

A chitin-binding domain in a marine bacterial chitinase and other microbial chitinases: implications for the ecology and evolution of 1,4- β -glycanases

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To examine the ecology and evolution of microbial chitinases, especially the chitin-binding domain, one of the chitinase genes (*chiA*) from the marine bacterium *Vibrio harveyi* was analysed. The deduced amino acid sequence of ChiA is not very similar overall to other proteins, except for two regions, the putative catalytic and chitin-binding domains. Among all bacterial chitinases sequenced to date, there is no relationship between percentage similarity of catalytic domains and chitin-binding domains in pairwise comparisons, suggesting that these two domains have evolved separately. The chitin-binding domain appears to be evolutionarily conserved among many bacterial chitinases and is also somewhat similar to cellulose-binding domains found in microbial cellulases and xylanases. To investigate the role of the chitin-binding domain, clones producing versions of ChiA with or without this domain were examined. One version with the domain (ChiA1) bound to and hydrolysed chitin, whereas a truncated ChiA without the putative chitin-binding domain (ChiA2) did not bind to chitin, but it could hydrolyse chitin, although not as well. ChiA1 diffused more slowly in agarose containing colloidal chitin than ChiA2, but diffusion of the two proteins in agarose without colloidal chitin was similar. These results indicate that the chitin-binding domain helps determine the movement of chitinase along *N*-acetylglucosamine strands and within environments containing chitin.

Keywords: *Vibrio harveyi*, chitinase, chitin-binding domain, cellulose-binding domain

INTRODUCTION

Cellulose and chitin, the first and second most abundant biopolymers in nature, are composed of 1,4- β -linked pyranose units whose glycosidic bonds are hydrolysed by cellulases and chitinases, respectively. These enzymes consist of discrete domains, which can be arranged in different orders in different proteins (for reviews, see Béguin, 1990; Gilkes *et al.*, 1991; Warren, 1996). Perhaps the two most important domains are those involved in catalysing the hydrolysis of the glycosidic bonds (the catalytic domain) and in mediating the binding of the

enzyme to the substrate (the carbohydrate-binding domain). Cellulose-binding domains are not essential for catalytic activity, but they do control the specific activities of the enzyme on different forms of cellulose (Coutinho *et al.*, 1993). Less is known about chitin-binding domains, especially in bacterial proteins (Graham & Sticklen, 1994; Warren, 1996).

Chitin-binding domains in plant proteins have been examined extensively (Graham & Sticklen, 1994). Certain positions within the chitin-binding domain, notably the eight cysteines, are highly conserved among plant chitinases (Graham & Sticklen, 1994). In addition to chitinases, plants have chitin-binding proteins (lectins) without chitinase activity, which also contain the cysteine-rich chitin-binding domain (Graham & Sticklen, 1994). In contrast, the chitin-binding domain of chitinase A1 from *Bacillus circulans* WL-12 contains

Abbreviation: MUF-diNAG, 4-methylumbelliferyl β -D-N,N'-diacetylchitobioside.

The GenBank accession number for the sequence reported in this paper is U81496.

only one cysteine and is not similar to that of plants (Watanabe *et al.*, 1994). Recently, Morimoto *et al.* (1997) found several other similar residues among chitin-binding domains from bacterial chitinases and observed that removal of the binding domain decreased hydrolysis of unprocessed chitin by a chitinase from *Clostridium paraputrificum*. The role of the chitin-binding domain in hydrolysing chitin by other chitinases is not clear.

In addition to the chitin-binding domain, other regions of bacterial chitinases are not very similar to plant chitinases nor to each other (Watanabe *et al.*, 1993). Watanabe *et al.* (1993) identified only four amino acids in the catalytic domain that are conserved between bacterial and plant class III chitinases. The diversity in chitinases is not unexpected because the structure of chitin is very diverse due to variation in arrangement of strands, degree of deacetylation and cross-linking to other structural components (Gooday, 1990). However, recently Chernin *et al.* (1997) did find several similar regions among six bacterial chitinases and suggested that enzymes from closely related bacteria are more similar than enzymes from distantly related bacteria. In contrast, catalytic domains of cellulases do not appear to follow phylogeny, although cellulose-binding domains in cellulases from a single bacterium can be quite similar (Gilkes *et al.*, 1991).

To gain a better understanding of bacterial chitinases and especially the chitin-binding domain, we examined a chitinase (ChiA) from the marine bacterium *Vibrio harveyi*. Chitin is potentially an important nutrient source for marine bacteria since it is produced by many marine organisms, including zooplankton and several phytoplankton species (Gooday, 1990). Previous work on *V. harveyi* has identified six separate chitinase genes (Svitil *et al.*, 1997), and two genes encoding chitin degradation enzymes have been at least partially sequenced (Jannatipour *et al.*, 1987; Soto-Gil, 1988). One of the partially sequenced genes encodes a chitinase, ChiA (Soto-Gil, 1988). Here we analysed the complete sequence of *chiA* and identified a chitin-binding domain specific to bacteria. The binding domain appears to help determine the movement of the enzyme along chitin strands and in its environment.

METHODS

Strains and vectors. *V. harveyi* strain BB7 was grown in LM (1% tryptone, 0.5% yeast extract, 2% NaCl). *Escherichia coli* strains XL-1 Blue MRF' and SOLR were grown in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl), unless otherwise noted. The phagemid pBluescript II KS(−) (Stratagene) was used for subcloning.

Genomic library construction and screening. Previous work by Soto-Gil (1988) had partially sequenced what was identified as the gene encoding the main chitinase in *V. harveyi* BB7, *chiA*. To complete the sequencing and analysis of this chitinase we constructed and screened two *V. harveyi* genomic libraries. The first library was constructed in pBluescript II KS(−), while the second was a plaque library prepared in λ ZAP II (both vectors from Stratagene) (Svitil *et al.*, 1997).

