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Three *Neocallimastix patriciarum* esterases associated with the degradation of complex polysaccharides are members of a new family of hydrolases

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Acetylesterase and cinnamoyl ester hydrolase activities were demonstrated in culture supernatant of the anaerobic ruminal fungus *Neocallimastix patriciarum*. A cDNA expression library from *N. patriciarum* was screened for esterases using β-naphthyl acetate and a model cinnamoyl ester compound. cDNA clones representing four different esterase genes (*bnaA–D*) were isolated. None of the enzymes had cinnamoyl ester hydrolase activity, but two of the enzymes (BnaA and BnaC) had acetylxylan esterase activity. *bnaA*, *bnaB* and *bnaC* encode proteins with several distinct domains. Carboxy-terminal repeats in BnaA and BnaC are homologous to protein-docking domains in other enzymes from *Neocallimastix* species and another anaerobic fungus, a *Piromyces* sp. The catalytic domains of BnaB and BnaC are members of a recently described family of Ser/His active site hydrolases [Upton, C. & Buckley, J. T. (1995). *Trends Biochem Sci* 20, 178–179]. BnaB exhibits 40% amino acid identity to a domain of unknown function in the CelE cellulase from *Clostridium thermocellum* and BnaC exhibits 52% amino acid identity to a domain of unknown function in the XynB xylanase from *Ruminococcus flavefaciens*. BnaA, whilst exhibiting less than 10% overall amino acid identity to BnaB or BnaC, or to any other known protein, appears to be a member of the same family of hydrolases, having the three universally conserved amino acid sequence motifs. Several other previously described esterases are also shown to be members of this family, including a rhamnogalacturonan acetylesterase from *Aspergillus aculeatus.* However, none of the other previously described enzymes with acetylxylan esterase activity are members of this family of hydrolases.

Keywords: acetylxylan esterase, *Neocallimastix patriciarum*, hydrolases, polysaccharide degradation

INTRODUCTION

Anaerobic ruminal fungi are known to degrade plant cell walls (Akin *et al.*, 1983) and a number of fibrolytic enzymes have been described from members of the genus *Neocallimastix*. Xylanases and cellulases have been purified from *N. frontalis* (Gomez de Segura & Fèvre, 1993; Li & Calza, 1991) and genes for such enzymes have been cloned from *N. patriciarum* (Gilbert *et al.*, 1992; Xue *et al.*, 1992a, b; Tamblyn Lee *et al.*, 1993; Black *et al.*, 1994; Zhou *et al.*, 1994). A common feature of these enzymes is their modular organization. One of the modules is a protein-docking domain (Fanutti *et al.*, 1995), implicated in the formation of cellulosomes (Ali *et al.*, 1995). A very similar domain

Abbreviations: ABX, acetylated birchwood xylan; FAXX, *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl-(1,3)-*O*-β-D-xylopyranosyl-(1,4)-D-xylopyranose; MUTMAC, 4-methylumbelliferyl-7-(*p*-trimethylammonium cinnamate) chloride; α-NA, α-naphthyl acetate; β-NA, β-naphthyl acetate; PAXX, *O*-[5-*O*-(*trans*-*p*-coumaroyl)-α-L-arabinofuranosyl-(1,3)-*O*-β-D-xylopyranosyl-(1,4)-D-xylopyranose; RGAE, rhamnogalacturonan acetylesterase.

The GenBank accession numbers for the nucleotide sequences reported in this paper are U66251–U66253.

also occurs in a xylanase and a mannanase from another anaerobic fungus, a *Piromyces* sp. (Fanutti *et al*., 1995). Feruloyl and *p*-coumaroyl esterases have been purified from *Neocallimastix* strain MC2 (Borneman *et al*., 1991, 1992). Significant extracellular acetylesterase activity was also detected in the MC2 strain (Borneman *et al*., 1990). Acetylesterases able to remove *O*-acetyl groups from xylose residues in xylan and xylo-oligomers are classified as acetylxylan esterases (Biely *et al*., 1985). With 22–50 % of xylose residues being acetylated at the *O*-2 and/or *O*-3 positions (Chesson & Forsberg, 1988), acetylation is an important factor influencing the digestibility of plant cell-wall material in ruminants (Bacon *et al*., 1975). Furthermore, it has been shown that chemical deacetylation of certain xylans increases biodegradability and renders cellulose more accessible (Grohmann *et al*., 1989). Biely *et al*. (1985) suggested that enzymic deacetylation may be a prerequisite for the breakdown of acetylxylan or may enhance the rate of its hydrolysis by other enzymes.

The occurrence of acetylxylan esterases in various micro-organisms has been summarized by Christov & Prior (1993). Only a few fungal acetylxylan esterases have been purified and characterized, including those from *Trichoderma reesei* (Sundberg & Poutanen, 1991) *Penicillium purpurogenum* (Egaña *et al*., 1996; Pangborn *et al*., 1996) and *Schizophyllum commune* (Halgasova *et al*., 1994). Acetylxylan esterase genes have been cloned from the aerobic fungi *Aspergillus niger* (European patent no. 92200685) and *T. reesei* (Margolles-Clark *et al*., 1996), but no acetylxylan esterase genes have been cloned from any ruminal fungi to date.

The limited number of cloned acetylxylan esterases is partly due to the difficulties encountered in finding suitable substrates for screening of libraries for acetylxylan-esterase-expressing clones. Although a technique has been described for detecting acetylxylan esterase activity directly using acetylated xylan (Poutanen *et al*., 1990), this method is cumbersome and was not considered to be suitable for screening a cDNA library. However, a number of acetylxylan esterases have been reported as having activity against α-naphthyl acetate (α-NA) and other artificial substrates (McDermid *et al*., 1990; Sundberg & Poutanen, 1991). A convenient method for detecting such esterase activity using general esterase substrates such as α-NA was described by Rosenberg *et al*. (1975). Although not all esterases that hydrolyse such substrates are acetylxylan esterases, it is likely that under conditions of high levels of synthesis of fibre-degrading enzymes, a significant proportion of esterases able to hydrolyse α-NA, or similar compounds, may be acetylxylan esterases.

