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A novel thermostable multidomain 1,4-β-xylanase from '*Caldibacillus cellulovorans*' and effect of its xylan-binding domain on enzyme activity

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The nucleotide sequence of the complete *xynA* gene, encoding a novel multidomain xylanase XynA of '*Caldibacillus cellulovorans*', was determined by genomic-walking PCR. The putative XynA comprises an N-terminal domain (D1), recently identified as a xylan-binding domain (XBD), homologous to non-catalytic thermostabilizing domains from other xylanases. D1 is followed by a xylanase catalytic domain (D2) homologous to family 10 glycosyl hydrolases. Downstream of this domain two cellulose-binding domains (CBD), D3 and D4, were found linked via proline-threonine (PT)-rich peptides. Both CBDs showed sequence similarity to family IIIb CBDs. Upstream of *xynA* an incomplete open reading frame was identified, encoding a putative C-terminal CBD homologous to family IIIb CBDs. Two expression plasmids encoding the N-terminal XBD plus the catalytic domain (XynAd1/2) and the xylanase catalytic domain alone (XynAd2) were constructed and the biochemical properties of the recombinant enzymes compared. The absence of the XBD resulted in a decrease in thermostability of the catalytic domain from 70 °C (XynAd1/2) to 60 °C (XynAd2). Substrate-specificity experiments and analysis of the main products released from xylan hydrolysis indicate that both recombinant enzymes act as endo-1,4-β-xylanases, but differ in their ability to cleave small xylooligosaccharides.

Keywords: thermophilic bacteria, thermostabilizing domain, *xynA*, hemicellulose degradation

INTRODUCTION

Hemicelluloses are non-cellulosic low-molecular-mass polysaccharides that are found together with cellulose in plant tissues. Xylan is the major component of the plant cell wall and the most abundant renewable hemicellulose (Timell, 1967). Xylans are heterogeneous polysaccharides consisting of a main chain of 1,4-linked β-D-xylopyranosyl residues that often carry acetyl, arabinosyl and glucuronosyl substituents. The action of the main xylanolytic enzyme, β-endoxylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is to convert polymeric xylan to xylooligosaccharides (Biely, 1985).

Many xylanases from fungi and bacteria have been characterized and cloned (Kulkarni *et al*., 1999; Sunna & Antranikian, 1997). However, substantial interest has been focused on thermostable xylanases due to their potential application in the development of environmentally friendly technologies in the paper and pulp industry (Viikari, 1991; Viikari *et al*., 1994). Xylanases are classified into two families, 10 and 11, according to the similarity of amino acid sequences of their catalytic domain in hydrophobic cluster analyses (Henrissat, 1991). Sequence analysis of glycosyl hydrolase family 10 and 11 xylanase genes shows that they often encode multidomain structures comprising a catalytic domain associated with discrete non-catalytic domains of various functions (Gilkes *et al*., 1991; Tomme *et al*., 1995).

Abbreviations: CBD, cellulose-binding domain; PT-linker, proline-threonine linker; TSD, thermostabilizing domain; XBD, xylan-binding domain; XU, xylanase unit.

The GenBank accession number for the sequence reported in this paper is AF200304.

Family 10 xylanases of thermophilic origin often have associated duplicated family IX cellulose-binding domains (CBDs) at the C terminus of the catalytic domain and thermostabilizing domains (TSDs, typically found in tandem domains), at the N terminus (Bergquist *et al*., 1999; Lee *et al*., 1993; Morris *et al*., 1999; Winterhalter *et al*., 1995; Zverlov *et al*., 1996).

'*Caldibacillus cellulovorans*' is a new thermophilic spore-forming aerobic bacterium isolated from an artificial compost that is able to grow on crystalline cellulose (X. P. Huang, unpublished). Here, we report the cloning, sequencing and expression of a glycosyl hydrolase family 10 β-xylanase gene (Henrissat, 1991) from '*C. cellulovorans*' that encodes a protein with an unusual structure and which is covalently associated with a single xylan-binding domain (XBD) and two CBDs. The effect of the XBD on thermostability and substrate hydrolysis is also discussed.

METHODS

Bacterial strain and genomic DNA. *Escherichia coli* JM101 [Δ(*lac-proAB*) *thi-1 supE44* F′ (*traD36 proAB*$^+$ *lacZ*ΔM15)] was used as the bacterial host for all DNA cloning and expression studies. '*Caldibacillus cellulovorans*' cells were kindly provided by Professor Hugh W. Morgan, University of Waikato, New Zealand. Genomic DNA was prepared as described previously (Morris *et al*., 1995). Media and other reagents are described by Croft *et al*. (1987).