The libraries were screened using a digoxigenin-labelled *chiA1* probe, which corresponds to the portion of *chiA* sequenced by Soto-Gil (1988). Positive clones or plaques were detected by colour (alkaline phosphatase) using the Genus kit (Boehringer Mannheim). After isolating positive plaques, the plasmid [pBluescript II SK(−) containing the insert] was excised from the phage according to the manufacturer's instructions. The plasmids obtained were reprobated to verify the positive results. DNA hybridizations were performed at 65 °C as recommended in the Genus kit manual. The probe was prepared by PCR following the procedure of Lion & Haas (1990), except 10 × DIG DNA labelling mixture (Boehringer Mannheim) was substituted for the nucleotides. PCR was performed with an annealing temperature of 45 °C on a DNA Thermal Cycler (Perkin Elmer Cetus) using *Taq* polymerase from either Perkin Elmer Cetus or Promega. The primers (5'-ATGTTAAACGTAAAGCTCTACAA and 5'-CTGCAGACCAGACACGCTCAGTTC), which were based on the sequence data from Soto-Gil (1988), were used to amplify most of the coding region (see Fig. 1). The single PCR product was 1.7 kb, the same length as predicted from the sequence information. Primers were synthesized by Genosys.

Colonies and plaques which hybridized to the probe were also screened for chitinase activity using a chitin analogue, 4-methylumbelliferyl β -D-N,N'-diacetylchitobioside (MUF-diNAG; Sigma), which fluoresces when hydrolysed. Activity in plaques or colonies on agar plates was detected by spraying or spotting 50 μ M MUF-diNAG (5 mM stock in N,N-dimethylformamide; Sigma) in 0.1 M phosphate buffer, pH 7.5. The plates were incubated at 37 °C for 10–30 min and then viewed under UV light. Phage in plaques exposed to this screening method are still able to infect *E. coli* and be amplified.

Sequencing of *chiA*. DNA sequencing was performed using a *Taq* DyeDeoxy Cycle Sequencing kit (Applied Biosystems). Nested deletions from pALS101 (see Results) were prepared in both directions using a Stratagene Exo/Mung bean kit. Sequencing reactions were then performed using primers for the vector. The plasmid pJM2 was used to obtain the missing C-terminal portion of *chiA*. The first strand was sequenced by 'walking' (Giesecke *et al.*, 1992) downstream from the end of *chiA1* using primers synthesized by either Genosys or DNAgency. The second strand was then sequenced using primers synthesized from the first strand. The region of *chiA* between the end of pALS101 to the end of *chiA1* was sequenced once to confirm the previous sequence data (Soto-Gil, 1988). Both strands from all other regions were sequenced.

Sequence analysis was performed using SeqEd (Applied Biosystems), GDE and GCG programs. Database searches were performed using FASTA from the European Bioinformatics Institute World Wide Web server. Default settings were used with the programs.

Construction of pChiA1 and pChiA2. A plasmid, pChiA1, encoding a protein containing both the putative catalytic and chitin-binding regions was prepared using genomic DNA from *V. harveyi* BB7. The DNA was amplified using the *chiA1* primers mentioned above, made blunt-ended with Mung bean nuclease and ligated into blunt-ended, alkaline-phosphatase-treated pBluescript II KS(−). Another plasmid, pChiA2, was prepared by ligating the *Pst*I–*Fsp*I fragment from pChiA1, containing most of the gene, into pBluescript II KS(−) digested with *Pst*I and *Sma*I. After electroporating the ligation reactions into XL-1 Blue MRF' (Svitil *et al.*, 1997), colonies were screened for chitinase activity with MUF-diNAG.

To examine the chitinases produced by these clones in further detail, crude protein extracts were prepared from IPTG-induced cells as described before (Svitil *et al.*, 1997). Briefly, sonicated cells were incubated with sarcosyl (0.1%, w/v, final concentration) for 1 h on ice, then centrifuged to remove cell debris. These crude protein extracts were used to test whether or not the two versions of ChiA could specifically bind to chitin. The procedure was as previously described (Montgomery & Kirchman, 1993). Protein extracts were also tested for the capacity to hydrolyse two types of chitin. Degradation of glycol chitin after SDS-PAGE was tested as previously described (Svitil *et al.*, 1997). Degradation of colloidal chitin was examined by incubating cell extracts with various concentrations of chitin at 37 °C for 4 h, after which 3 M HCl was added. The mixture was then centrifuged to remove precipitated protein and undegraded chitin. The supernatant containing soluble by-products from the degraded chitin was hydrolysed at 95 °C for 4 h in vacuum-sealed ampules. These conditions were effective in hydrolysing oligomers of N-acetylglucosamine to glucosamine, which was measured by HPLC with pulse-amperometric detection (Borch & Kirchman, 1997). To examine the by-products of hydrolysis by ChiA, the supernatant containing soluble by-products was analysed by HPLC without heat and acid hydrolysis. The HPLC method could separate glucosamine, N-acetylglucosamine and the dimer of N-acetylglucosamine; the trimer and tetramer could be resolved from the other compounds, but they formed two overlapping peaks on the chromatogram. Samples were diluted 500- to 1000-fold with deionized water before HPLC analysis. Fucose was used as an internal standard.

To examine the relative diffusion rate of the two forms of ChiA, protein extracts were added to wells in agarose plates with and without colloidal chitin. After 24 h, melted agarose containing 10 µM MUF-diNAG was poured over the plate, incubated for 1 h, then observed with UV light to detect the presence of the enzyme, especially in the agarose plate without colloidal chitin. The diameter of the fluorescence area around the centre well was measured. In all degradation and diffusion experiments, equal amounts of protein extract of clones containing either ChiA1 or ChiA2 were used.