In this communication, we report the screening of a cDNA library from *N. patriciarum* for expression of esterases, using the artificial substrate β-naphthyl acetate (β-NA). Preliminary characterization of the activities of four esterases cloned from this organism, including two with acetylxylan esterase activity, is also described, together with an analysis of the sequences of genes encoding three of the enzymes.

METHODS

Microbial strains, vectors and culture media. Host strains for cDNA cloning were *Escherichia coli* XL1-Blue and JM109 (Stratagene). The vectors were lambda ZAP II and pBluescript SK(−) (Stratagene). Recombinant *E. coli* strains were cultured in Luria broth containing 100 μg ampicillin ml^{-1} to select transformants.

Screening of a cDNA library. A cDNA expression library of *N. patriciarum* constructed in lambda ZAP II, described by Xue *et al*. (1992a), was plated in 0·7 % (w/v) agarose overlays containing 10 mM IPTG to induce gene expression using *E. coli* XL1-Blue cells as the host strain. Esterase activity was detected by a method adapted from that described by Rosenberg *et al*. (1975). The plaques were transferred to Whatman no. 1 filter papers, which were then soaked in 0·5 mg β-NA (Sigma) ml^{-1} dissolved in DMSO and 0·5 mg tetrazotized *o*-dianisidine ml^{-1} (Diazo Blue B; Sigma) in 50 mM sodium phosphate buffer (pH 7·4). Positive plaques were harvested and purified.

DNA from all phages expressing esterase activity was amplified by PCR using T3 and T7 sequencing primers. The resulting DNA was digested with restriction endonucleases as recommended by the manufacturer (Promega) for generating the restriction maps of cDNA. The cDNA inserts of selected β-NA esterase-positive phage were recovered from the lambda phage in the form of pBluescript SK(−) in *E. coli* XL1-Blue cells using ExAssist helper phage (Stratagene). Nucleotide sequences were determined by the dideoxynucleotide chain-termination procedure using the Sequenase sequencing kit (Amersham). Synthesis was primed using the T3 and T7 primers, or with internal primers synthesized on an Oligo 1000 DNA synthesizer (Beckman). Computer analysis of DNA and protein sequences was carried out using MacVector (IBI) and the ANGIS (Australian National Genomic Information Service) facility.

Enzyme assays. Enzyme assays were carried out on total cell lysates consisting of *E. coli* JM109 cells grown and collected by centrifugation (10000 ***g*** for 5 min). The cell pellets were then resuspended in 1/50 vol. PBS (137 mM NaCl, 2·7 mM KCl, 10·1 mM Na_2HPO_4, 1·8 mM KH_2PO_4, pH 7·4) and lysed by sonication.

The protein concentration was measured using the BCA protein assay (Pierce) with BSA as the standard. The following substrates were used: α-NA and β-NA, α- and β-naphthyl propionate, α- and β-naphthyl butyrate, α- and β-naphthyl caprate, 4-methylumbelliferyl-7-(*p*-trimethylammonium cinnamate) chloride (MUTMAC), all from Sigma; acetylated birchwood xylan (ABX), prepared by the acetic anhydride/DMSO method of Johnson *et al*. (1988) from birchwood xylan (Sigma); *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl-(1,3)-*O*-β-D-xylopyranosyl-(1,4)-D-xylopyranose (FAXX) and *O*-[5-*O*-(*trans*-*p*-coumaroyl)-α-L-arabinofuranosyl-(1,3)-*O*-β-D-xylopyranosyl-(1,4)-D-xylopyranose (PAXX), prepared from spear grass (*Heteropogon contortus*) by the method of Borneman *et al*. (1990).

The release of α- and β-naphthol was detected by the method of Rosenberg *et al*. (1975). The release of 4-methylumbelliferone was observed by fluorescence using a UV light source. The release of acetate from acetylated substrates was measured using a Boehringer Mannheim acetic acid assay kit (no. 148261) or by HPLC using an Aminex HPX-87H column, eluting with 0·01 M H_2SO_4 at 0·6 ml min^{-1} and detecting at a wavelength of 210 nm. Acetate was released from acetylated substrates by treatment with NaOH at a final concentration of

0·1 M and total acetate content estimated using sodium acetate standards for comparison. Cinnamoyl ester hydrolase activity was assayed in 100 mM bis-Tris/HCl (pH 7·0) with 0·08 mM FAXX or PAXX. The release of ferulic and *p*-coumaric acids was analysed by HPLC using a reverse phase analytical column (8 × 100 mm, Waters Nova-Pak C18 Radial-PAK cartridge, 4 μm pore size) with isocratic elution by water/acetic acid/butanol (350:1:7) at 2 ml min^{-1} and detection at a wavelength of 254 nm. One unit of enzyme is defined as the amount of enzyme liberating 1 μmol product min^{-1} at pH 6·0 and 39 °C.

α-NA and β-NA zymograms were prepared from native acrylamide gels run in 25 mM Tris/250 mM glycine running buffer. Gels were then equilibrated in 0·1 mM phosphate buffer (pH 7·4) and soaked in α-NA or β-NA and Diazo Blue B to detect bands of activity as described by Rosenberg *et al.* (1975). Gels for MUTMAC zymograms were run in 40 mM Tris/40 mM boric acid running buffer. They were then equilibrated in phosphate buffer (pH 7·4) and overlaid with 0·7 % agarose containing 0·2 mg MUTMAC ml^{-1}.