Consensus and genomic-walking PCR. A consensus PCR fragment from a glycosyl hydrolase family 10 xylanase was amplified from the '*Caldibacillus cellulovorans*' genomic DNA using the XYNFB and XYNFR consensus primer pair (Table 1). PCRs were performed as described by Morris *et al*. (1998). Linker assembly, linker library construction and genomic-walking PCR were performed according to Morris *et al*. (1995, 1998). The forward and reverse genomic-walking primers used in this study are shown in Table 1. When necessary, genomic-walking PCR was performed using the GC-Rich PCR System (Roche Diagnostics). PCRs were performed as recommended by the manufacturer with an annealing temperature of 55 °C and an extension time of 3 min at 72 °C.

DNA sequencing. DNA sequencing was carried out on an Applied Biosystems 377 DNA sequencer using Big Dye-terminator chemistry. Computer analysis of sequence data were carried out with the Genetics Computer Group (GCG) software package (Devereux *et al*., 1984).

Construction of a xynAd1/2 recombinant plasmid. The catalytic or non-catalytic domains are numbered from the N terminus and the linkers are ignored in this nomenclature (see Fig. 1). The specific primers XYNACBD4BF and XYNA-CBD4BR (Table 1) were designed to allow PCR amplification of DNA encoding the first two N-terminal domains (D1 and D2) of *xynA* from '*Caldibacillus cellulovorans*'. The primers XYNACBD4BF and XYNACBD4BR incorporate the restriction sites *Sph*I and *Bam*HI, to allow directional in-frame ligation of the *xynAd1/2* PCR fragment into complementary sites of the expression plasmid pJLA602 (Schauder *et al*., 1987), to give the recombinant plasmid pSUN18. Xylanase-positive transformants were identified after induction of enzyme production at 42 °C for 3 h on plates containing birchwood xylan (Sigma) using Congo red staining (Teather & Wood, 1982). The recombinant plasmid encoding XynAd1/2 was sequenced on both strands to confirm that there were no PCR-derived base changes in the DNA except for those introduced in the engineered restrictions sites of the PCR primers (Pro→Met change at the N terminus, and Thr→Ser change at the C terminus of the XynAd1/2 peptide). Translation of XynAd1/2 was terminated using a stop codon in the multiple cloning site of pJLA602 directly adjacent to the *Bam*HI site.

Table 1. Oligonucleotides used in this study

Primer	Sequence (5′–3′)
Family 10 xylanase consensus primers*	
XYNFB	CAT ACK TTK GTT TGG CA H T F/L V W
XYNFR†	TGG GAY GTK GTM AAY GA W D V V N
Genomic-walking primers	
GW1F	GACACATTAAAACCGTCGTCAGCC
GW1R	TTCCTTTGTAGCGGCTGACGACGG
GW2F	GACTGGCCGCTGTTGTTCGATGAGC
GW2R	TCAGCACGTTTCCAGGCGTCACAC
3NEWGW2F	GCGGAAGTTCCCAGTCG
GW6R	CCGAACCTTCTCGGCACGGACGTTGTGCGT
***xynAd1/2* and *xynAd2* specific primers‡**	
XYNACBD4BF	CGATTCCGTGCATGCATGTCGCTTCAGCGG
XYNACBD4BR	GTGACGGAACGGATCCGACTACCGCCCAATATGC
XYNA-CBD4BF	GGTAGAAGCCATGGTTCCGTCCCTGAAAGATG

* The sequences below the primer sequences are the amino acid sequences encoded.

† Reverse complement of synthesized oligonucleotide.

‡ Engineered restriction sites are underlined.

Construction of a xynAd2 recombinant plasmid. The primers XYNA-CBD4BF and XYNACBD4BR (Table 1) were used to amplify *xynAd2* encoding the xylanase catalytic domain from the recombinant plasmid pSUN18. The primers XYNA-CBD4BF and XYNACBD4BR incorporate the restriction sites *Nco*I and *Bam*HI, respectively, allowing the directional in-frame ligation of *xynAd2* into pJLA602 to give the recombinant plasmid pSUN24. Xylanase-positive transformants were identified as described above. The recombinant plasmid encoding XynAd2 was sequenced on both strands to confirm that there were no PCR-derived base changes in the DNA, except for those introduced in the engineered restrictions sites of the PCR primers (Asn→Met and Ile→Val change at the N terminus, and Thr→Ser change at the C terminus of the XynAd2 peptide). Translation was terminated as described for pSUN18.

Protein purification. XynAd1/2 and XynAd2 were produced from *E. coli* strains harbouring the recombinant pSUN18 and pSUN24 plasmids, respectively, as described previously (Morris *et al*., 1998). XynAd1/2 was purified by anion-exchange chromatography in a similar fashion to that reported earlier (Morris *et al*., 1998), whilst XynAd2 was purified by anion-exchange chromatography on a HiTrapQ column (Amersham Pharmacia Biotech) pre-equilibrated with 20 mM Tris/HCl buffer, pH 8·0. More than 90 % of contaminating proteins bound to the anion exchanger, whereas the recombinant XynAd2 was found in the flow-through in a highly purified form (data not shown). Both XynAd1/2 and XynAd2 were concentrated, desalted and stored in 50 mM sodium phosphate buffer (pH 6·0) at 4 °C.

