of this bacterium and to other bacterial chitinases, a more systematic analysis using increased numbers of sequences was performed.

To compare amino acid sequences of all bacterial family 18 chitinases, we first attempted to align the amino acid sequences of the entire catalytic domains available from protein data banks and from the literature. However, due to the extensive diversity of the amino acid sequences of some chitinases, alignment by conventional methods was found to be impractical. Therefore, the sequence segments corresponding to the β-strands in the catalytic domain of each chitinase were first delineated by hydrophobic cluster analysis (HCA) [41,42] by comparison with the 3D-structure of the catalytic domain of chitinase A of *Ser. marcescens*

QMB1466 [3]. This chitinase consists of three domains: an all-β N-terminal domain, a catalytic (β/α)8-barrel domain and a small α + β-fold domain (third domain). Then, an alignment was made using subsequences corresponding to the regions extending from the first to the sixth β-strand in a manner such that each of the respective β-strands was aligned, as shown in Figure 6. The other regions of the catalytic domains of these chitinases were not included because the extensive divergence in these regions would reduce the reliability of the alignment. In particular, the region corresponding to the sequences between the seventh and eighth β-strands sometimes (but not always) contains a large insertion corresponding to the small α + β-fold region of chitinase A from *Ser. marcescens* QMB1466 inserted in the (β/α)8-barrel [3].

In spite of the extensive diversity of some chitinases, several amino acids in the segments corresponding to β-strands were very well conserved in this alignment. Short conserved regions of family 18 chitinases containing SXGG and DXDXE are located at the ends of the third and fourth β-strands respectively. A

Chitinase A | Subfamily A

Chitinase B | Subfamily A

Chitinase C1 | Subfamily B

Chitinase C2 | Subfamily B

▨ ⧄ Catalytic domain
⬛ ▪ Chitin-binding domain
⬚ Signal sequence
➡ Fibronectin Type III-like domain
▨ N-terminal domain
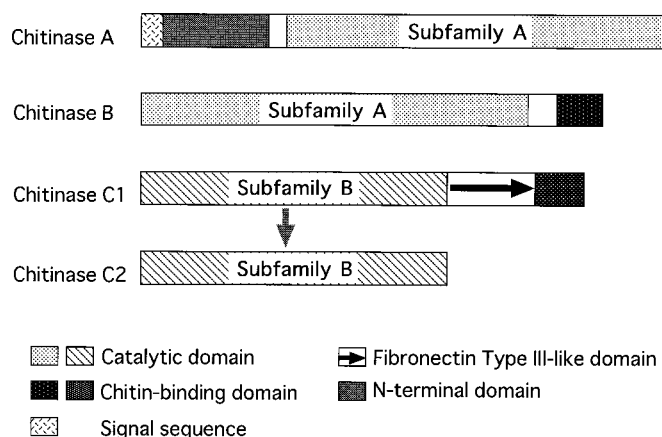
Figure 9 Domain organization of chitinases of *S. marcescens* 2170

phylogenetic tree was then constructed (Figure 7) from this alignment using the neighbour-joining method [43]. Bacterial chitinases are clearly separated into three major subfamilies A, B and C, suggesting divergence at a relatively early stage of bacterial chitinase evolution. Chitinase C1 is found in subfamily B, whereas chitinases A and B are both in subfamily A, showing that *Ser. marcescens* 2170 contains two types of highly divergent chitinases. A similar situation is observed with several other chitinolytic bacteria, including *B. circulans* WL-12 [10], *Strep. lividans* [11] and *Aeromonas* sp. [12], which also possess chitinases belonging to different subfamilies.

HCA analysis of the other regions of the catalytic domains revealed the presence of a third domain in all subfamily A chitinases, corresponding to the insertion of an $\alpha + \beta$-fold region between the seventh and eighth β-strands of the $(\beta/\alpha)8$-barrel observed in the 3D-structure of chitinase A from *Ser. marcescens* QMB1466 [3]. On the other hand, none of the chitinases in subfamilies B and C have this insertion. Therefore the presence or absence of the $\alpha + \beta$-fold insertion domain appears to be subfamily specific. Examples of HCA plots of subfamilies A and B chitinases are shown in Figure 8. A deep substrate-binding cleft is located at the top of the catalytic $(\beta/\alpha)8$-barrel domain of chitinase A, and the $\alpha + \beta$-fold insertion domain forms a wall on one side of the cleft, making the cleft deeper. Therefore, although no 3D-structure is available for chitinases from subfamilies B or C, one can easily imagine that the substrate-binding cleft of chitinases from subfamilies B and C is not as deep as that of chitinases from subfamily A.