RESULTS AND DISCUSSION

Esterase activity of *N. patriciarum*

Esterase activity in *N. patriciarum* culture supernatant was demonstrated using α-NA and β-NA as substrates. Zymograms of α-NA- and β-NA-hydrolysing activity in *N. patriciarum* supernatant identified several identical bands. An additional band was very prominent with β-NA (Fig. 1). Therefore, in an attempt to isolate the maximum number of different esterase clones, β-NA was chosen as the substrate to screen the library. *N. patriciarum* culture supernatant was also demonstrated to release ferulic and *p*-coumaric acids from FAXX and PAXX, respectively [specific activities of 3·8 and 0·8 m units (mg protein)$^{-1}$]. A zymogram of cinnamoyl ester hydrolase activity in *N. patriciarum* culture supernatant using the model substrate MUTMAC revealed a number of bands of activity (not shown). As this substrate has been shown to be cleaved by an enzyme cloned from *Butyrivibrio fibrisolvens* that releases ferulic acid from FAXX (Dalrymple *et al.*, 1996), MUTMAC was chosen to screen the library.

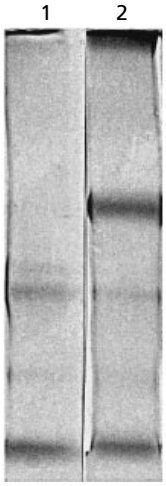

Fig. 1. Zymograms of non-denaturing polyacrylamide gels (9 % acrylamide) of 20 μl 50× concentrate *N. patriciarum* culture supernatant. Lanes: 1, α-NA; 2, β-NA.

Isolation of *N. patriciarum* cDNAs expressing esterase activity

A total of 10^5 plaques were screened for expression of esterases using β-NA as a substrate and 36 positive plaques were isolated. Initial characterization of PCR-amplified cDNA inserts from phage DNA by restriction mapping indicated that there were four unrelated classes of esterase cDNAs derived from four genes, designated *bnaA–D*. The plasmids pCX30, pCX39, pCX16 and pCX31 were chosen as representative clones of *bnaA*, *bnaB*, *bnaC* and *bnaD*, respectively. The inserts ranged in length from 1·4 kb (*bnaA*) to 2·7 kb (*bnaB*). The frequency of isolation varied widely for the different classes of enzymes. Thirty clones were derived from *bnaA* (isolation rate 1 in 3300) while only two clones were derived from each of the other three genes (isolation rate 1 in 50000). The high rate of isolation of cDNA clones expressing BnaA was similar to the rate of isolation of a xylanase (XynA) from the same library (approximately 1 in 3000) (Gilbert *et al.*, 1992) and twofold greater than the rate of isolation of all cellulases from the library (approximately 1 in 6000) (Xue *et al.*, 1992a). The *N. patriciarum* library was also screened for cinnamoyl esterases using MUTMAC (10^5 plaques screened). However, no positive plaques were detected using this substrate.

Characterization of enzymes

The enzymes produced by representative clones derived from the four genes were designated BnaA–D. Total cell lysates of *E. coli* containing these enzymes were then assayed for esterase activity against a number of substrates. As shown in Table 1, all enzymes except BnaD cleaved α- and β-naphthyl propionate at a greatly reduced rate relative to α-NA and β-NA. Activity against the α-naphthyl esters was higher than against the β-naphthyl esters for BnaA and BnaD, similar for BnaC and significantly lower for BnaB, as shown by the ratios of β-NA to α-NA activity for BnaA, -B, -C and -D, which were 0·79, 3·86, 1·02 and 0·57, respectively. This suggests that BnaB may have been the enzyme responsible for the additional band observed in the zymograms of *N. patriciarum* culture supernatant using β-NA. The lack of esterase activity against longer chain α- and β-naphthyl fatty acid esters rules out the possibility that one or more of the enzymes may have been a lipase. Additionally, acetylxylan esterase activity was demonstrated for BnaA and C by their ability to release acetate from ABX (Table 1).

It has been reported that some purified fungal acetylxylan enzymes also hydrolyse cinnamoyl esters (Ten-

Table 1. Specific activities of cloned esterases with various substrates

Values shown are the means of at least two separate assays and are expressed as units (mg protein of total cell lysate)$^{-1}$.

Substrate	*E. coli* lysate containing:				
	BnaA	BnaB	BnaC	BnaD	–
α-NA	3·75 ± 0·12	0·21 ± 0·03	12·38 ± 1·70	2·91 ± 0·07	< 0·01
α-Naphthyl propionate	0·56 ± 0·03	0·10 ± 0·01	1·77 ± 0·01	2·79 ± 0·03	< 0·01
α-Naphthyl butyrate	< 0·10	< 0·10	< 0·10	0·19 ± 0·01	< 0·01
α-Naphthyl caprate	< 0·10	< 0·10	< 0·10	< 0·10	< 0·01
β-NA	2·96 ± 0·13	0·81 ± 0·13	12·71 ± 0·79	1·65 ± 0·04	< 0·01
β-Naphthyl propionate	0·31 ± 0·01	0·22 ± 0·02	1·37 ± 0·14	1·86 ± 0·09	< 0·01
β-Naphthyl butyrate	< 0·10	< 0·10	< 0·10	0·12 ± 0·004	< 0·01
β-Naphthyl caprate	< 0·10	< 0·10	< 0·10	< 0·10	< 0·01
ABX	1·10 ± 0·03	UD	2·40 ± 0·07	UD	UD
MUTMAC*	UD	UD	UD	UD	UD
FAXX*	UD	UD	UD	UD	UD
PAXX*	UD	UD	UD	UD	UD

UD, Undetectable.

