**The Conserved Noncatalytic 40-Residue Sequence in Cellulases and Hemicellulases from Anaerobic Fungi Functions as a Protein Docking Domain**

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Two cDNAs, designated xynA and manA, encoding xylanase A (XYLA) and mannanase A (MANA), respectively, were isolated from a cDNA library derived from mRNA extracted from the anaerobic fungus, *Piromyces* Xyla and MANA displayed properties typical of endo-β-1,4-xylanases and mannanases, respectively. Neither enzyme hydrolyzed cellulose substrates. The nucleotide sequences of xynA and manA revealed open reading frames of 1875 and 1818 bases pairs, respectively, coding for proteins of M, 68,049 (XYLA) and 68,055 (MANA). The deduced primary structure of MANA revealed a 458-amino acid sequence that exhibited identity with Bacillus and Pseudomonas fluorescens subsp. cellulosa mannanases belonging to glycosyl hydrolase Family 26. A 40-residue reiterated sequence, which was homologous to duplicated noncatalytic domains previously observed in *Neocallimastix patricia*; xylanase A and endoglucanase B, was located at the C terminus of MANA. Xyla contained two regions that exhibited sequence identity with the catalytic domains of glycosyl hydrolase Family 11 xylanases and were separated by a duplicated 40-residue sequence that exhibited strong homology to the C terminus of MANA. Analysis of truncated derivatives of MANA confirmed that the N-terminal 458-residue sequence constituted the catalytic domain, while the C-terminal domain was not essential for the retention of catalytic activity. Similar deletion analysis of Xyla showed that the C-terminal catalytic domain homologue exhibited catalytic activity, but the corresponding putative N-terminal catalytic domain did not function as a xylanase. Fusion of the reiterated noncatalytic 40-residue sequence conserved in Xyla and MANA to glutathione S-transferase, generated a hybrid protein that did not associate with cellulose, but bound to 97- and 116-kDa polypeptides that are components of the multienzyme cellulase-hemicellulase complexes of *Pirrmyces* and *Neocallimastix patricia*; xyla, respectively. The role of this domain in the assembly of the enzyme complex is discussed.

Endo-β-1,4-xylanase (xylanase; EC 3.2.1.8) and endo-β-1,4-mannanase (mannanase; EC 3.2.1.78) hydrolyze, respectively, the β-1,4-linked polysaccharide backbones of xylans and mannans, which form the two major hemicellulose components of hardwoods and softwoods (1). Recent studies on the structure of xylanases have revealed that some enzymes are comprised of single catalytic domains while other xylanases are modular, consisting of single or multiple catalytic domains fused via linker sequences to noncatalytic sequences, some of which constitute cellulose binding domains (CBD; Refs. 2 and 3). Hemicellulases derived from aerobic microorganisms do not appear to associate, while anaerobic organisms often synthesize multienzyme cellulase-hemicellulase complexes (2). Many xylanases have been analyzed, but their catalytic domains appear to have evolved from only two progenitor sequences. In contrast, the primary structures of only seven mannanases have been determined, to date. Based on sequence alignments, two of the enzymes belong to glycosyl hydrolase Family 5 (4, 5), three of the mannanases are located in Family 26 (6–8), while the two enzymes from aerobic fungi exhibit significant sequence identity, although they have not been assigned to a specific family (9). The complexity of the molecular architecture of these enzymes is variable; the *Streptomyces* liivianus, Bacillus spp., and *Pseudomonas* fluorescens subsp. cellulosa (4, 6, 8) mannanases consist of single catalytic domains, while the corresponding *Caldocellum* saccharolyticum enzyme (5) is comprised of two catalytic domains, that exhibit mannanase and endo-β-1,4-glucanase activity, respectively, and are separated by a duplicated CBD homologue.

Recent studies in our laboratories have focussed on plant cell wall-degrading enzymes of anaerobic fungi that are particularly active against the more recalcitrant plant structural polysaccharides. These organisms produce cellulases and hemicellulases that associate into large molecular weight multienzyme complexes and bind tightly to cellulose (10, 11). Recently, the molecular architecture of cellulases and xylanases from *Neocallimastix* patricia has been analyzed. Two of the enzymes contain catalytic domains that are linked to a duplicated 40-residue noncatalytic C-terminal domain (12, 13) of unknown function. In general, there is a paucity of information on the structure/function relationship of plant cell wall hydrolases of rumen chytridiomycetes. Specifically, it remains to be established whether the 40-residue noncatalytic domain is conserved between different species of fungi and if so, whether the formation of cellulase/hemicellulase complexes in
these organisms is mediated by a common mechanism involving this sequence; in addition, the molecular architecture of rumen fungal plant cell wall hydrolases, other than cellulases and xylanases, remains to be elucidated, and it is unclear to what extent the plant cell wall degrading systems in these organisms arose through horizontal gene transfer between rumen organisms. We have used the cloned sequences to characterize the encoded enzymes. In this report we describe gus,

encoding plant cell wall hydrolases from another rumen fungus, Piromyces, and we have used the cloned sequences to characterize the encoded enzymes. In this report we describe gus,