Enzyme assay and protein determination. Xylanase activity was determined by the dinitrosalicylic acid method of Bernfeld (1955), using birchwood xylan as substrate. The standard assay reaction mixture consisted of 0·5 % (w/v) xylan supplemented with 120 mM universal buffer (Britton & Robinson, 1931), pH 6·0, and enzyme to give a final volume of 0·1 ml. The reaction mixture was incubated at 90 °C (XynAd1/2) or 70 °C (XynAd2) for 15 min. One xylanase unit (XU) is defined as the amount of enzyme required to liberate 1 μmol xylose per minute at the assay temperature. Protein concentrations were determined using the BCA protein quantification kit (Pierce).

Effects of temperature, pH and thermostability on xylanase activity. The effects of temperature, pH and thermostability on the activity of XynAd1/2 and XynAd2 were determined as described by Sunna *et al*. (2000b). Universal buffer was used for determination of optimal pH for activity and accordingly, all the pH values were adjusted at 90 °C and 70 °C.

Protein electrophoresis. SDS-PAGE was performed in 4–20 % precast gradient gels (Gradipore) by the method of Laemmli (1970). Proteins were stained with Coomassie brilliant blue R-250 (Sigma). Low-molecular-mass marker proteins (Amersham Pharmacia Biotech) were used to determine the molecular mass of the enzymes.

Kinetic determinations. The initial rate of xylan hydrolysis using the purified enzymes was determined with birchwood and oat spelts xylan at various concentrations, under the standard assay conditions. The values of the Michaelis–Menten constant (K_m) and the maximum velocity (V_{max}) were estimated from linear regression of Hanes–Woolf plots.

Substrate specificity and mode of action of XynAd1/2 and XynAd2. The substrate specificity of XynAd1/2 and XynAd2 were determined by incubating the enzymes with different polymeric substrates under the standard assay conditions. Hydroxyethylcellulose was from Merck and all other substrates were from Sigma. The reaction mixtures consisted of 160 μl 0·5 % (w/v) solutions of either birchwood or oat spelts xylan in 120 mM universal buffer (pH 6·0) and 40 μl purified enzyme. The mixtures were incubated at 70 °C (XynAd1/2) or 60 °C (XynAd2) for 3 h. Products from enzymic hydrolysis were extracted and desalted as described previously (Gibbs *et al*., 1999). The ability of the purified enzymes to hydrolyse short xylooligosaccharides was also investigated as described by Sunna *et al*. (2000b). Sugars liberated from xylan and xylooligosaccharide hydrolysis at 70 °C (XynAd1/2) or 60 °C (XynAd2) for 3 h were separated on silica gel plates as described previously (Sunna *et al*., 2000b). Reference xylooligosaccharides were obtained from Megazyme International.

RESULTS AND DISCUSSION

Analysis of consensus and genomic-walking PCR nucleotide sequence

A single 160 bp PCR fragment was amplified from '*Caldibacillus cellulovorans*' genomic DNA with the XYNFB–XYNFR consensus PCR primer pair (Table 1). The nucleotide sequence of this fragment revealed homology to bacterial xylanases belonging to the glycosyl hydrolase family 10 (Henrissat, 1991). The amplification of the DNA fragments upstream and downstream of the *xynA* consensus fragment region was achieved using genomic-walking primers (see Table 1) and genomic-walking PCR. The nucleotide sequence data were combined to generate a 3237 bp sequence. A GC-rich specific PCR was required to successfully amplify the C-terminal portion of *xynA* due to the relatively high G+C content (65 mol % mean) of the DNA encoding the PT-linkers and type IIIb CBDs compared to the G+C content (44 mol % mean) of the DNA encoding the catalytic domain (see Fig. 1b). Overall, '*Caldibacillus cellulovorans*' has a mol % G+C content of 62·4 (X. P. Huang, unpublished). These sequence data have been submitted to the GenBank database under accession number AF200304.

Analysis revealed the presence of two ORFs, ORF1 and *xynA*. The partial ORF1 consists of a sequence encoding a putative C-terminal type IIIb CBD (1–279 bp). Downstream of the ORF1 terminator codon TAA, a 104 bp intergenic region was identified (280–383 bp), containing a 14 bp palindromic repeat sequence, which may serve as a transcription-terminator signal. The second ORF, *xynA* (384–3149 bp), encodes a multidomain β-xylanase, XynA, with a putative size of 921 amino acids. The initiation codon ATG was preceded at a spacing of 7 bp by a potential ribosome-binding sequence (GAGGA).