* See Methods section.

kanen *et al*., 1991; Castanares *et al*., 1992; McCrae *et al*., 1994). However, no cinnamoyl ester hydrolase activity could be demonstrated for BnaA, -B, -C or -D as indicated by the failure of the lysates to cleave MUTMAC, FAXX or PAXX. This suggests either that BnaA and BnaC are significantly different from the enzymes with cinnamoyl ester hydrolase activity, or that the fungal enzyme preparations were contaminated with cinnamoyl ester hydrolases.

The nucleotide sequences of *bnaA*, *-B* and *-C* and deduced amino acid sequences

The complete DNA sequence of the *bnaA* cDNA in plasmid pCX30 was determined. A single ORF encoding a protein of 393 amino acids was identified. The predicted protein contained a probable signal sequence, followed by the presumed catalytic domain, a 23 amino acid linker rich in threonine and a cysteine-rich duplicated carboxy-terminal domain (Figs 2–4). The coding region was flanked by extremely A/T-rich sequences suggesting that the complete ORF had been identified. The complete DNA sequence of the *bnaB* cDNA in plasmid pCX39 was determined, except for a long array of repeated sequences in the 3′ half of the clone. In the 5′ end of the insert a single ORF was identified encoding a protein of 392 amino acids that was incomplete at the amino-terminal end. The predicted protein contained a 155 amino acid sequence of unknown function (no significant matches identified from database searches), followed by the presumed catalytic domain of 237 amino acids (Figs 2, 3). The complete DNA sequence of the *bnaC* cDNA in plasmid pCX16 was determined. A single ORF encoding a protein of 389 amino acids was identified. The predicted protein contained a probable signal sequence, a 33 amino acid linker rich in glycine, followed by the presumed catalytic domain, a 17 amino acid linker rich in threonine and a cysteine-rich duplicated carboxy-terminal domain (Figs 2–4). As for *bnaA*, the coding region was flanked by extremely A/T-rich sequences.

DNA sequence analysis of *bnaD* identified BnaD as an intracellular enzyme belonging to the acetylcholinesterase-containing family of esterases and lipases described by Cygler *et al*. (1993) (data not shown).

The carboxy-terminal domains of BnaA and BnaC contain a conserved probable protein-docking domain

The carboxy-terminal domains of BnaA and C are homologous to carboxy-terminal domains described from CelB and XylA from *N. patriciarum* (Gilbert *et al*., 1992), CelA from *N. frontalis* (universal ID:g1051285) and XynA, ManA, ManB and ManC from a *Piromyces* sp. (Fanutti *et al*., 1995; Millward-Sadler *et al*., 1996). All except ManA (three repeats) contain two copies of a 40–46 amino acid repeat containing eleven invariant residues, including four cysteines (Fig. 4). However, on the basis of amino acid sequence similarities two distinct sub-

families of repeats can be identified (Fig. 5). A member of this family of repeats from ManA has been demonstrated to bind to a large protein in both a *Piromyces* sp. and *N. patriciarum* (Fanutti *et al*., 1995). It has been proposed that this domain is a protein-docking domain required for the formation of extracellular multienzyme complexes. It is likely that BnaA and BnaC are com-