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RESULTS

Isolation of manA and xynA—In a previous study cDNAs encoding functional cellulases and hemicellulases were isolated from a cDNA library constructed using mRNA derived from Piromyces cells cultured with Avicel and soluble xylan as carbon sources (10). Restriction maps of the longest cDNAs encoding a functional mannanase and a xylanase, designated manA and xynA, respectively, are displayed in Fig. 1. Nucleic acid probes consisting of the 5' regions of manA and xynA hybridized to single Piromyces mRNA species. The sizes of the hybridizing transcripts suggest that the two cDNAs were almost full length (data not shown).

Characterization of XYLA and MANA—The enzymes encoded by xynA and manA were designated XYLA and MANA, respectively. XYLA produced by E. coli harboring pGX1 (Fig. 1) hydrolyzed oat spelt xylan, wheat arabinoxylan, and rye arabinoxylan (Table I). The major products generated during prolonged hydrolysis of each substrate were xylobiose and xylotriose. No arabinose was detected among the reaction products, indicating that the enzyme does not exhibit arabinofuranosidase activity. The enzyme displayed typical endo activity promoting a rapid decline in the viscosity of the substrate (data not shown). XYLA was more active against xylpentose than xylotetraose, displayed trace activity against xylotriose, but did not hydrolyze xylobiose. The major products released from the two xyloligosaccharides, after prolonged incubation, were xylotriose and xylobiose (Table I). Interestingly, during the early phase of xylotetraose hydrolysis, a significant quantity of xylo-ribose was generated, which was not associated with the production of xylose (Table II). This pattern of product release indicates that either (i) the enzyme is releasing xylrose from xylotetraose, which then participates in transglycosylation reactions or (ii) the xylose released from the substrate trans-
glycosylates with the tetramer to form a hexamer, which is then very rapidly hydrolyzed to release the triose. XYLA displayed no detectable activity against mannan, arabinan, galactan, or cellulolic polysaccharides.

MANA hydrolyzed carob and locust bean galactomannan and ivory nut mannann. The major products released from each substrate were mannose and mannobose. The enzyme exhibited no activity against other plant structural polysaccharides (Table I). To evaluate whether MANA had a preference for polymeric substrates, the rate at which the enzyme hydrolyzed a range of manno oligosaccharides was evaluated. The data (Table II) showed that the enzyme had very high activity against mannohexaose compared with mannopentaose and smaller manno oligosaccharides. The major products generated when the reaction had gone to completion were mannose and manno-biose (data not shown). MANA displayed no detectable transglycosylating activity. These data indicate that the active site of MANA has a minimum of six sugar-binding sites and that substrates which occupy five or less of these sites exhibit low affinity for the enzyme. This is in contrast to the mannannase from P. fluorescens subsp. cellulosa which displays similar activity toward mannohexaose compared with mannopentaose and smaller manno oligosaccharides.

The codon utilization and the A-T richness (approximately 90% A-T) for both cDNAs was very similar; 18 codon types were not utilized and there was a marked preference for T and an exclusion of G from the wobble position. The noncoding regions flanking the two open reading frames were extremely A + T rich (approximately 90% A + T for both sequences), while the A + T content of the protein coding sequences was 58 and 56% for xynA and manA, respectively. The codon utilization and the A + T-rich character of the noncoding sequences of manA and xynA cDNAs are similar to other DNA sequences encoding cellulases, xylanases, and phosphoene pyruvate carboxykinase from Neocallichlamis (12, 13, 25, 26).

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Nucleotide sequences of xynA and manA.

The nucleotide sequences of xynA and manA and the deduced primary structures of the encoded enzymes are shown in A and B, respectively. The proposed linker sequences of XYLA and MANA are boxed.