RESULTS

Identification of *chiA*-containing clones

Two genomic libraries were constructed from *V. harveyi* BB7 and screened to identify clones containing the gene for a major chitinase, *chiA*. To identify an intact *chiA* gene the libraries were probed with *chiA1*, which was made from a part of the coding region (map of *chiA* and surrounding regions in Fig. 1). In the plasmid library (550 colonies), the probe hybridized to a single clone (pPV1) with a 7 kb insert; this clone did not have chitinase activity. Using Southern blots, the region of pPV1 hybridized by *chiA1* was identified and subcloned (pALS101; Fig. 1). Sequence data from the subclone and a restriction map of pPV1 revealed that only the 5'-terminal portion of *chiA* was present in pALS101 and pPV1. In the plaque library (4000 plaques), the *chiA1* probe hybridized to at least six plaques, none of which had chitinase activity. Restriction mapping and partial sequencing identified clones containing the beginning (pJM1) or end (pJM2) of *chiA*, but none with the entire

gene (Fig. 1). Attempts to construct a full-length *chiA*, by joining the cloned portions or amplification from primers, were unsuccessful, but the truncated portion originally obtained by Soto-Gil (1988) was successfully produced using PCR and was cloned (see below).

Sequence analysis of *chiA*

The complete sequence of *chiA* was obtained by sequencing the clones outlined in Fig. 1. The ORF of *chiA* encodes a 729 residue protein with a calculated molecular mass of 92 kDa. Analysis of the predicted amino acid sequence revealed characteristic features of a signal peptide which is required for membrane translocation (Inouye & Halegoua, 1980; von Heijne, 1986). The putative site for cleavage of this signal peptide is after the twenty-fourth amino acid. The resulting peptide is calculated to be 89 kDa which is close to the molecular mass of the excreted chitinase, 85 kDa (Soto-Gil, 1988; Montgomery & Kirchman, 1993). A clone (pChiA1; see below) with a portion of *chiA* produces a protein which is recognized by antibodies raised against the 85 kDa excreted chitinase (Svitil *et al.*, 1997). These data confirmed that the sequenced ORF does encode the excreted chitinase, ChiA.

The sequence contains the elements necessary for transcription. A promoter region is located upstream of the ORF. The initiation codon is preceded by a ribosome-binding site. Upstream of the ribosome-binding site is a region very similar to the *E. coli* consensus -35/-10 sites, and the AT content is high (81%) where strand-opening is predicted to occur, from the -10 region through the first three codons of the ORF.

Comparison with other chitinases

Catalytic domain. A search of the protein database by FASTA revealed several chitinases with portions similar to two regions in ChiA (Fig. 2). One section in the N-terminus appears to be the catalytic domain, based on its similarity (24–29%) to the active portions in several plant, fungal and bacterial chitinases (Watanabe *et al.*, 1993). For example, the *V. harveyi* ChiA is 29% similar to the catalytic domain of ChiD from *B. circulans* WL-12 (Watanabe *et al.*, 1992; ChiA residues 5–320) (Fig. 2). The catalytic site for chitinases contains four residues, two of which are essential for activity, and which are conserved among various prokaryotic, yeast and plant class III chitinases (Watanabe *et al.*, 1993). ChiA of *V. harveyi* contains three of the four residues in this conserved region, amino acids 110–158 (Fig. 3). This conserved area is located in the region similar to *B. circulans* ChiD. Additional residues appear conserved among the bacterial chitinases (Fig. 3), although overall these chitinases share little similarity. We determined a consensus catalytic domain consisting of 13 residues which are present in at least 69% of the sequences (Fig. 3).

Pairwise comparisons using BESTFIT revealed that the

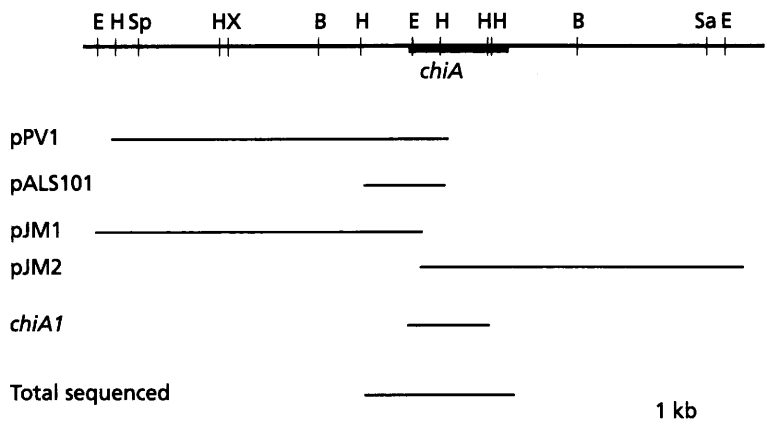


Fig. 1. Restriction map of *chiA* and surrounding DNA. Clone inserts and sequenced regions are given. B, *Bam*HI; E, *Eco*RI; H, *Hind*III (not all sites identified); Sa, *Sal*I; Sp, *Spe*I; X, *Bst*XI.

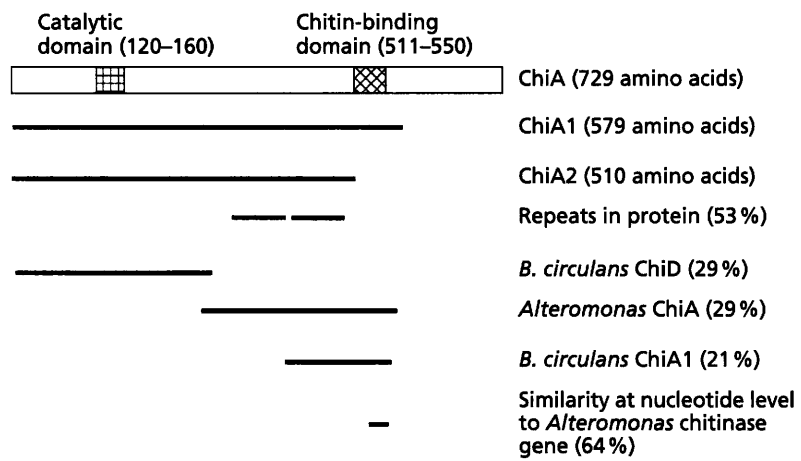


Fig. 2. Schematic representation of regions identified in *ChiA*. The regions with similarities to chitinases from other bacteria are identified; the percentage identity is given in parentheses.