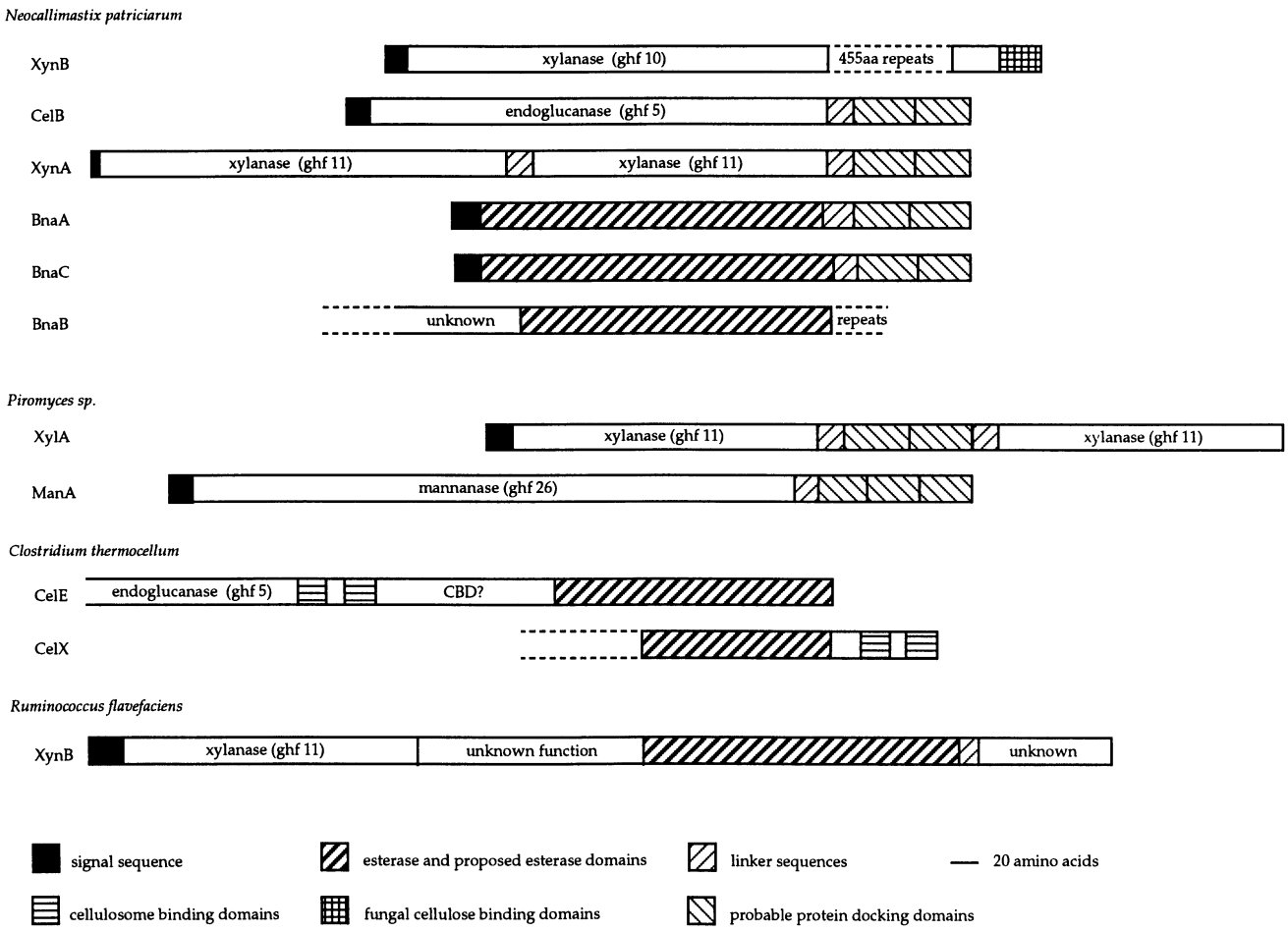

Fig. 2. Domain structure of glycosyl hydrolases and esterases from anaerobic fungi and glycosyl hydrolases with domains similar to the esterases. The glycosyl hydrolase families (ghf) are numbered according to the scheme of Henrissat & Bairoch (1993).

ponents of these cellulosome-like complexes involved in the degradation of plant fibre.

Amino acid sequence alignment of the catalytic domains of BnaA, -B and -C with similar proteins

The complete amino acid sequences of the probable catalytic domains of BnaA–C were screened against the non-redundant protein sequence database using BLAST (Altschul & Lipman, 1990). No significant matches were identified with BnaA. The sequence of BnaB between amino acids 156 and 392 has 40% identity to the carboxy-terminal domain of unknown function in the CelE cellulase of *Clostridium thermocellum* (Hall *et al.*, 1988) (Figs 2, 3). The sequence of BnaC, between amino acids 55 and 287, has 52% amino acid identity to the carboxy-terminal domain of unknown function in the XynB xylanase of *Ruminococcus flavefaciens* (Zhang *et al.*, 1994) and 24% amino acid identity to the partial sequence of a domain of unknown function in the CelX cellulase of *C. thermocellum* (Hall *et al.*, 1988) (Figs 2, 3). The equivalent domains in XynB and CelX have 26% amino acid identity. CelE is a multidomain protein (Hall *et al.*, 1988; Durrant *et al.*, 1991). *celX*, described as encoding a cellulase, has only been partially sequenced. The available amino acid sequence commences approximately one third into the proposed esterase domain, which is followed by a probable cellulosome-binding domain (Hall *et al.*, 1988).

Sequence alignment indicates that CelE and CelX, components of the cellulosome of *C. thermocellum*, are likely to have associated esterase activity. Since cellulose is a homopolymer without any substitutions there is no clear role for esterases in the breakdown of cellulose itself. The most likely role for these catalytic domains is in the cleavage of ester linkages in surrounding polysaccharides, increasing the extent of exposure of the cellulose to the cellulases.

A number of different families of esterases have been identified (Ettinger *et al.*, 1987; Cygler *et al.*, 1993; Cruz *et al.*, 1994; Atlan *et al.*, 1994; Upton & Buckley, 1995; Brick *et al.*, 1995). The sequences described above, in particular the conserved regions containing serine, aspartic acid and histidine residues (possible catalytic residues), were compared with these families. Three of