Analysis of the deduced amino acid sequences

Fig. 1(a) shows a diagrammatic representation of the elements encoded by the two ORFs of the 3237 bp sequence. Similarity searches were carried out between the deduced amino acid sequences of ORF1 and XynA against entries in the GenBank and SWISS-PROT databases. The incomplete nucleotide sequence of ORF1 encodes a partial C-terminal CBD homologous to family

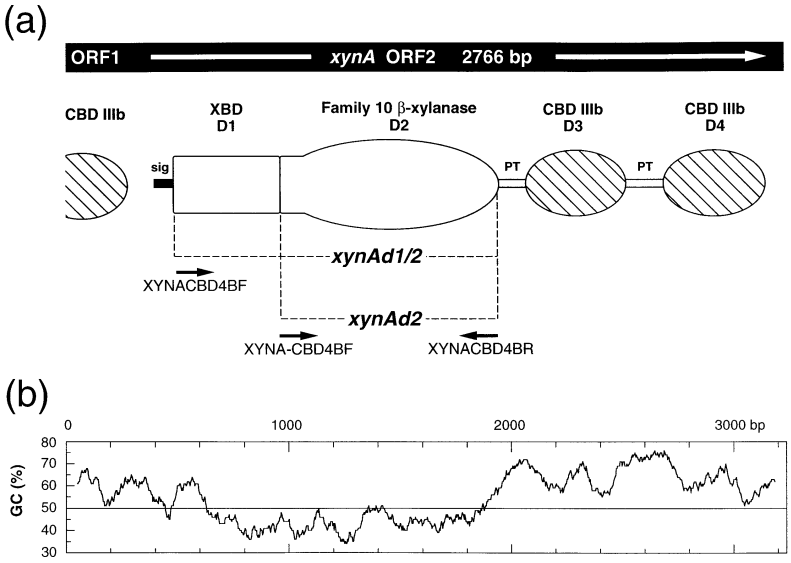

Fig. 1. (a) Diagrammatic representation of the domains encoded by the gene products of ORF1 and *xynA*. The position of the PCR primer pair XYNACBD4BF–XYNACBD4BR used to amplify the *xynAd1/2* fragment from the genomic DNA of '*Caldibacillus cellulovorans*' for construction of pSUN18 is shown. The PCR primer pair XYNA-CBD4BF–XYNACBD4BR used to amplify the *xynAd2* fragment from pSUN18 for the construction of the expression pSUN24 is also shown. sig, signal peptide; *, stop codon; PT, proline-threonine-rich linker peptide; D, domain. (b) A plot of the relative mol% G+C content across the DNA sequence encoding the various domains of XynA.

IIIb CBDs (Fig. 1a), as classified by Tormo *et al*. (1996). It showed highest homology (% identity) to a partial ORF encoding a C-terminal family IIIb CBD upstream of '*Caldibacillus cellulovorans*' ManA and to the C-terminal IIIb CBD (ManAd4) from ManA of the same organism (99%; Sunna *et al*., 2000b). ORF1 exhibited only moderate sequence identity to the internal CBD of *Caldicellulosiruptor* sp. Rt69B.1 multidomain XynC (55%; Morris *et al*., 1999), a partial ORF encoding a CBD directly upstream of *Bacillus lautus* CelA (53%; Hansen *et al*., 1992), and the C-terminal CBD from *Clostridium stercorarium* CelY (52%; Bronnenmeier *et al*., 1997).

Analysis of XynA revealed a multidomain structure. The N-terminal sequence has a putative signal peptide sequence with a predicted cleavage site between position 33 (Ala) and position 34 (Glu). Removal of the signal peptide yields a mature protein with a predicted molecular mass of 98·8 kDa. The mature protein has a XBD, XynAd1, at its N terminus (483–965 bp, Fig. 1a). XynAd1 shows only low sequence identity (34%) to the N-terminal TSD of *Clostridium thermocellum* XynC (Hayashi *et al*., 1997) and the internal TSD of *Caldicellulosiruptor* sp. Rt69B.1 XynC (Morris *et al*., 1999).