catalytic domains can be divided into at least four groups (Fig. 3). Group I (93 % mean similarity) consists of 4 chitinases, all from γ -proteobacteria (Gram-negative). Members of Group II (85 % mean similarity) include a chitinase from a γ -proteobacterium and three chitinases from Gram-positive high-G + C-content bacteria. Group III (83 % mean similarity) is comprised of five chitinases all from Gram-positive low-G + C-content bacteria. Group IV (71 % mean similarity) contains three chitinases from vibrios (including *ChiA* from *V. harveyi*) and three chitinases from Gram-positive bacteria. The domains which could not be placed into a group were assigned to Group IV (51 % mean similarity). Clearly, these comparisons indicate that the evolution of the catalytic domain does not follow bacterial phylogeny and that taxonomically related bacteria may have dissimilar catalytic domains. In fact, three chitinases from a single species (*Aeromonas* sp.) were not very similar (46 % mean similarity). In pairwise comparisons, there was no correlation ($r = 0.192$; $n = 136$) between percentage similarity of catalytic domains and of 16S rRNA genes.

Chitin-binding domain. The second region in *V. harveyi* *ChiA* which is similar to other proteins as revealed by

FASTA appears to be the chitin-binding domain (Fig. 2). Watanabe *et al.* (1990) first identified a chitin-binding domain in *ChiA1* from *B. circulans* and later found a similar domain in another *B. circulans* chitinase, *ChiD*, by sequence analysis (Watanabe *et al.*, 1992). A search of *V. harveyi* *ChiA* revealed a region (amino acids 511–551) which is 37 % and 26 % identical to *B. circulans* *ChiA1* (CA) and *ChiD* (ND) binding regions, respectively (Fig. 4). This region is also similar to the *Alteromonas* chitinase (Figs 2, 4), even at the nucleotide level, suggesting that it is conserved because of chitin-binding capacities. Searches using this proposed chitin-binding region revealed several chitinases containing this domain (Fig. 4). Because many residues are conserved in this domain, we were able to generate a consensus binding sequence, which consists of 15 residues (17 including conserved aromatic residues) conserved in at least 69 % of the sequences (Fig. 4). The latter portion of this domain, which is absent from the *B. circulans* chitinases and present in only the Gram-negative bacteria, contains a conserved aromatic region with at least one tryptophan (corresponding to *ChiA* residues 537 and 538).

The chitin-binding domains cannot be classified into the same groups as defined by the catalytic domains, based

				↓ ↓			↓ ↓ *
I (93%)	γV	Alt A	261	PDLKILPSVGGWTLSD ----- PFHGFTNKAN-RDTFVASVKQFLKT -- WK-F --- YDGV DDIDWE FPGGD			
	γV	Aeo c	263	PDLKILPSVGGWTLSD ----- PFYFLGDKTK-RDTFVASVKEFLQT -- WK-F --- FDGV DDIDWE FPGGQ			
	γE	Sma A	263	PDLKILPSIGGWTLSD ----- PFFFMGDKVK-RDRFVGSVKEFLQT -- WK-F --- FDGV DDIDWE FPGGK			
	γE	Egg	263	PDLKILPSIGGWTLSD ----- PFFFMGDKVK-RDRFVGSVKEFLQT -- WK-F --- FDGV DDIDWE FPGGG			
II (85%)	γE	Sma B	85	PSLRIMFSIGGWYY SNDLG VSHANYVNAVKTPAARTKFAQSCVRIMKD -- YG --- FDGV DDIDWE YPQAA			
	hi	Stm 63	330	PHIKILYSFGGW TWSGGF ----- PDAVKNPAAFAK SCHDLVEDPRWADV -- FDGID LDWE YPNAC			
	hi	Stm C	329	PNIKILYSFGGW TWSGGF ----- PDAVKNPAAFAK SCHDLVEDPRWADV -- FDGID LDWE YPNAC			
	hi	Stm 40	137	PHIKVLWSFGGW TWSGGF ----- ADAAKRPAAFAQSCYNLVHDP RWDGV -- FDGID -- WEYPNAC			
III (83%)	lo	Bcc A1	52	PNLKTIISVGGW TWSN ----- RFSDVAATAATREVFANSAVD FLRK -- YN --- FDGV DLDE WYFPVSG			
	lo	Bcl	149	PHLKTIFISVGGW TWSN ----- RFSDVAADPVARGNFASAVE FLRK -- YG --- FDGV DLDE WYFPVSG			
	lo	Bct	157	PHLKTIIISVGGW TWSN ----- RFSDMAADEKTRKVF FAESTVAFLPA -- YG --- FDGV DLDE WYFPVSG			
	lo	Clf C	112	PHVKTLISVGGW TESK ----- YFSDVALTEESRNTFADSC VEFIRT -- YR --- FDGV DDIDWE YPVSG			
	lo	Kuz	153	PNLKTIISVGGW TWSN ----- RFSDVAATAATREVFANSAVD FLRK -- YN --- FDGV DLDE WYFPVSG			
IV (71%)	γV	ChiA	110	QGRSVLLALGG ----- ADAHVELETGDE-R-AFADE --- IRLTERYG --- FDGLD IDLE QAAVT			
	γV	Aeo 1	302	RGKKVLISIGG ----- QNGEIQLTTTAA-RDNFVNSISAI IDK -- WS --- LDGLD IDFEGH SLQ			
	γV	Aeo 3	318	KGKKVLSLGG ----- QDGSITLNTASD-GDNFVNSLYGILT K -- YG --- FDGID LDLE RAARE			
	lo	Bcc D	254	QGKKVLISMGG ----- ANGRIELTATKKRQ QFEDSLKSIIST -- YG --- FNGLD IDLE GSSLS			
	hi	Stm X	336	AGKKVLISIGG ----- QNGQVQLTTTAA-RDTFVSSVSKI ID -- YG --- LDGLD IDFEGH SLS			
V (51%)	γV	Aeo II	96	QGGRLIISFGG ----- AAVPMWKPAVPAPRWPRW MPCCNAPACV ----- PLDFD IEGS QLS			
	γV	Aeo 2	285	LGGGVIISSGGW NASD ----- IVRTCTDAR ----- SIATVYENV LER -- FGA -- DHLD SDPE HGDQE			
	γE	Ewa	80	ANRQVIVSFGG ----- ASNADISTKFTVDQLVQTYT DVVQKFKAKQ ----- LD FDLE NGQYD			
	β	Jan	357	PNLKLFI SLGGWS SKNFSVGSATDALRKQ MVRSCIDIYIKGNLPVQGGV GAAANI FDGID IDWE YPVGG			
	lo	Bcc C	104	PDLKVLLSVGGW G ----- ANGFSDAALTDASRTTFAD SIVQLVTSNN ----- LDGV DLDE WYPTNP			
	hi	Sae	69	AGGDVIPSIGG YSGSKL ----- GEVCQDSQSLAGAYQK -- VIDAYGLK ----- A --- IDVD IEATE FE			
consensus				K S GG	F S	@	FDG D D E