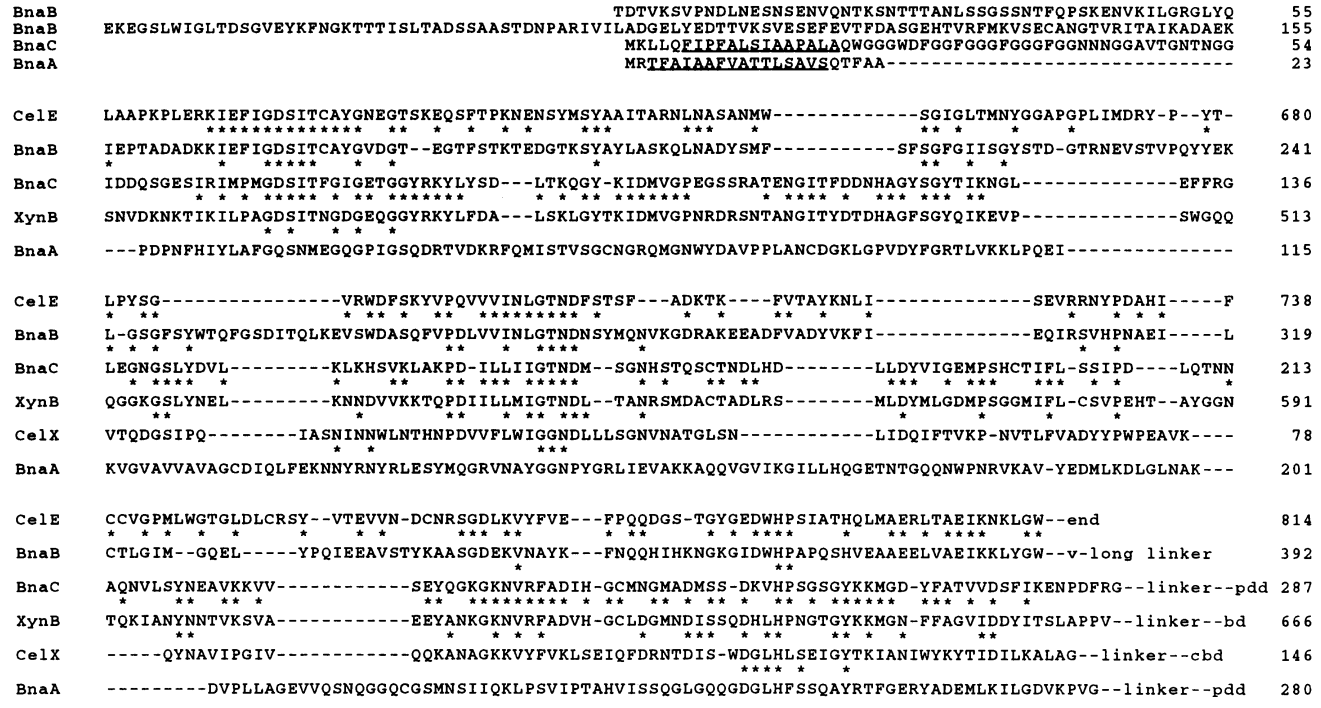

Fig. 3. Alignment of the amino acid sequences of BnaA–C, CelE, CelX and XynB. Asterisks indicate amino acid identity between pairs of sequences. Probable signal sequences are underlined. pdd, putative protein-docking domain; bd, putative binding domain; cbd, cellulosome-binding domain. The sequences of CelE and CelX are from Hall *et al.* (1988). The sequence of XynB is from Zhang *et al.* (1994).

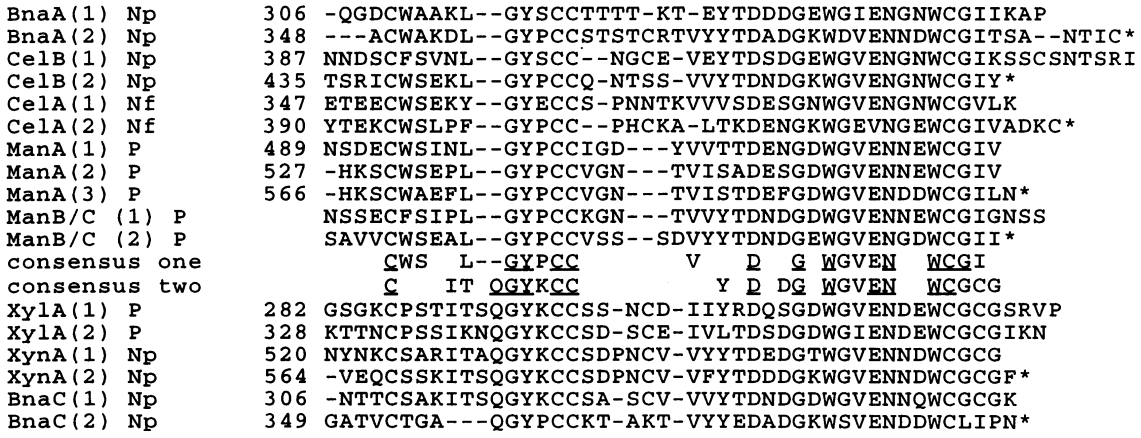

Fig. 4. Alignment of the amino acid sequences of the putative protein-docking domains from glycosyl hydrolases and esterases from anaerobic fungi. Dashes indicate gaps introduced to improve the alignment. Asterisks indicate carboxy-termini of the amino acid sequences. Amino acid residues conserved in at least half of the sequences in each subfamily are shown in the consensus sequence. Invariant amino acids are underlined. Np, *N. patriciarum*; Nf, *N. frontalis*; P, *Piromyces* sp. CelB is from Zhou *et al.* (1994); CelA is g1051285; ManA and XylA are from Fanutti *et al.* (1995); ManB and ManC are from Millward-Sadler *et al.* (1996); XynA is from Gilbert *et al.* (1992).

the five blocks of similar sequence (blocks I, III and V) identified in the hydrolases recently grouped into a family of lipases (Upton & Buckley, 1995; Brick *et al.*, 1995) also appear to be conserved in BnaB, BnaC, CelE, CelX and XynB (Fig. 5). No regions clearly homologous to blocks II and IV could be identified.

Visual inspection of the sequence of BnaA also identified regions of similarity corresponding to blocks I, III and V of the family described by Upton & Buckley (1995). Although the overall level of similarity of BnaA with any of the other amino acid sequences is less than 10%, GQS in the equivalent position of the GDS motif in the other