XynAd1 is followed by a second domain, XynAd2 (966–1946 bp, Fig. 1a), homologous to glycosyl hydrolase family 10 β-1,4-xylanases (Henrissat, 1991). The xylanase domain shares 51% sequence identity with the xylanase catalytic domains of *Caldicellulosiruptor saccharolyticus* XynE (V. S. J. Te'o, unpublished), *Caldicellulosiruptor* sp. Rt69B.1 XynC (Morris *et al*., 1999) and *Thermotoga maritima* XynA (Winterhalter *et al*., 1995). The XynAd2 catalytic domain is linked to a family IIIb CBD, XynAd3 (2076–2519 bp, Fig. 1a), through a 43 amino acid PT-linker. This CBD shares greatest sequence identity (97%) with the internal IIIb CBD (ManAd2) of '*Caldibacillus cellulovorans*' ManA, but only 53% sequence identity to the C-terminal IIIb CBD (ManAd4) of the same enzyme (Sunna *et al*., 2000b). Furthermore, XynAd3 exhibited only 43% sequence identity with the internal CBD of *Caldicellulosiruptor* sp. Rt69B.1 XynC (Morris *et al*., 1999) and the C-terminal CBD of *Bacillus subtilis* endoglucanase EglS (MacKay *et al*., 1986). XynAd3 is linked via a 59 amino acid PT-linker to a second family IIIb CBD, XynAd4 (2697–3149 bp, Fig. 1a), at the C terminus of the protein that is almost identical (93% sequence identity) to the partial C-terminal CBD of ORF1, but shows only 51% sequence identity to the internal XynAd3 CBD. In addition, XynAd4 exhibited 93% sequence identity to the partial ORF encoding a C-terminal family IIIb CBD upstream of '*Caldibacillus cellulovorans*' ManA, and 95% sequence identity to the C-terminal IIIb CBD (ManAd4) from ManA of the same organism (Sunna *et al*., 2000b). XynAd4 was homologous to the internal CBD of *Caldicellulosiruptor* sp. Rt69B.1 XynC (55% sequence identity; Morris *et al*., 1999), the C-terminal CBD from *Clostridium stercorarium* CelY (53% sequence identity; Bronnenmeier *et al*., 1997) and the partial ORF encoding a CBD directly upstream of *B. lautus* CelA (52% sequence identity; Hansen *et al*., 1992).

Recently, we have identified two further putative family IIIb CBDs in a gene sequence that codes for a multidomain cellulase in '*Caldibacillus cellulovorans*' (A. Sunna, unpublished). Interestingly, all family IIIb CBDs

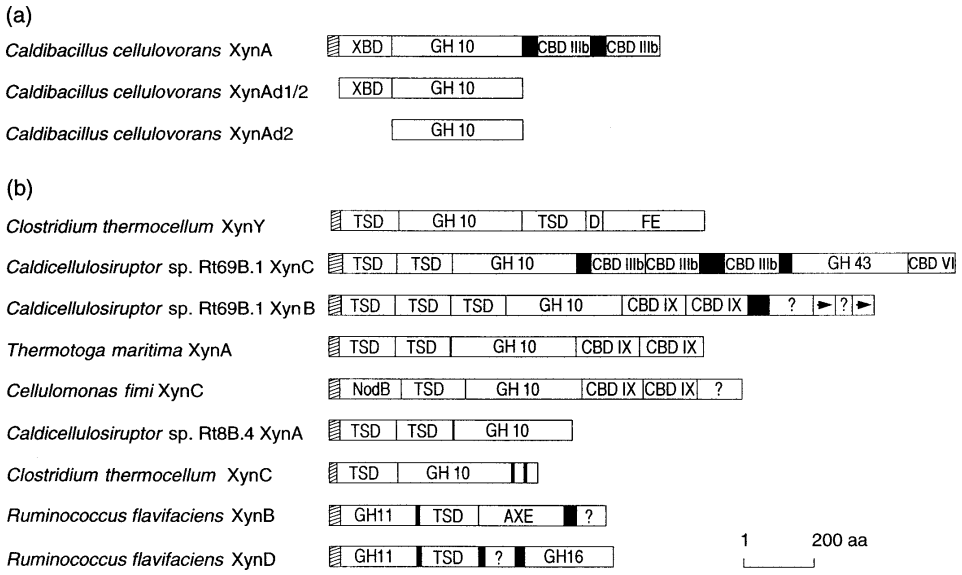

Fig. 2. Domain composition of '*Caldibacillus cellulovorans*' XynA and representative xylanases with related domain structures. (a) Full-length XynA, and recombinant XynAd1/2, XynAd2. (b) *Thermotoga maritima* XynA (Winterhalter *et al.*, 1995); *Caldicellulosiruptor* Rt69B.1 XynB and XynC (Morris *et al.*, 1999); *Clostridium thermocellum* XynC (Hayashi *et al.*, 1997) and XynY (Blum *et al.*, 2000; Fontes *et al.*, 1995); *Cellulomonas fimi* XynC (Clarke *et al.*, 1996); *Caldicellulosiruptor* Rt8B.4 XynA (Dwivedi *et al.*, 1996); *Ruminococcus flavefaciens* XynB (Zhang *et al.*, 1994) and XynD (Flint *et al.*, 1993). XBD, xylan-binding domain; TSD, thermostabilizing domain; CBD IX, family IX CBD; CBD IIIb, family IIIb CBD; ?, domain of unknown function. Other glycosyl hydrolase domain functions are: GH11, family 11 xylanase domain; GH16, family 16 β-glucanase domain; GH43, family 43 domain (reported xylosidase/arabinofuranosidase activities). AXE, acetylxylan esterase; FE, feruloyl esterase; D, dockering domain. Signal peptides are indicated as striped boxes; repeated SLH (S-layer-homology) domains are indicated by arrowheads, whilst the interdomain linker peptides are indicated by black lines.

identified so far in the modular glycosyl hydrolases from '*Caldibacillus cellulovorans*' can be grouped into two distinct groups. Type 1 family IIIb CBDs are all C-terminal, preceded by a PT-linker region and end with a stop codon. This subclass shares more than 93% sequence identity at the amino acid level. Type 2 family IIIb CBDs are all internal and flanked by two PT-linker regions. Type 2 IIIb CBDs share more than 97% amino acid sequence identity. Type 1 and 2 family IIIb CBDs exhibit only around 50% sequence identity.