Fig. 2. Nucleotide sequences of xynA and manA. The nucleotide sequences of xynA and manA and the deduced primary structures of the encoded enzymes are shown in A and B, respectively. The proposed linker sequences of XYLA and MANA are boxed.
derivatives of xynA and manA were constructed and the capacity of the modified genes to express functional enzymes was evaluated. Data presented in Fig. 1 show that a truncated form of manA, in which 176 bp of the 3'-coding region (pGM3), encoding the 40 amino acid C-terminal repeat, had been removed, encoded a mannanase which retained catalytic activity. In contrast, no functional mannanase was produced from a derivative of manA lacking 483 bp of 3'-coding sequence (pGM4). Similarly, cloning a truncated form of manA lacking 543 bp of 5'-coding sequence, into pMtl23p such that the mannanase gene was in-frame with the vector’s lacZ’ translational start codon, did not generate a plasmid encoding a functional mannanase (pGM5). These data support the view that MANA comprises of a 450 residue N-terminal catalytic domain which is linked to a C-terminal noncatalytic reiterated sequence.

Derivatives of xynA, lacking only 48 bp of 3'-coding sequence (Fig. 1; pGx10) did not encode a functional xylanase. Insertion of the 5' 1539 bp of xynA into pMtl23p on a SstI/EcoRI restriction fragment, such that the ATG start codon was in-frame with the vector’s lacZ’, generated a plasmid (pGX3) that also did not direct the synthesis of a functional xylanase. In contrast, cloning 1090 bp of the 3'-coding region of xynA into pMtl23p, such that the xylanase gene was in-frame with the vector’s lacZ’, generated a plasmid (pGX7) that encoded a functional xylanase. These data suggest that while the C-terminal catalytic domain homologue exhibited catalytic activity, the putative N-terminal catalytic domain was inactive.

Analysis of the Role of the Conserved Duplicated 40-Residue Noncatalytic Domain—The conservation of the noncatalytic duplicated sequence within both cellulosases and hemicellulases from different species of anaerobic fungi suggests that this domain plays an important role in enzyme function. In view of the observation that the plant cell wall hydrolases of these eukaryotes associate into large multienzyme complexes that bind to cellulose (9, 10), two possible roles for the reiterated noncatalytic domain can be envisaged: (i) it could constitute a protein docking region which plays a pivotal role in the assembly of the complex; (ii) the sequence could function as a CBD. To distinguish between these two possibilities, the region of xynA encoding the 40-residue repeated sequence was amplified by polymerase chain reaction and cloned into pGEX-2T such that the inserted DNA was in-frame with the GST gene. The encoded fusion protein was used to probe Western blots of the polypeptides contained in the multienzyme cellulose-binding complexes from N. patriciarum and Piromyces, respectively. Data presented in Fig. 6 show that the GST-Xyla’ hybrid protein bound selectively to 116- and 97-kDa polypeptides derived from Neocallimastix and Piromyces, respectively.

The two domains of Piromyces Xyla were compared with enzymes from glycosyl hydrolase Family 11 which exhibited at least 30% sequence identity with one of the putative catalytic domains of the Piromyces enzyme, I, sequence identity; S, sequence similarity.

**Table III**

Sequence identity between Xyla and other glycosyl hydrolase Family 11 enzymes.

| Family 11 enzyme | Sequence identity and similarity |
|------------------|---------------------------------|
|                  | N-terminal catalytic domain C-terminal catalytic domain |
|                  | % % % % % % % % % % % % % % % % % % % % % |
| Clostridium acetobutylicum Xyla | 38 56 40 54 |
| Bacillus pumilus Xyla | 36 53 38 54 |
| N. patriciarum Xyla N-terminal | 34 56 37 58 |
| N. patriciarum Xyla C-terminal | 36 57 38 58 |
| R. flavifaeciens Xyla | 34 51 34 52 |
| S. lividans XYL B | 32 53 30 52 |
| S. lividans XYL C | 31 54 29 51 |

**Fig. 3. Sizes of recombinant Xyla and Manana.** Cell-free extracts derived from E. coli MB8 harboring pGX1 (A) and pGM1 (B) were subjected to SDS-PAGE using 10% (w/v) polyacrylamide gels containing soluble xylan or carob galactomannan, respectively. Zymogram analysis was conducted as described under “Materials and Methods.” Sizes of standard proteins and the largest polypeptides that exhibited xylanase (A) and mannanase (B) activity, respectively, are shown in kilodaltons.