Fig. 3. Comparison of putative catalytic region in bacterial chitinases. Conserved residues are in bold. Arrows indicate the four residues identified by Watanabe et al. (1993) as being conserved among bacterial and plant class III chitinases; *, residue necessary for activity (Watanabe et al., 1993); @, conserved aromatic residue; γE, Gram-negative γ-subdivision, family Enterobacteriaceae; γV, Gram-negative γ-subdivision, family Vibrionaceae; β, Gram-negative β-subdivision; lo, Gram-positive low-G+C-content; hi, Gram-positive high-G+C-content; Alt A, *Alteromonas* sp. strain O-7 chitinase (accession number D13762; Tsujibo et al., 1993b); Aeo c, *Aeromonas caviae* chitinase (U09139; Sitrit et al., 1995); Sma A, *Serratia marcescens* chitinase A (X03657; Jones et al., 1986); Egg, *Enterobacter agglomerans* chitinase (U59304; Chernin et al., 1997); Sma B, *S. marcescens* ChiB (X15208; Harpster & Dunsmuir, 1989); Stm 63, *Streptomyces plicatus* chitinase 63 (M82804; Robbins et al., 1992); Stm C, *Streptomyces lividans* chitinase C (D12647; Fujii & Miyashita, 1993); Stm 40, *Streptomyces thermoviolaceus* chitinase 40 (D14536; Tsujibo et al., 1993a); Bcc A1, *Bacillus circulans* WL-12 chitinase A1 (J05599; Watanabe et al., 1990); Bcl, *Bacillus licheniformis* TP chitinase (U71214); Bct, *Bacillus thuringiensis* chitinase (U89796); Clf A, *Clostridium thermocellum* ChiA (Z68924); Kuz, *Kurthia zopfii* chitinase (D63702); ChiA, *Vibrio harveyi* BB7 chitinase A (this study); Aeo 1 and 3, *Aeromonas* sp. NO. 105-24 chitinases ORF 1 and 3, respectively (D63139, D63141; Shiro et al., 1996); Bcc D, *B. circulans* WL-12 chitinase D (D90534; Watanabe et al., 1992); Stm X, *Streptomyces olivaceoviridis* exochitinase (X71080; Blaak et al., 1993); Stm A, *St. lividans* 66 ChiA (D13775; Miyashita & Fujii, 1993); Aeo II, *Aeromonas* sp. NO. 105-24 chitinase II (D31818; Ueda et al., 1994); Aeo 2, *Aeromonas* sp. NO. 105-24 chitinases ORF 2 (D63140; Shiro et al., 1996); Ewa, *Ewingella americana* chitinase (X90562); Jan, *Janthinobacterium lividum* chitinase chitin-binding domain 2 (U07025; Gleave et al., 1995); Bcc C, *B. circulans* WL-12 ChiC (D89568; Alam et al., 1995); Sae, *Saccharopolyspora (Streptomyces) erythraeus* chitinase (P14529; Kamei et al., 1989).

on pairwise comparisons using BESTFIT. There is no correlation between percentage similarities in the catalytic and chitin-binding domains ($r = 0.183$; $n = 65$) in pairwise comparisons. However, unlike the catalytic domain, chitin-binding domains in chitinases from a single organism can be similar. For example, the five chitinases from *Aeromonas* sp. all share high similarity (mean 88%).

To verify that the putative chitin-binding domain is necessary for binding to chitin, two clones expressing

modified functional versions of ChiA were produced (Fig. 5). The protein produced from the first plasmid, pChiA1, contains the proposed catalytic and chitin-binding domains, while the other plasmid, pChiA2, encodes a protein lacking the putative chitin-binding domain. Sequencing of the second plasmid (pChiA2) confirmed the 3' end of the gene and the lack of the putative chitin-binding domain (Fig. 5). The chitinases encoded by the two plasmids hydrolyse glycol chitin after SDS-PAGE, with apparent sizes of 60 and 67 kDa for ChiA2 and ChiA1, respectively (Fig. 6a). These sizes

γ	ChiA	511	AAAWDANTVYVEGDQVSHD GATWVAGWYTRGEEPG--TT--GE---W
γ	Alt A	777	-PTWDRSTVYVGGDRVIHNSNVFEAKWWTQGEPEG--TAD----VW
γ	Aeo c1	772	-- AWSAGTVYINTNDKVS HKQLVWQAKYWTQGNEPS--RTAD----QW
γ	Aeo c2	820	---WDAGVVYINGGDTVSHNGRKWKAKYWTQ DEPG ---KAA----VW
γ	Aeo II	392	AATWSSSTAYNGGATVAY NGHNYQAKWWTQGNVPSSST---GDGQPW
γ	Aeo 1	122	APVWSSSTAYNGGWQVS YNGHTYTAKWWTQGNVPSSST---GDGSPW
γ	Aeo 2	139	AAAWSAGTAYNGGTQVS YNGRNYTAKWWTQGNVPSSST---GEGQPW
γ	Aeo 3	122	-- AWSSGTAYNGGAQVS YNGHLYTAKWWTQGNVPSSST---GDGQPW
γ	Aeo 4	146	-- AWNTGTAYNGGVQVS YNGHTYTAKWWTQGNIPSSST---GDGQPW
γ	Sma B	453	APAYVPGTTYAQ ALVSYQGYVWQTKWGYITSAPGS-----D-SAW
β	Jan	151	ALAWAAGTAYSAGATVS YAGTNYRANYWTQGDNPSTSSGGAGTGKPW
lo	Bcc CA	653	VSAWQVNTAYTAGQLV TYNGKTYKCL-----QPH--TSLAG----W
lo	Bcc ND	31	AAQWQAGTAYKQ GDLYTYLNKDYECI-----QPH--TALTG----W
consensus			W---T-Y--G--VS--G--@-A-W@T-G--P(-)T(-)G(--)W