Comparison with other family 10 glycosyl hydrolases

The N-terminal region of '*Caldibacillus cellulovorans*' XynA is homologous to regions found in several thermostable xylanases. These regions have been described as TSDs as their removal has been associated with loss of thermostability of the adjacent catalytic domain (Fontes *et al.*, 1995). The molecular architecture of thermophilic family 10 multidomain xylanases is usually based on the domain arrangement TSD–TSD–catalytic domain–CBDIX–CBDIX (Bergquist *et al.*, 1999). This arrangement is shared by XynB and XynC from *Thermotoga* FjSS3B.1 (Reeves *et al.*, 2000), XynA from *Thermotoga maritima* (Winterhalter *et al.*, 1995), XynA from *Thermotoga neapolitana* (Zverlov *et al.*, 1996), and XynA from *Thermoanaerobacterium saccharolyticum* B6A-RI (Lee *et al.*, 1993). In the case of XynB from *Caldicellulosiruptor* sp. Rt69B.1 (Morris *et al.*, 1999), three TSDs are found associated with the catalytic domain at the N terminus, while at the C terminus the catalytic domain is associated with two type IX CBDs. The modular XBD–catalytic domain–PT-linker–CBDIIIb–PT-linker–CBDIIIb structure of XynA from '*Caldibacillus cellulovorans*' is unique when compared to other thermophilic family 10 xylanases. The only xylanases from this family associated with type IIIb CBDs are XynC from *Caldicellulosiruptor* sp. Rt69B.1 (Morris *et al.*, 1999) and CelB from *Caldicellulosiruptor saccharolyticus* (Saul *et al.*, 1990). It should be noted that *Caldibacillus* is not closely related to *Caldicellulosiruptor*, a low-percentage G/C anaerobe. A selection of xylanases with domain structures related to '*Caldibacillus cellulovorans*' XynA is shown in Fig. 2.

Purification and properties of the recombinant XynAd1/2 and XynAd2

XynAd1/2 and XynAd2 were purified to electrophoretic homogeneity with a respective final specific activity of 177 XU mg^{-1} and 14 XU mg^{-1}. The apparent molecular mass of XynAd1/2 and XynAd2 was estimated to be 57 and 38 kDa (data not shown), respectively, which is consistent with the respective 56 and 37 kDa deduced from their translated amino acid sequences.

The recombinant XynAd1/2 enzyme was active between 40 and 100 °C, with an optimal temperature for activity

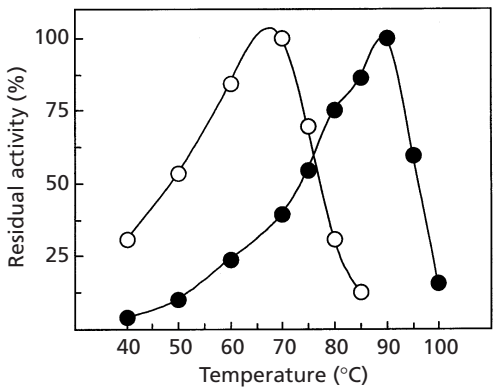

Fig. 3. Effect of temperature on the enzyme activity of the purified recombinant XynAd1/2 (●) and XynAd2 (○). Assays were performed for 15 min at the indicated temperature.

at 90 °C (15 min assay, Fig. 3), which is comparable to that reported for thermostable xylanases from other hyperthermophilic bacteria (Sunna *et al*., 1997). XynAd2 was active between 40 and 85 °C and displayed an optimal temperature for activity of only 70 °C (15 min assay, Fig. 3). At 95 and 100 °C, 60 and 15% of the initial activity of XynAd1/2 was detected, respectively, whilst XynAd2 displayed only 12% of its initial activity at 85 °C. The optimal pH for activity of the XynAd1/2 (at 90 °C) and XynAd2 (at 70 °C) enzymes was 6·0 (data not shown). Both enzymes displayed 40% of initial activity when assayed at pH 8·0, whilst no activity was observed at either pH 4·0 or 9·0.

