A modular xylanase containing a novel non-catalytic xylan-specific binding domain

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Xylanase D (XYLD) from *Cellulomonas fimi* contains a C-terminal cellulose-binding domain (CBD) and an internal domain that exhibits 65% sequence identity with the C-terminal CBD. Full-length XYLD binds to both cellulose and xylan. Deletion of the C-terminal CBD from XYLD abolishes the capacity of the enzyme to bind to cellulose, although the truncated xylanase retains its xylan-binding properties. A derivative of XYLD lacking both the C-terminal CBD and the internal CBD homologue did not bind to either cellulose or xylan. A fusion protein consisting of the XYLD internal CBD homologue linked to the C-terminus of glutathione S-transferase (GST) bound to xylan, but not to cellulose, while GST bound to neither of the polysaccharides. The K_m and specific activity of full-length XYLD and truncated derivatives of the enzyme lacking the C-terminal CBD (XYLDcbd), and both the CBD and the internal CBD homologue (XYLDcd), were determined with soluble and insoluble xylan as the substrates. The data showed that the specific activities of the three enzymes were similar for both substrates, as were the K_m values for soluble substrate. However, the K_m values of XYLD and XYLDcbd for insoluble xylan were significantly lower than the K_m of XYLDcd. Overall, these data indicate that the internal CBD homologue in XYLD constitutes a discrete xylan-binding domain which influences the affinity of the enzyme for insoluble xylan but does not directly affect the catalytic activity of the xylanase. The rationale for the evolution of this domain is discussed.

INTRODUCTION

Plant cell-wall hydrolases are unusual enzymes in that they often comprise both catalytic and non-catalytic domains linked by sequences rich in hydroxy amino acids [1]. The majority of non-catalytic domains constitute cellulose-binding domains (CBDs) that are located, not only in cellulases, but also in endo-β1,4-xylanases and other hemicellulases that remove side chains from the xylan backbone [2,3]. Although most CBDs exhibit absolute specificity for cellulose, the CBD of xylanase A from *Thermomonospora fusca* appears to display equal affinity for cellulose and xylan [4]. However, no xylanase with a discrete non-catalytic xylan-binding domain (XBD) that exhibits no affinity for cellulose has been identified. The role of the CBDs located in hemicellulases is unclear, although it is possible that the CBD, by bringing an array of enzymes into close contact with the plant cell wall, enhances the synergism that is known to exist between the plant cell-wall hydrolases. The rationale for the evolution of xylanases with CBDs, rather than XBDs, could be a consequence of the composition of the two polysaccharides; although the structure of xylan varies, depending on the origin of the plant cell wall, the structure and chemical nature of cellulose is invariant. Thus the acquisition of a CBD would enable a xylanase to adhere to plant cell walls regardless of their origin [1]. However, some plant cell walls, for example the husks of cereals, contain low levels of β-1,4-linked glucose polymers, and for substrates such as these it would be rational for xylanases to contain a XBD. In the course of investigating whether there are xylanases that contain non-catalytic XBDs, we have analysed the role of the non-catalytic domains of xylanase D (XYLD) from *Cellulomonas fimi*. In a previous study [5] we showed that this 64 kDa enzyme contains an N-terminal Family G catalytic domain followed by a CBD homologue which does not bind to cellulose, a NodB-like sequence (NodB is a *Rhizobium*-derived protein which is involved in generating plant-specific nodulation signals; [6]) and a C-terminal functional CBD. The objective of the present study was to determine the function of the internal CBD homologue of XYLD. Data presented here show that the domain binds to xylan, but not to cellulose, either when a component of XYLD or when fused to a heterologous reporter protein. The domain appears to increase the affinity of XYLD for its substrate. Thus the present results provide evidence for the presence of a discrete XBD in a xylanase. The rationale for the evolution of both an XBD and a CBD in XYLD is discussed.

MATERIALS AND METHODS

Microbial strains, vectors and culture conditions

The *Escherichia coli* strain employed in the present study was JM83. The vector used was pGEX-2T (Pharmacia). Full-length *xynD* (encodes XYLD) and derivatives of the gene that encode the catalytic domain only (XYLDcd) and the catalytic domain fused to the internal CBD homologue (XYLDcbd), were encoded by pCF9, pJS5 and pJS8 respectively [5]. *E. coli* cells were cultured in Luria broth at 37 or 30 °C. Ampicillin (100 μg/ml) was used to select for transformants. Functional xylanase was detected by the addition of 4-*O*-methyl-*O*-glucurono-D-xylan-Remazol Brilliant Blue R (500 μg/ml) to solid media.

Recombinant DNA methodology

Agarose-gel electrophoresis, plasmid isolation, use of DNA-modifying enzymes and *E. coli* transformations were performed as described by Sambrook et al. [7]. DNA sequencing was performed as described previously by Millward-Sadler et al. [5].

Abbreviations used: XYLD, xylanase D; CBD, cellulose-binding domain; GST, glutathione S-transferase; XYLDcbd, truncated XYLD derivatives lacking the C-terminal CBD; XYLDcd, truncated XYLD derivative lacking the C-terminal CBD and the internal-CBD homologue; XBD, xylan-binding domain.