**Fig. 7** revealed that the probe bound to polypeptides in the Piromyces complex, but not to the bacterial proteins. To establish whether the duplicated domain bound to the nonadenatured form of the Piromyces polypeptide, different quantities of native proteins from E. coli, the C. thermocellum cellulose, and the Piromyces complex were dot-blotted onto Immobilon-P and probed with the 40-residue sequence. The data, presented in Fig. 7, showed that although the duplicated sequence bound to the 97-kDa Piromyces polypeptide, it did not bind to any polypeptide in the E. coli extract or the C. thermocellum cellulose. These data suggest that the capacity of the 40-residue sequence to bind to the Piromyces-Neocallimastix-derived polypeptides is a specific interaction. To establish whether the duplicated domain bound to the nondenatured form of the Piromyces polypeptide, different quantities of native proteins from E. coli, the C. thermocellum cellulose, and the Piromyces complex were dot-blotted onto Immobilon-P and probed with the 40-residue sequence. The data, presented in Fig. 7, showed that although the duplicated sequence bound to the 97-kDa Piromyces polypeptide, it did not bind to any polypeptide in the E. coli extract or the C. thermocellum cellulose. These data suggest that the capacity of the 40-residue sequence to bind to the Piromyces-Neocallimastix-derived polypeptides is a specific interaction. To establish whether the duplicated domain bound to the nonadenatured form of the Piromyces polypeptide, different quantities of native proteins from E. coli, the C. thermocellum cellulose, and the Piromyces complex were dot-blotted onto Immobilon-P and probed with the 40-residue sequence. The data, presented in Fig. 7, showed that although the duplicated sequence bound to the 97-kDa Piromyces polypeptide, it did not bind to any polypeptide in the E. coli extract or the C. thermocellum cellulose. These data suggest that the capacity of the 40-residue sequence to bind to the Piromyces-Neocallimastix-derived polypeptides is a specific interaction. To establish whether the duplicated domain bound to the nonadenatured form of the Piromyces polypeptide, different quantities of native proteins from E. coli, the C. thermocellum cellulose, and the Piromyces complex were dot-blotted onto Immobilon-P and probed with the 40-residue sequence. The data, presented in Fig. 7, showed that although the duplicated sequence bound to the 97-kDa Piromyces polypeptide, it did not bind to any polypeptide in the E. coli extract or the C. thermocellum cellulose. These data suggest that the capacity of the 40-residue sequence to bind to the Piromyces-Neocallimastix-derived polypeptides is a specific interaction. To establish whether the duplicated domain bound to the nonadenatured form of the Piromyces polypeptide, different quantities of native proteins from E. coli, the C. thermocellum cellulose, and the Piromyces complex were dot-blotted onto Immobilon-P and probed with the 40-residue sequence. The data, presented in Fig. 7, showed that although the duplicated sequence bound to the 97-kDa Piromyces polypeptide, it did not bind to any polypeptide in the E. coli extract or the C. thermocellum cellulose. These data suggest that the capacity of the 40-residue sequence to bind to the Piromyces-Neocallimastix-derived polypeptides is a specific interaction.
Piromyces Xylanase and Mannanase

Fig. 4. Comparison of the reiterated 40-residue sequences found in cellulases and hemicellulases from \textit{N. patriciarum} and \textit{Piromyces}. The position of the first residue of each reiterated amino acid sequence within the primary structure of \textit{N. patriciarum} Xyla (12), \textit{N. patriciarum} CelB (13), \textit{Piromyces} Xyla and \textit{Piromyces} MANA is shown. Those residues which exhibit identity or similarity in at least five of the five sequences are boxed.

Fig. 5. Comparison of the primary structure of the catalytic domain of \textit{Piromyces} MANA with the corresponding mannanases (MANA) of \textit{Bacillus} and \textit{P. fluorescens} subsp. \textit{cellulosa} and an endoglucanase (EG) of \textit{Bacteroides ruminicola}. The positions of the first and last residues in the primary structures of the \textit{Bacillus} mannanases (8, 27), \textit{Pseudomonas} MANA (6), and the \textit{B. ruminicola} EG' are shown. Those amino acids that exhibit identity or similarity in four of the five sequences are boxed.