The domain organization of chitinases C1 and C2 is summarized together with those of chitinases A and B of this bacterium (*Ser. marcescens* 2170) in Figure 9. Chitinase A of this strain probably displays a 3D-structure identical with that determined for chitinase A of strain QMB1466 [3], as the two enzymes display 95.4 % amino acid identity. Chitinase B was suggested to consist of a catalytic domain and a C-terminal ChBD; this putative ChBD displays significant sequence similarity to well-characterized as well as putative ChBDs of other chitinases (Figure 4). Chitinase C1 is the only *Ser. marcescens* chitinase having an Fn3 domain and a catalytic domain from subfamily B. The reason why this bacterium and other chitinolytic bacteria produce mutiple chitinases is unknown. Brurberg et al. [44] reported that chitinase A of *Ser. marcescens* BJL200 has a higher specific activity towards chitin than chitinase B, and a synergistic effect on chitin degradation was observed upon

combining the two enzymes. Our recent results show that a combination of three chitinases of strain 2170 greatly enhanced the hydrolytic activity with respect to powdered chitin (K. Suzuki, N. Sugawara, M. Suzuki, N. Nikaidou and T. Watanabe, unpublished work). Similar results were also observed with chitinases of *B. circulans* WL-12 (T. Mizutani and T. Watanabe, unpublished work). These observations suggest that multiple chitinases with different properties are necessary for an efficient synergistic hydrolysis of chitinous substrates, a situation analogous to that encountered in the degradation of cellulose by cellulases. In addition to the synergistic effects on a particular substrate, different chitinases may also be beneficial for the digestion of various types of chitin characterized by different degrees of acetylation, crystallinity, crystalline form, etc.

The multiplicity of chitinase genes appears to be a widespread phenomenon, as is the co-production of chitinases belonging to different subfamilies in chitinolytic bacteria. Detailed analysis of the enzymic properties, individual roles and 3D-structures of individual chitinases and their synergism in binary or tertiary mixtures should shed light on the significance of the presence of multiple chitinases in chitinolytic bacteria.

REFERENCES

1 Henrissat, B. (1991) Biochem. J. **280**, 309–316
2 Ohno, T., Armand, S., Hata, T., Nikaidou, N., Henrissat, B., Mitsutomi, M. and Watanabe, T. (1996) J. Bacteriol. **178**, 5065–5070
3 Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A. B., Chet, I., Wilson, K. S. and Vorgias, C. E. (1994) Structure **2**, 1169–1180
4 Terwisscha van Scheltinga, A. C., Kalk, K. H., Beintema, J. J. and Dijkstra, B. W. (1994) Structure **2**, 1181–1189
5 Hart, P. J., Pfluger, H. D., Monzingo, A. F., Hollis, T. and Robertus, J. D. (1995) J. Mol. Biol. **248**, 402–413
6 Blaak, H. and Schrempf, H. (1995) Eur. J. Biochem. **229**, 132–139
7 Morimoto, K., Karita, S., Kimura, T., Sakka, K. and Ohmiya, K. (1997) J. Bacteriol. **179**, 7306–7314
8 Svitil, A. L. and Kirchman, D. L. (1998) Microbiology **144**, 1299–1308
9 Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S. and Tanaka, H. (1994) J. Bacteriol. **176**, 4465–4472
10 Alam, M. M., Mizutani, T., Isono, M., Nikaidou, N. and Watanabe, T. (1996) J. Ferment. Bioeng. **82**, 28–36
11 Miyashita, K., Fujii, T., Watanabe, A. and Ueno, H. (1997) J. Ferment. Bioeng. **83**, 26–31
12 Shiro, M., Ueda, M., Kawaguchi, T. and Arai, M. (1996) Biochim. Biophys. Acta **1305**, 44–48
13 Harpster, M. H. and Dunsmuir, P. (1989) Nucleic Acids Res. **17**, 5395
14 Jones, J. D. G., Grady, K. L., Suslow, T. V. and Bedbrook, J. R. (1986) EMBO J. **5**, 467–473
15 Brurberg, M. B., Eijsink, V. G., Haandrikman, A. J., Venema, G. and Nes, I. F. (1995) Microbiology **141**, 123–131
16 Brurberg, M. B., Eijsink, V. G. and Nes, I. F. (1994) FEMS Microbiol. Lett. **124**, 399–404
17 Gal, S. W., Choi, J. Y., Kim, C. Y., Cheong, Y. H., Choi, Y. J., Bahk, J. D., Lee, S. Y. and Cho, M. J. (1997) FEMS Microbiol. Lett. **151**, 197–204
18 Watanabe, T., Kimura, K., Sumiya, T., Nikaidou, N., Suzuki, K., Suzuki, M., Taiyoji, M., Ferrer, S. and Regue, M. (1997) J. Bacteriol. **179**, 7111–7117
19 Gal, S. W., Choi, J. Y., Kim, C. Y., Cheong, Y. H., Choi, Y. J., Lee, S. Y., Bahk, J. D. and Cho, M. J. (1998) FEMS Microbiol. Lett. **160**, 151–158
20 Suzuki, K., Suzuki, M., Taiyoji, M., Nikaidou, N. and Watanabe, T. (1998) Biosci. Biotechnol. Biochem. **62**, 128–135
21 Fuchs, R. L., McPherson, S. A. and Drahos, D. J. (1986) Appl. Environ. Microbiol. **51**, 504–509
22 Palomar, J., Guasch, J. F., Regue, M. and Vinas, M. (1990) FEMS Microbiol. Lett. **57**, 255–258
23 Monreal, J. and Reese, E. T. (1969) Can. J. Microbiol. **15**, 689–696
24 Watanabe, T., Oyanagi, W., Suzuki, K. and Tanaka, H. (1990) J. Bacteriol. **172**, 4017–4022