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The PCR was used to amplify the region of *xynD* encoding the internal CBD homologue. The primers used were:

5′-CTCGGATCCGGCGGCGACACGGGCGGAGGC-3′

and

5′-CTCGAATTCGGGCGTCGGCGTCGGCGTCGG-3′

which showed sequence identity with nucleotides 709–729 and 1030–1050 respectively of the *xynD* open reading frame (accession number X76729 in the EMBL Nucleotide Sequence Database). The conditions for the PCR reactions were as follows. The concentration of the plasmid pJS8 and the primers were 1 μg/ml and 100 nM respectively in a buffer consisting of 10 mM Tris/HCl, pH 8.0, containing 1 mM $MgCl_2$, 50 mM KCl, 10 μg of gelatin/ml, and 1.25 units of *Taq* polymerase (Applied Biosystems Instruments) in a final volume of 100 μl. The reaction conditions consisted of a hot start at 94 °C for 3 min, followed by the addition of the enzyme and then 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. The amplified DNA was digested with *Bam*HI and *Eco*RI and cloned into suitably digested pGEX-2T to generate pGB1.

Purification of full-length and derivatives of XYLD

Full-length XYLD was purified by cellulose affinity chromatography as described by Millward-Sadler et al. [5]. XYLDcbd and XYLDcd were purified by anion-exchange chromatography from cell-free extracts, prepared from 3-litre cultures of *E. coli* JM83 harbouring pJS8 and pJS5 respectively. The cell-free extracts were dialysed against 10 mM Tris/HCl buffer, pH 8.0, loaded on to a column of DEAE-Trisacryl M (26 mm × 240 mm), equilibrated with the same buffer, and eluted at 30 ml/h with a gradient containing from 0 to 400 mM NaCl in 10 mM Tris/HCl buffer, pH 8.0. To obtain pure XYLDcd and XYLDcbd, column fractions containing xylanase activity were pooled, dialysed against 10 mM Tris/HCl buffer, pH 8.0, and rechromatographed on the same column using a gradient from 0–300 mM NaCl. GST–XYLD fusion proteins were purified by GSH–Sepharose affinity chromatography [8] following the protocol suggested by the supplier of the pGEX-2T vector (Pharmacia). The degree of purification and the amount of enzyme recovered after purification were as follows: full-length XYLD, 150-fold and 0.56 mg; XYLDcd, 71-fold and 2.59 mg; XYLDcbd, 79-fold and 3.88 mg; GST–XBD, 9-fold, 50 mg.

Assays

Xylanase assays were performed as described previously [9], using the soluble and insoluble xylan fractions of oat spelt xylan (insoluble xylan contains α1,2- and α1,3-linked arabinofuranoside branches and was purchased from Sigma Chemical Co.) as substrates [10]. One unit of enzyme activity released 1 μmol of product/min at 37 °C. The binding of XYLD and its derivatives to cellulose and insoluble xylan was assessed as described by Ferreira et al. [11]. The size and purity of enzymes bound to Avicel or insoluble xylan were evaluated using SDS/PAGE [12]. Protein was determined by the method of Sedmak and Grossberg [13]. GST activity was measured as described by Habig et al. [14] using 1-chloro-2,4-dinitrobenzene (purchased from Sigma Chemical Co.) as substrate.

RESULTS

Binding of full-length and truncated derivatives of XYLD to polysaccharides

Previously, Millward-Sadler et al. [5] demonstrated that XYLD contains two CBD homologues, of which only the C-terminal domain exhibited cellulose-binding capacity. To evaluate the role of the internal CBD homologue, the capacity of full-length XYLD, XYLDcbd and XYLDcd (Figure 1) to bind to xylan and Avicel was evaluated. The data (Figure 2; Table 1) show that *E. coli* harbouring pCF9 (encodes full-length XYLD) expressed a 64 kDa functional xylanase that bound to cellulose and xylan, while *E. coli* containing pJS8 (codes for XYLDcbd) synthesized a 37 kDa active xylanase that bound to xylan but not to cellulose. N-terminal sequence analysis confirmed that the 64 and 37 kDa polypeptides constituted full-length XYLD and XYLDcbd, respectively. Although *E. coli* containing pJS5 (encodes

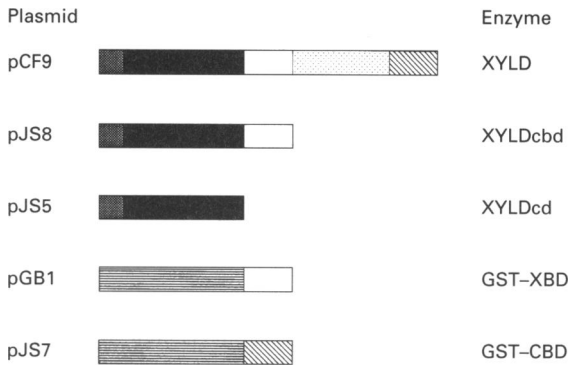

Figure 1 Molecular architecture of XYLD, XYLDcbd, XYLDcd, GST–CBD and GST–XBD

The functional domains in full-length and truncated derivatives of XYLD, and the GST–XBD and GST–CBD fusion proteins were as follows: ■, signal peptide; ■, XYLD catalytic domain; □, internal CBD homologue; ⊡, NodB sequence; ▨, functional CBD; and ▤, GST. The plasmids encoding the various enzymes are also defined.