Influence of XBD on enzyme activity

Thermostability of the purified XynAd1/2 enzyme in the absence of substrate was studied at 70 and 80 °C while purified XynAd2 was studied at 60 and 70 °C (data not shown). XynAd1/2 was almost completely stable at 70 °C (85% residual activity) for the complete assay period of 240 min, whilst at this temperature the half-life of XynAd2 was 25 min. XynAd2 was completely stable at 60 °C for the complete assay period of 120 min. The half-life of the XynAd1/2 enzyme at 80 °C was 35 min, whilst XynAd2 was completely inactivated in less than 5 min.

The lack of the XBD in XynAd2 resulted in a decrease in the optimum temperature for activity to 70 °C, which is closer to the 65 °C growth temperature of '*Caldibacillus cellulovorans*' (X. P. Huang, unpublished). In addition, XynAd2 was less thermostable in the absence of substrate when compared to XynAd1/2. The fact that no other major changes in the biochemical properties of the two enzymes were observed indicates that the deleted region does thermostabilize the catalytic domain of XynA. Removal of the TSD region has been reported to result in both a decrease in optimum temperature for activity and a dramatic reduction in thermostability of the thermophilic XynY from *Clostridium thermocellum* (Fontes *et al*., 1995) and XynA from *Thermotoga maritima* (Winterhalter *et al*., 1995). A reduction in thermostability in XynA family 10 xylanase from *Thermoanaerobacterium saccharolyticum* B6A-RI has been associated to the removal of its N-terminal TSDs (Lee *et al*., 1993). However, in this case the decrease in thermostability could be ascribed to deletion of a portion of the xylanase catalytic domain, rather than to the removal of the TSDs. TSDs are not a prerequisite for thermal stability as several hyperthermophilic bacteria produce single-domain family 10 xylanases that in many cases are more thermostable than their respective multidomain counterpart containing TSDs. For example the single-domain family 10 XynB from *Thermotoga maritima* has an optimum temperature for activity of 105 °C compared to 92 °C for the family 10 multidomain (tandem N-terminal TSDs) XynA (Winterhalter *et al*., 1995; Winterhalter & Liebl, 1995).

TSDs have mainly been found in association with thermophilic family 10 xylanases from bacteria and no TSD has yet been reported in either family 10 or 11 fungal xylanases. However, TSD domains have been reported in the XynB and XynD family 11 xylanases from the mesophilic bacterium *Ruminococcus flavefaciens* (Flint *et al*., 1993; Zhang *et al*., 1994) and the XylC family 10 xylanase from *Cellulomonas fimi* (Clarke *et al*., 1996). XylC was most active at 60 °C and displayed 50% residual activity at 70 °C (Clarke *et al*., 1996), unlike the other xylanases characterized from *Cellulomonas fimi* (Khanna & Gauri, 1993), which are active at 40–45 °C. Recently, the multidomain XynC from the mesophilic *Bacillus* sp. BP-23 was reported to have the same TSD–TSD–catalytic domain–CBDIX–CBDIX commonly found in thermophilic xylanases (Blanco *et al*., 1999). Removal of the tandem N-terminal TSD from XynC resulted in a 10 °C decrease (from 45 to 35 °C) in the optimal temperature for activity of the xylanase, as well as a decrease in its thermostability. There seems to be no obvious reason for the presence of TSD in enzymes from mesophilic bacteria and a more general role for TSDs in conferring protection against proteolytic attack and extremes of pH has been postulated (Clarke *et al*., 1996; Fontes *et al*., 1995). Recently, we have shown that the XBD of '*Caldibacillus cellulovorans*', which is homologous to TSDs associated with glycosyl hydrolase family 10 xylanases, has the ability to selectively bind to xylan. Furthermore, it was suggested that the thermostabilizing function of this class of domain is a result of a lack of discrete linker peptides separating the XBD (or their homologous TSDs) from the adjacent catalytic domain (Sunna *et al*., 2000a).

Substrate specificity and hydrolysis pattern

The action of purified XynAd1/2 and XynAd2 enzymes was tested against different polymeric substrates (data not shown). The recombinant enzymes were active against different xylans (β-1,4-linkages), including beechwood, birchwood, larchwood and oat spelts xylan. Of these, oat spelts xylan gave the highest reducing sugar values. Both enzymes were inactive against barley β-glucan (β-1,4/β-1,3-linkages), lichenan (β-1,4/β-1,3-