Fig. 4. Alignment of protein sequences to identify similarities in the putative bacterial chitin-binding domains. Conserved residues are in bold and closely related amino acids are underlined. Abbreviations and symbols are the same as in Fig. 3, except that dashes indicate a variable number of intervening residues; Aeo c1 and c2, regions 1 and 2 in *Aeromonas caviae* chitinase (Sitrit *et al.*, 1995); Aeo 4, *Aeromonas* sp. NO. 105-24 ORF 4 (D63142; Shiro *et al.*, 1996); Bcc CA, chitin-binding domain identified in *Bacillus circulans* WL-12 chitinase A1 (Watanabe *et al.*, 1990); Bcc ND, chitin-binding domain identified in *B. circulans* WL-12 chitinase D (Watanabe *et al.*, 1992).

2341	TGTGACTGACGGTTCGAAC	↓	TGCGCAGCAGCATGGGATGCTAATACAGTTTATGTCGAAGG
	V T D G S N C		A A A W D A N T V Y V E G
2401	TGATCAGGTATCCACGATGGAGCAACTTGGGTGGCGGGTGGTACACTCGTGGTGAAGA		
	D O V S H D G A T W V A G W Y T R G E E		
2461	GCCGGGAACAACCTGGTGAGTGGGCGTTTGGAAAGAAAGCGTCAGACAGTTCATGTGGTGG		
	P G T T G E W G V W K K A S D S S C G G		
2521	CAACCCAAGTCCAGGTGGTGACGTAGAACTGAGCGTGTCTGGTCTGCAGTCTGAATACAT	↓	
	N P S P G G D V E L S V S G L Q S E Y I		

Fig. 5. Partial nucleotide sequence of *chiA*, showing the end of pChiA1 and pChiA2. The numbering corresponds to that in the sequence reported to GenBank, accession number U81496. The deduced amino acid sequence is given below the nucleotide sequence. The end of pChiA1 (↓) and pChiA2 (↓) are shown above the sequence. The nucleotide region similar to *Alteromonas* sp. strain O-7 chitinase (64%; Tsujibo *et al.*, 1993b) is underlined once and the putative chitin-binding domain is underlined twice.

are close to the size predicted for these clones, 62 and 70 kDa, respectively.

To test the role of the putative chitin-binding domain in mediating binding of ChiA to chitin, extracts of *E. coli* expressing either ChiA1 or ChiA2 were exposed to chitin and proteins binding to chitin were analysed by SDS-PAGE. Fig. 6(b) shows the binding of ChiA1 to chitin whereas ChiA2 does not appear to bind; proteins removed from chitin exposed to ChiA2-containing extracts (Fig. 6, lane 2) are nearly the same as those from *E. coli* bearing the vector without an insert, i.e. the negative control (Fig. 6, lane K). These data confirm the role of the chitin-binding domain in ChiA.

The role of the chitin-binding domain in chitin degradation was examined in greater detail in two series of experiments. In addition to hydrolysing glycol chitin, cell extracts containing ChiA2 degraded colloidal chitin as evident from clearing zones on colloidal chitin plates, but the zones were more opaque. Additional experiments confirmed that loss of the chitin-binding domain decreased the effectiveness of the enzyme. Cell extracts containing ChiA1 hydrolysed colloidal chitin about fivefold more effectively than ChiA2 extracts (Fig. 7). Preliminary HPLC analysis indicated that the major by-

product of ChiA1 hydrolysis was the dimer of *N*-acetylglucosamine (80%), with much smaller amounts of the monomer (10%) and larger oligomers (10%) also present. In contrast, ChiA2 appeared to release relatively more large oligomers (trimers and tetramers) than ChiA1, suggesting that removal of the chitin-binding domain increased relative endochitinase activity of ChiA.

Diffusion of the two forms of ChiA was examined with colloidal chitin plates. The diameter of the clearing zone produced by ChiA2 on colloidal chitin plates was 20% wider than that of the clearing zone produced by ChiA1. The extent of diffusion on agarose plates without colloidal chitin was the same for cell extracts containing either ChiA1 or ChiA2. These results suggest that the chitin-binding domain inhibited diffusion of ChiA1 in the presence of colloidal chitin.

DISCUSSION

Sequences of bacterial chitinases can differ greatly, except for possibly two regions, the catalytic domain and the chitin-binding domain. ChiA from the marine bacterium *V. harveyi* contains these two regions. Like

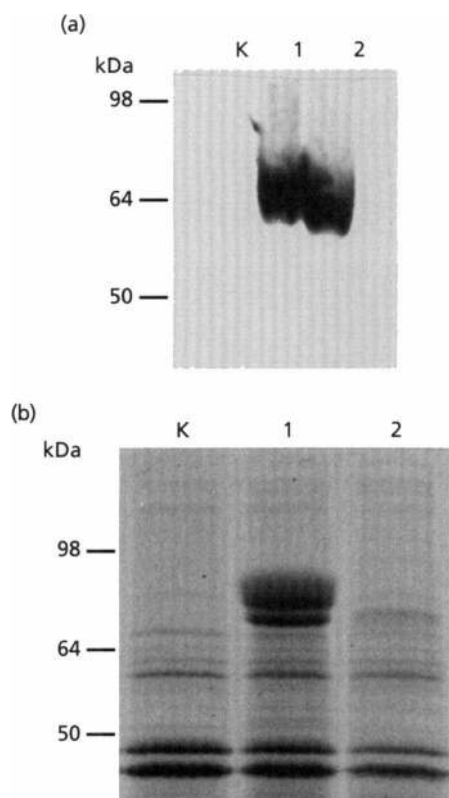


Fig. 6. Chitinase activity and chitin-binding capacity of truncated ChiA proteins. (a) Glycol chitin gel. Dark area indicates hydrolysis of chitin after SDS-PAGE. (b) SDS-PAGE of chitin-binding proteins. Protein extracts were added to chitin, then bound proteins were eluted and analysed by SDS-PAGE. Lanes: K, vector alone [pBluescript II KS(-)]; 1, pChiA1; 2, pChiA2 (missing presumptive chitin-binding domain).