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            BlockI                                  BlockIII                                BlockV
CelE        RKIEFIGDSITCAYGNEGTSKEQSFTP  (58)   SKYVPQ-VVVINLGTNDFSTSFADKTKFV  (64)   STGYGED-WHPSIATHQLMAER
BnaB        KKIEFIGDSITCAYGVDGT--EGTFST  (78)   SQFVPD-LVVINLGTNDNSYMQNVKGDRA  (68)   KNGKGID-WHPAPQSHVEAAEE
BnaC        IRIMPMGDSITFGIGETGGYRKYLYSD  (64)   KLAKPD--ILLIIGTNDMSGNHSTQSCTN  (68)   ADMSS-DKVHPSGSGYKKMGDY
XynB        IKILPAGDSITNGDGEQGGYRKYLFDA  (63)   KKTQPD-IILLMIGTNDLTANRSMDACTA  (70)   NDISSQDHLHPNGTGYKKMGNF
CelX                                             NTHNPD-VVFLWIGGNDLLLSGNVNATGL  (62)   TDIS-WDGLHLSEIGYTKIANI
EST2        EKFLLFGDSITEFAFNTRPTEDGKDQY  (37)   KHESNIVMATIFLGANDACSAGPQSVPLP  (84)   WQQLLTDGLHPSGKGYKIFHDE
YcsK        LRYTALGDSLTTGRGSGLFSPGFVQRF  (38)   CIRDAD-MITITGCGNDLIDSVLAYQTSK  (84)   QEYLSFDGVHPNSKGYQAMAEA
YacA        IVIAAVGDSLTEGVGDPDGKGYVGKVA  (38)   GIKDAD-YVFFTIGGNDLMKILRQNFLQL  (87)   SRISEEDDFHPHGTGYSLIAKR
Syn         LKVLAMGDSLVYGYGDPIGGGWAERLR  (38)   RNKVPD-LLILSVGVNDSPRLGRPDGRCF  (82)   NDHLMADGLHPNVAGYKALLDD
Vib ae      EKLLVLGDSLSAGYQMPIEKSWPSLLP  (33)   DQHTPD-LVLIELGANDGLRGFPPKVITS  (61)   PEWMMDDGLHPKPEAQPWIAEF
Tesa        DTLLILGDSLSAGYRMSASAAWPALLN  (30)   KQHQPR-WVLVELGGNDGLRGFQPQQTEQ  (61)   PQWMQDDGIHPNRDAQPFIADW
Pp          SGMIVFGDSLSDAGQFGGVRFTNLDAN  (84)   LRADPNALYYLTGGGNDFLQGLVNSPADA  (119)  TKLLFNDLVHPTIAGQQLIADY
Lip1        NNLYVFGDSLSDGGNNGRYTVDGINGT  (56)   NRADHNGMYVHWIGGNDVDAAL-RNPADA  (184)  KPFLFADDFHPTPEAHHIVSQY
Vibh        NKVVALGDSLSDTGNIFNASQWRFPNP  (61)   NYKPANTLFTLEFGLNDF-MNYNRGVPEV  (123)  EKFVFWNVTHPTTATHRYVAEK
GCAT        SRIVMFGDSLSDTGKMYSKMRGYLPSS  (65)   DSFKPDDLVILWVGANDY-LAYGWNTEQD  (154)  EGKMFWDQVHPTTVVHAALSEP
Paerg                                                                                    DQYYFWDEWHPTRRVHQLAGEA
BnaA        HIYLAFGQSNMEGQGPIGSQDRTVDKR  (75)   RLESYMQGRVNAYGGNPYGRLIEVAKKAQ  (83)   GLGQQGDGLHFSSQAYRTFGER
RGAE        TVYLA-GDSTMAKNG-GGSGT-NGWGE  (34)   VVTAGD-YVIVEFGHNDGGSLSTDNGRTD  (98)   NSYFPIDHTHTSPAGAEVVAEA
YxiM        TIYVG-GDSTVCNYYPLNSSKQAGWGQ  (37)   YIKPGD-YFMLQLGINDTNPKHKESEAEF  (77)   GLYMDGDTLHPNRAGADALARL
SDEstA      LQWVALGDSYTAGVIRAAGDAIDYPRD  (68)   VGPGTDVITVG-VGGNTLGFADILTKCQE  (133)  NQDPQLSFVHPNASGHRNAADH
SSEstA      VPTVFFGDSYTANFGIAPVTNQDSERG  (59)   LKQDTQL-TVGSLGGNTLGFNRILKQCSD  (154)  LGTKIPWYAHPNDKGRDIQAKQ
beta-PAF    PDVLFVGDSMVQLMQQYEIWRELFSPL  (24)   ENIKPK-VIVVWVGTNNHENTAEEVAGGI  (68)   SCHDMFDFLHLTGGGYAKICKP
cons              GDS                                      G ND                              D  HP
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Fig. 5. Alignment of the amino acid sequences of selected members of the new family of hydrolases. For sequences of plant enzymes see Brick *et al.* (1995). Dashes indicate gaps introduced to improve the alignment. Numbers of intervening amino acids are indicated in brackets. Amino acids present in more than half of the sequences are shown in the consensus sequence (cons). Absolutely conserved amino acids are underlined. Blocks are numbered as described by Upton & Buckley (1995). Sources of sequences are as described in the legend to Fig. 4 and as follows: EST2, *Sacch. cerevisiae* isoamyl acetate esterase (Fukuda *et al.*, 1996); YcsK, *B. subtilis* protein of unknown function (g1175717); YacA, *B. subtilis* protein of unknown function (g1256648); Syn, *Synechocystis* sp. protein of unknown function (g1651708); Vib ae, *Vibrio mimicus* arylesterase (Shaw *et al.*, 1994); Tesa, *E. coli* thioesterase (Ichihara *et al.*, 1993); Pp, *Pseudomonas putida* protein of unknown function (Essar *et al.*, 1990); Lip1, *Photorhabdus luminescens* lipase (Wang & Dowds, 1993); Vibh, *Vibrio parahaemolyticus* haemolysin (Taniguchi *et al.*, 1986); GCAT, *Aeromonas hydrophilia* phospholipid–cholesterol acyltransferase (Thornton *et al.*, 1988); Paerg, *Pseudomonas aeruginosa* protein of unknown function (Hoitink *et al.*, 1990); RGAE (Kaupinnen *et al.*, 1995); YxiM, *B. subtilis* protein of unknown function (g1177009); SDEstA, *Strep. diastochromogenes* esterase (Tesch *et al.*, 1996); SSEstA, *Strep. scabies* esterase (Raymer *et al.*, 1990); beta-PAF, β-subunit of bovine brain platelet-activitating factor acetylhydrolase (Hattori *et al.*, 1995).

enzymes, GXN in the equivalent position of the GXND motif and DXXH in the equivalent position of the DXXHP motif were identified in BnaA (Figs 3, 5). We propose that BnaA is also a member of this family of hydrolases.