25 Schagger, H. and von Jagow, G. (1987) Anal. Biochem. **166**, 368–379

26 Silhavy, T. J., Berman, M. L. and Enquist, L. W. (1984) Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

27 Lipman, D. J. and Pearson, W. R. (1985) Science **227**, 1435–1441

28 Imoto, T. and Yagishita, K. (1971) Agr. Biol. Chem. **35**, 1154–1156

29 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275

30 Jeuniaux, C. (1966) Methods Enzymol. **8**, 644–650

31 Yamada, H. and Imoto, T. (1981) Carbohydr. Res. **92**, 160–162

32 Molano, J., Duran, A. and Cabib, E. (1977) Anal. Biochem. **83**, 648–656

33 Boulware, D. W., Goldsworthy, P. D., Nardella, F. A. and Mannik, M. (1985) Mol. Immunol. **22**, 1317–1322

34 Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M. and Tanaka, H. (1993) J. Biol. Chem. **268**, 18567–18572

35 Tormo, J., Lamed, R., Chirino, A. J., Morag, E., Bayer, E. A., Shoham, Y. and Steitz, T. A. (1996) EMBO J. **15**, 5739–5751

36 Watanabe, T., Suzuki, K., Oyanagi, W., Ohnishi, K. and Tanaka, H. (1990) J. Biol. Chem. **265**, 15659–15665

37 Little, E., Bork, P. and Doolittle, R. F. (1994) J. Mol. Evol. **39**, 631–643

38 Lètoffè, S., Ghigo, J. M. and Wandersman, C. (1993) J. Bacteriol. **175**, 7321–7328

39 Akatsuka, H., Kawai, E., Omori, K., Komatsubara, S., Shibatani, T. and Tosa, T. (1994) J. Bacteriol. **176**, 1949–1956

40 Binet, R., Létoffé, S., Ghigo, J. M., Delepelaire, P. and Wandersman, C. (1997) Gene **192**, 7–11

41 Gaboriaud, C., Bissery, V., Benchetrit, T. and Mornon, J. P. (1987) FEBS Lett. **224**, 149–155

42 Lemesle-Varloot, L., Henrissat, B., Gaboriaud, C., Bissery, V., Morgat, A. and Mornon, J. P. (1990) Biochimie **72**, 555–574

43 Saitou, N. and Nei, M. (1987) Mol. Biol. Evol. **4**, 406–425

44 Brurberg, M. B., Nes, I. F. and Eijsink, V. G. (1996) Microbiology **142**, 1581–1589

45 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) Nucleic Acids Res. **22**, 4673–4680