The anaerobic fungus \textit{Piromyces} were determined, and the relationship between the structure and function of these two enzymes was investigated. Data presented in this report indicate that XYLA contains two catalytic domain homologues. Although other plant cell wall hydrolases comprising of multiple catalytic domains, originating from distinct ancestral genes, have been reported from \textit{C. saccharolyticum} (5) and \textit{Ruminococcus flavefaciens} (29, 30), the reiteration of catalytic domains in cellulases and xylanases has only been observed previously in enzymes derived from the anaerobic fungus \textit{Neocallimastix} (12, 31). The similarity in the molecular organization of XYLA from \textit{Piromyces} and xylanase A from \textit{Neocallimastix} supports the view that \textit{Neocallimastix} and \textit{Piromyces} are closely related or that there has been extensive gene transfer between the two organisms. In contrast to the \textit{Neocallimastix} xylanase, which contained duplicated catalytic domains that were both functional, only the C-terminal domain of XYLA from \textit{Piromyces} appears to exhibit catalytic activity. However, this interpretation must be viewed with some caution as: (i) it is possible that the folding of the protein in \textit{E. coli} is different to that in the endogenous host, and the consequence of misfolding is an inactive N-terminal domain; (ii) a mutation in xynA, during the cloning of the cDNA, may have resulted in the inactivation of the first catalytic region. This is unlikely, because the same xylanase CDNA was isolated from a number of distinct recombinant phage, and each form of the xylanase exhibited the same properties. Inspection of the primary structure of the N-terminal catalytic domain of the \textit{Piromyces} xylanase did not provide an explanation for its lack of catalytic activity; residues that are conserved in all other Family 11 xylanases were also present within XYLA. Of particular note is the conservation of Glu133 and Glu218; these active site residues function as the nucleophile and proton donor (32), respectively, in the acid-base hydrolysis of glycosidic bonds mediated by Family 11 xylanases. It is difficult, therefore, to evaluate precisely why the N-terminal catalytic domain homologue is inactive. The rationale for the evolution of a xylanase with a second, but inactive catalytic domain is also not readily apparent. It is possible that early in evolution xynA encoded an enzyme with two active catalytic domains, and although a mutation occurred in the 5' region of the gene, resulting in the inactivation of the N-terminal domain, the xylanase activity of the C-terminal region of XYLA was sufficient selective pressure to ensure the retention of xynA within the \textit{Piromyces} genome.

This paper describes, for the first time, the primary structure of...
fractionated by SDS-PAGE, electroblotted onto Immobilon-P membrane.

Whether the ancestral sequence was an endoglucanase, mannanase, or β-glycanase with a broad substrate specificity remains to be elucidated. However, it is interesting to note that, while some endoglucanases display xylanase activity (33, 34), the endoglucanases and mannanases in Family 26 exhibit no detectable cross-specificity, even though the two enzyme species have evolved from the same ancestral sequences (7).

A further major objective of this study was to evaluate the function of the highly conserved noncatalytic 40-residue sequence located in anaerobic fungal cellulases and hemicellulases. Noncatalytic repeated sequences have been observed in a number of cellulases and xylanases (3, 35), and the role of these reiterated sequences has been defined for certain enzymes. For example, duplicated noncatalytic CBDs have been identified in cellulases from C. saccharolyticum, Cellulomonas fimicola, Clostridium cellulolyticum, and Clostridium stercorum (3). In addition, the duplicated 95-residue sequences, which exhibited 65% sequence identity, and are located in xylanase D from C. fimicola, constitute a CBD and noncatalytic xylan-binding domain, respectively (36, 37). In addition to repeated CBDs, a 23-residue duplicated sequence has been observed in cellulases and xylanases from C. thermocellum and C. cellulolyticum (3). This sequence plays an important role in the docking of the cellulases and xylanases to a noncatalytic scaffolding protein to form a multienzyme cellulase complex referred to as the “cellulosome” (28, 38). Data presented in this study clearly showed that the reiterated noncatalytic domain observed in anaerobic fungal plant cell wall hydrolases is not a polysaccharide binding domain, but functions as a protein docking sequence that interacts with polypeptides of 116 and 97 kDa that are present in the multienzyme cellulase-hemicellulase complexes of Neocalimastix and Piromyces, respectively. Although the role of this protein docking domain has not been defined, we suggest that the polypeptides to which the conserved noncatalytic domain binds are the scaffolding proteins and that this protein-protein interaction mediates the assembly of the multienzyme cellulase-hemicellulase complexes synthesized by these eukaryotic microorganisms. It should be emphasized, however, that other mechanisms could also contribute to the formation of the eukaryotic enzyme complexes. For example, it is possible that, in addition to the binding of the protein docking sequence to a scaffolding protein, direct interactions between the catalytic domains could also exist.

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