Identification of additional members of the new family of hydrolases

Visual inspection of the amino acid sequence of a rhamnogalacturonan acetylesterase (RGAE) from *Aspergillus aculeatus* (Kaupinnen *et al.*, 1995) also identified GDS, GXND and DXXH motifs in the correct regions of the amino acid sequence (Fig. 5). The sequence of RGAE was used to search the non-redundant amino acid sequence databases. One significantly similar protein was identified, YxiM from *Bacillus subtilis* (g1177009). The proposed active site motifs were also conserved in YxiM. The amino acid sequence of RGAE is not similar to the sequences of the other esterase domains associated with the degradation of complex polysaccharides outside of the small number of highly conserved amino acids. Thus RGAE appears to lie in a fourth cluster of hydrolases associated with the degradation of complex polysaccharides.

Visual inspection of further esterase sequences and database searches with amino acid sequences of members of the family also identified enzymes from *Bos taurus*, *Saccharomyces cerevisiae*, *Synechocystis* sp., *B. subtilis*, *Streptomyces scabies* and *Streptomyces diastochromogenes* as probable members of this family (Fig. 5). Site-directed mutagenesis of the conserved serine in the GXS motif in block I in the *Vibrio mimicus* (Shaw *et al.*, 1994), *B. taurus* (Hattori *et al.*, 1995) and *Strep. diastochromogenes* (Tesch *et al.*, 1996) enzymes, and structural studies with the *Strep. scabies* (Wei *et al.*, 1995) and *B. taurus* enzymes (Ho *et al.*, 1997) have demonstrated that this serine is involved in catalysis. Wei *et al.* (1995) and Ho *et al.* (1997) have also demonstrated that the conserved histidine in the DXXHP motif in block V is also involved in catalysis. However, the same studies have also shown that the *Strep. scabies* enzyme lacks a carboxylic acid as the third member of the catalytic triad whilst the conserved aspartic acid in the *B. taurus* enzyme has been proposed to be the third member of the catalytic triad. However, on the basis of the current sequence alignments there is no entirely conserved aspartic or glutamic acid residue in this family.

BnaA–C are not related to sequenced acetylxylan esterases

The amino acid sequences of a number of other acetylxylan esterases have been published. All are members of families of enzymes quite distinct from that described in this paper. AxeA from *Streptomyces lividans* and the similar domains of unknown function in xylanases from *Cellvibrio mixtus* and *Cellulomonas fimi* are members of the deacetylase family that includes nodulation proteins and chitin deacetylases (Shareck *et*

al., 1995). A diverse family of ser/asp/his active site hydrolases that includes a number of known and potential acetylxylan esterases has also been described (Dalrymple *et al*., 1996). An acetylxylan esterase from *T. reesei*, which contains a cellulose-binding domain, falls in the cutinase family of esterases (Margolles-Clark *et al*., 1996). A fourth distinct family of esterases includes the two other sequenced acetylxylan esterases, *A. niger* AxeA (g832903) and XylD from *P. fluorescens* subsp. *cellulosa* (Ferreira *et al*., 1993). The *A. niger* AxeA exhibits up to 30 % amino acid identity with members of the family of poly-(3-hydroxybutyrate) depolymerases described by Jendrossek *et al*. (1995) and XylD from *P. fluorescens* subsp. *cellulosa* (Ferreira *et al*., 1993) is most similar (28 % amino acid identity) to another probable member of the same family, a *Mycobacterium leprae* protein of unknown function (g466863) (data not shown). On the basis of homologies in the available amino-terminal sequence of AXEI from *Penicillium purpurogenum* (Egaña *et al*., 1996), it is also likely to be a member of this family. AXEII from the same organism (Egaña *et al*., 1996) has homologies in the available amino-terminal sequence with axe1 from *T. reesei* and therefore is also likely to be a member of the cutinase family (data not shown).

Concluding remarks

BnaB and BnaC are the most closely related of the new esterase domains described. They are also closely related to uncharacterized domains in *R. flavefaciens* XynB, and *C. thermocellum* CelE and CelX. All of these domains are members of a large and diverse family that includes known esterases with a range of specificities from acetyl to aryl esters and phospholipid–cholesterol acyltransferase. BnaA also appears to be a member of the same family, but is only distantly related to BnaB and BnaC. In contrast, BnaD belongs to a completely different family altogether, it does not contain docking domains, is predicted to be intracellular and is unlikely to be involved in the degradation of complex polysaccharides.

Whilst BnaA and BnaC have some acetylxylan esterase activity it cannot be ruled out that their main substrate(s) differ(s) from acetylxylan. However, the presence of the docking domains in both enzymes suggests that they would be present in macromolecular complexes with xylanases, cellulases and mannanases and that therefore they would participate in the degradation of plant cell walls. Since neither BnaA or -C appeared to cleave MUTMAC, FAXX or PAXX, hydrolysis of cinnamoyl ester linkages appears unlikely to be their function in this process. Although BnaB does not exhibit acetylxylan or cinnamoyl ester hydrolase activity, or contain protein-docking domains, the high homology with a domain in the CelE cellulase of *C. thermocellum* suggests that it plays an as yet unknown role in the degradation of plant fibre. Further work is now being undertaken to determine the roles of BnaA–C in the degradation of complex polysaccharides.

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