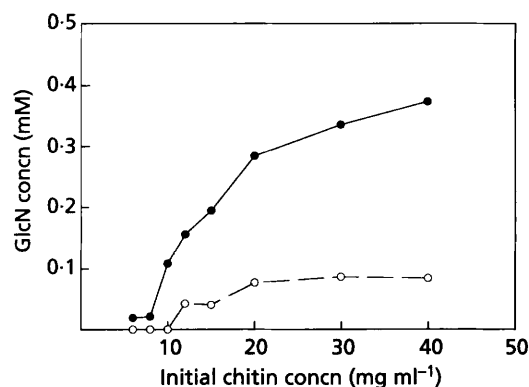


Fig. 7. Degradation of colloidal chitin by truncated ChiA proteins. Various concentrations of colloidal chitin were incubated for 4 h with extracts containing either ChiA1, with chitin-binding domain (●) or ChiA2, without chitin-binding domain (○). Hydrolysis by ChiA1 and ChiA2 was assessed by measuring glucosamine (GlcN) concentration after acid and heat hydrolysis of all soluble polymers.

the arrangement of domains in *B. circulans* ChiA1 (Watanabe *et al.*, 1993), ChiA from *V. harveyi* has its catalytic domain in the N-terminal portion and chitin-binding domain near the C-terminus. Although both regions share similarity with analogous domains in other microbial chitinases, we found more conserved residues (present in at least 69% of the sequences) in the chitin-binding domain (17) than in the catalytic domain (13). The sequence analysis presented here suggests that, in addition to the demonstrated chitin-binding domains in *V. harveyi* ChiA and two *B. circulans* chitinases, binding domains are also present in chitinases from *Alteromonas* sp., *Aeromonas* sp., *Janthinobacterium lividum* and *Serratia marcescens*. Recently, Morimoto *et al.* (1997) found a similar binding domain in ChiB from *C. paraputrificum*.

Chitin-binding domains appear to increase hydrolysis of specific forms of chitin, as has been found for cellulases (Coutinho *et al.*, 1993), but the binding domain may have other roles in chitin hydrolysis as well. Morimoto *et al.* (1997) found that removing the chitin-binding domain from *C. paraputrificum* ChiB reduced hydrolysis of unprocessed chitin but not of colloidal chitin. Apparently, the binding domain only impacts hydrolysis of the more tightly packed strands of unprocessed chitin. In contrast, we found that removing the chitin-binding domain from *V. harveyi* ChiA decreased hydrolysis of even colloidal chitin. However, *V. harveyi* ChiA does not appear to be the same type of chitinase as it produces more large oligomers (trimers and tetramers) of *N*-acetylglucosamine than reported by Morimoto *et al.* (1997) for *C. paraputrificum* ChiB. Even so, we found that removing the binding domain increased relative endochitinase activity of ChiA, suggesting that the chitin-binding domain affects how the enzyme accesses glycosidic bonds within the chitin strands, analogous to what Morimoto *et al.* (1997) observed.

Diffusion of chitinases in an organic matrix containing chitin may be affected by the chitin-binding domain, although this seems to vary with the chitinase. We observed that diffusion of *V. harveyi* ChiA in agarose with colloidal chitin was inhibited by the chitin-binding domain. In contrast, Watanabe *et al.* (1994) did not observe any difference in clearing zone size produced by a *B. circulans* chitinase with and without its chitin-binding domain, suggesting that the domain had no effect on diffusion of the enzyme. The original habitats of the two bacteria may explain the different results. Unlike the aqueous environment of *V. harveyi*, *B. circulans* was originally isolated from soil (Tanaka & Phaff, 1965) where diffusion is restricted. Chitinases of aquatic bacteria like *V. harveyi* may be selected to bind more tightly to chitin and thus diffuse more slowly in organic detritus suspended in aquatic habitats compared with chitinases selected for in soil environments.

The chitin-binding domain of bacterial chitinases is unlike the domain found in plant chitinases, which contains eight conserved cysteine residues. In contrast, several aromatic amino acids, principally tryptophan, are prominent among the residues conserved in the