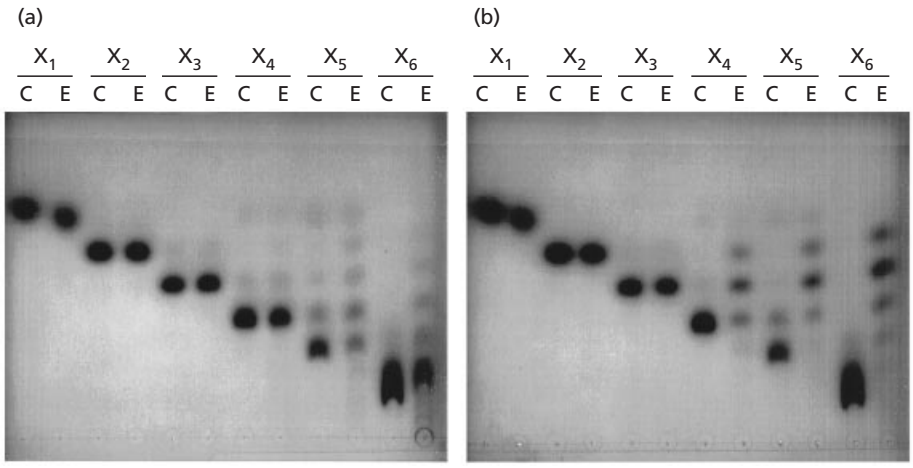

Fig. 4. Thin-layer chromatograms of xylooligosaccharide hydrolysis products. Purified XynAd1/2 and XynAd2 were incubated with xylooligosaccharides for 3 h at 70 and 60 °C, respectively. Xylooligosaccharide controls without enzyme were also incubated for 3 h at the above temperatures. (a) Hydrolysis products liberated by XynAd1/2. (b) Hydrolysis products liberated by XynAd2. C, xylooligosaccharide without enzyme; E, xylooligosaccharide with enzyme; X_1, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose; X_6, xylohexaose.

linkages), laminarin (β-1,3-linkages), soluble starch (α-1,4/α-1,6-linkages), pullulan (α-1,6-linkages), dextran (α-1,6/α-1,3-linkages) and galactomanann (β-1,4/α-1,6-linkages). In addition, no reducing sugars were released upon incubation of the purified xylanases with carboxymethyl- or hydroxyethylcellulose (β-1,4-linkages).

The apparent K_m and V_{max} values calculated for the purified XynAd1/2 xylanase in the presence of birchwood xylan were 1·8 mg ml^{-1} and 256 XU mg^{-1}, respectively, while in the presence of oat spelts xylan the apparent K_m and V_{max} values were 2·7 mg ml^{-1} and 333 XU mg^{-1}, respectively. In the presence of birchwood xylan, XynAd2 displayed K_m and V_{max} values of 1·0 mg ml^{-1} and 15·7 XU mg^{-1}, respectively. When oat spelts xylan was used as substrate, the K_m and V_{max} values calculated for XynAd2 were 2·1 mg ml^{-1} and 21·3 XU mg^{-1}, respectively. The kinetic parameters of the two recombinant enzymes are within the range estimated from kinetic data reported for other xylanases (Sunna & Antranikian, 1997).

The action of purified XynAd1/2 (70 °C) and XynAd2 (60 °C) xylanases on partially soluble birchwood and oat spelts xylan and xylooligosaccharides was analysed qualitatively by thin-layer chromatography. Equivalent units of each enzyme (40 mXU) were used for each assay. After 3 h incubation, the major products liberated from both xylans by XynAd1/2 were xylobiose, xylotriose and xylotetraose (data not shown). The action of XynAd2 on these two substrates liberated mainly xylotriose and xylotetraose (data not shown). The ability of the purified enzymes to hydrolyse short xylooligosaccharides was also investigated (Fig. 4). Equivalent units of each enzyme (1 mXU) were used for each assay. After 3 h incubation at 70 °C, XynAd1/2 was unable to hydrolyse xylobiose, xylotriose or xylotetraose (Fig. 4a). There was minimal release of xylobiose, xylotriose and xylotetraose when xylopentaose was treated with XynAd1/2. It incompletely hydrolysed xylohexaose, releasing xylobiose, xylotriose, xylotetraose and xylopentaose. Assays using five times excess (5 mXU) enzyme did not improve the efficiency of xylooligomer hydrolysis by XynAd1/2. XynAd2 was unable to hydrolyse xylobiose and xylotriose but in contrast to XynAd1/2, it almost completely hydrolysed xylotetraose to mainly xylotriose and xylobiose (Fig. 4b). Xylopentaose and xylohexaose were completely hydrolysed by XynAd2 to mainly xylotriose and xylobiose as end products, accompanied by lesser amounts of xylotetraose. Thus, based on their substrate specificity and xylan hydrolysis pattern, both purified recombinant enzymes act as endo-1,4-β-xylanases. The inability of XynAd1/2 to efficiently hydrolyse xylooligomers, and the efficient release of xylobiose, xylotriose and xylotetraose from xylan, may indicate the requirement of this enzyme for side group substitutions (i.e. acetyl, arabinosyl or glucuronosyl groups) in the xylan backbone or the inability of the active site to accommodate oligomers of equal or less than six xylose residues. In either case the substrate cleavage pattern of the xylanase is changed by the presence of its XBD.

This is the first report on a XBD domain with a dual function of conferring thermostability and also affecting the substrate cleavage pattern of a xylanase.

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