Chitinases	ChiA	514	<u>W</u> DANTVY- <u>V</u> EGDQV- <u>S</u> HDGATWVAGW- <u>Y</u> TRGEEP <u>G</u> T <u>T</u> GEW
	consensus		W---T-Y---G--V-S--G--*-A-W-*T-G--P-T-G-W
	Cex	391	<u>W</u> NTGFTAN <u>V</u> TVKNT <u>S</u> SAPVDG <u>W</u> T <u>L</u> TFSF <u>S</u> PGQO <u>V</u> - <u>T</u> Q-AW
Cellulases	CenA	45	<u>W</u> PGGFGAN <u>V</u> TITNLGD-PVSSW <u>K</u> LDW <u>T</u> YTAG <u>Q</u> RI-QQ <u>L</u> -W
	CenB	956	<u>W</u> NVGFTGS <u>V</u> KITNTGT <u>T</u> PLT- <u>W</u> T <u>L</u> GFAP <u>S</u> PGQO <u>V</u> - <u>T</u> QG-W
	Cf1X	215	<u>W</u> NTGFTAS <u>V</u> RVNTGT <u>T</u> ALNGW <u>T</u> L <u>T</u> FPFANG <u>Q</u> TV-QQG-W
	CelA	370	<u>W</u> PGGFQAE <u>V</u> TVKNT <u>G</u> SSPINGW <u>T</u> VQW <u>T</u> LP <u>S</u> GQ <u>S</u> I- <u>T</u> Q <u>L</u> -W
	EndA	874	<u>W</u> NNQFTAVIRVRNNG <u>S</u> SAINRWSVNW <u>S</u> YSDG <u>S</u> RI- <u>T</u> N-SW
	EndB	40	<u>W</u> GSGFTASIRITNNG <u>S</u> SSTINGW <u>S</u> VS <u>W</u> NY <u>T</u> DG <u>S</u> RV- <u>T</u> S-SW
	End1	464	<u>W</u> GSGYQVLIKVKNDSASRV <u>D</u> GW <u>T</u> LKIS-KSEVKI- <u>D</u> S-SW
Xylanases	XynB/C	47	<u>W</u> STGFTANITLKNDTGAAIN <u>N</u> WNVN <u>W</u> QY <u>S</u> -SNRM- <u>T</u> SG-W
	XynA	39	<u>W</u> NTGYTGDITIT <u>N</u> RGS <u>S</u> AINGW <u>S</u> VNWQY <u>A</u> - <u>T</u> NRL-SS-SW

Fig. 8. Comparison of ChiA and consensus chitin-binding domain with bacterial cellulose- and xylan-binding domains. The bacterial cellulose-binding domain alignment is from Gilkes *et al.* (1991). Residues conserved or closely related to amino acids in the ChiA binding domain are in bold or underlined, respectively. The arrows indicate the tryptophan residues shown to be essential for cellulose binding in XynA (Poole *et al.*, 1993). ChiA, *Vibrio harveyi* chitinase A (this study); consensus, chitin-binding consensus sequence, see Fig. 5; Cex, *Cellulomonas fimi* exoglucanase (O'Neill *et al.*, 1986); CenA, *C. fimi* endoglucanase A (Wong *et al.*, 1986); CenB, *C. fimi* endoglucanase B (Meinke *et al.*, 1991a); CflX, *Cellulomonas flavigena* ORF X (Al-Tawheed, 1988); CelA, *Microbispora bispora* endoglucanase A (Yablonsky *et al.*, 1988); EndA, *Pseudomonas fluorescens* subsp. *cellulosa* endoglucanase A (Hall & Gilbert, 1988); EndB, *P. fluorescens* subsp. *cellulosa* endoglucanase B (Gilbert *et al.*, 1990); End1, *Butyrivibrio fibrisolvens* endoglucanase 1 (Berger *et al.*, 1990); XynB/C, *P. fluorescens* subsp. *cellulosa* xylanases B and C (Kellett *et al.*, 1990); XynA, *P. fluorescens* subsp. *cellulosa* xylanase A (Hall *et al.*, 1989).

bacterial chitin-binding domain. Tryptophans have been shown to be involved in the binding of a cellulase to cellulose (Poole *et al.*, 1993) and have been implicated in the binding of a non-catalytic chitin-binding protein to chitin (Schnellmann *et al.*, 1994). Some of the Gram-negative bacteria have another aromatic amino acid (phenylalanine or tyrosine) in place of the second or third tryptophan in the chitin-binding domain. Aromatic amino acids appear to be essential for hydrophobic binding of 1,4- β -glycanases to polysaccharides (Poole *et al.*, 1993).

Fig. 8 illustrates the similarities between the consensus microbial chitin-binding domain and the carbohydrate-binding domains for cellulose and another 1,4- β -glycan, xylan. In addition to conserved tryptophans, several other residues (or conserved replacements) appear in both domains (Fig. 8). The role of these other residues in the binding of 1,4- β -glycanases to their respective substrates is not known. Regardless, the similarity between the two carbohydrate-binding domains suggests that these 1,4- β -glycanases bind to carbohydrates by a similar mechanism. In further support of this hypothesis, it is worthwhile noting that some cellulose-binding domains can bind to chitin (Warren, 1996) and a chitinase has been shown to bind to cellulose (Mori-moto *et al.*, 1997).

ChiA from *V. harveyi* does not have all the domains found in other chitinases. Unlike the chitinases in *B. circulans* (Watanabe *et al.*, 1990, 1992) and *Streptomyces olivaceoviridis* (Blaak *et al.*, 1993), ChiA from *V. harveyi* does not have a fibronectin type III domain, which occurs in a number of prokaryotic extracellular enzymes

(including cellulases) that degrade insoluble polymers (Meinke *et al.*, 1991b; Bork & Doolittle, 1992). However, the *V. harveyi* ChiA may have another region that serves the same purpose. In the N-terminal region of the chitin-binding domain, ChiA from *V. harveyi* contains a pair of repeats which are 68 residues long and 53% identical (Fig. 2). These repeats were found to be cadherin-like domains by Morimoto *et al.* (1997). Cadherins are animal cell proteins involved in calcium-dependent cell adhesion (Takeichi *et al.*, 1988). As Watanabe *et al.* (1994) hypothesized for the fibronectin type III repeats, the repeats in ChiA may help maintain the optimal distance and orientation between catalytic and chitin-binding domains.

This study adds to other evidence indicating that 1,4- β -glycanases arose by mutation and domain shuffling (Gilkes *et al.*, 1991) and that the catalytic and carbohydrate-binding domains of bacterial 1,4- β -glycanases have evolved separately. As in cellulases (Gilkes *et al.*, 1991), the catalytic domains of chitinases from phylogenetically diverse bacteria share some similarity and can be divided into groups independent of phylogeny. Variation in chitin-binding domains also does not appear to follow phylogeny, although the binding domains of chitinases from a single bacterium can be quite similar, unlike the catalytic domains, again analogous to cellulases (Gilkes *et al.*, 1991). Although further work is needed, our results suggest that chitin-binding domains have evolved not only to aid in hydrolysis of different forms of chitin but also in response to factors that affect movement of the enzyme along the substrate and in different environments.

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