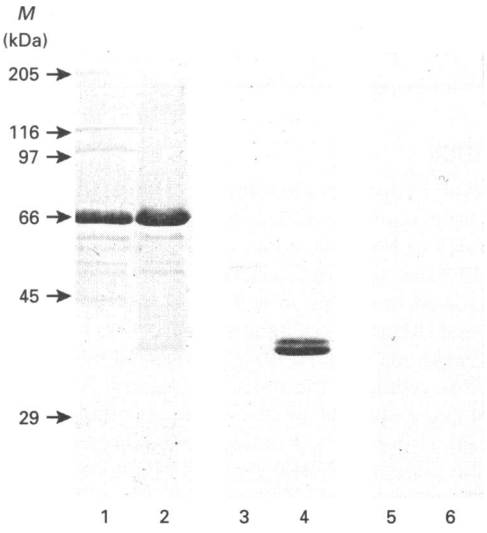

Figure 2 Binding of full-length and truncated derivatives of XYLD to cellulose and xylan

Cell-free extracts derived from *E. coli* strains harbouring pCF9 (XYLD; lanes 1 and 2), pJS8 (XYLDcbd; lanes 3 and 4) and pJS5 (XYLDcd; lanes 5 and 6) were incubated with Avicel and xylan and the polypeptides which bound after repeated washing were eluted from the polysaccharides with 10% SDS and analysed by SDS/PAGE using a 10% polyacrylamide gel. Lanes 1, 3, and 5 contained cellulose-bound material, while lanes 2, 4 and 6 contained xylan-bound material. The molecular masses (*M*, in kDa) of standard proteins are shown.

Table 1 Binding of XYLD, XYLDcd and XYLDcbd to cellulose and xylan

The incubation of cell-free extracts, containing the various forms of XYLD, with cellulose and xylan, and subsequent washing of the polysaccharides were as described in Figure 2. At the end of the final wash, the polysaccharides, which had been incubated with the various cell-free extracts, were assayed for xylanase activity using soluble oat-spelt xylan as the substrate.

	Polysaccharide-bound xylanase activity (total units)	
Enzyme	Avicel	Xylan
XYLD	35	42
XYLDcbd	1	24
XYLDcd	0	0

Table 2 Binding of GST, GST–CBD and GST–XBD to cellulose and xylan

	Enzyme associated with polysaccharide (units/g of polysaccharide)*	
Enzyme	Insoluble xylan	Avicel
GST	ND†	ND
GST–CBD	3.1	2.7
GST–XBD	5.8	0.1

* Equal quantities (25 mg) of cellulose and xylan were incubated with cell-free extracts derived from appropriate *E. coli* strains cultured at 30 °C, containing GST, GST–XBD and GST–CBD respectively for 1 h at 4 °C, and after extensive washing with ice-cold 50 mM Tris/HCl buffer, pH 8.0, the activity that remained bound to the polysaccharide was assayed as described in the Materials and methods section.

† ND, no activity detected.

XYLDcd) produced a 23 kDa polypeptide (size of XYLDcd) that exhibited xylanase activity, the enzyme did not bind to cellulose or to xylan. These data suggest that the internal CBD homologue of XYLD binds to xylan and is therefore classified as an XBD.

Polysaccharide-binding properties of GST–XBD and GST–CBD

To evaluate whether the internal XBD can function independently of the catalytic domain, the region of *xynD* that encodes the XBD was fused in-frame with the GST gene (encoded by pGEX-2T), to generate the plasmid pGB1. The capacity of *E. coli* harbouring pGB1 and pGEX-2T (encodes GST alone) to express polypeptides that bind to cellulose and/or insoluble xylan was assessed. In initial experiments the GST–XBD hybrid was insoluble when expressed by *E. coli* cultured at 37 °C. However, when produced by *E. coli* grown at 30 °C, the fusion protein was soluble (results not shown). The data, presented in Figure 3 and Table 2 show that *E. coli* harbouring pGB1, when grown at 30 °C, synthesized a polypeptide that bound to xylan but not to Avicel, displayed GST activity, and was identical in size with the GST–XBD hybrid purified by GSH affinity chromatography. No polypeptide expressed by *E. coli* JM83 containing pGEX-2T bound to either cellulose or xylan (Table 2; Figure 3). The binding of the GST–XBD hybrid polypeptide to xylan increased with increasing xylan/enzyme ratios (Figure 4). In contrast, there was negligible retention of GST–XBD on Avicel, even at a very high cellulose/enzyme ratio (Figure 4). The K_D and relative affinity (K_r) for the interaction of the GST–XBD with insoluble xylan were 4 μM and 11 litres/g respectively. The K_r for the XBD was within the range of values [15–17] reported for the binding of bacterial CBDs to crystalline and amorphous cellulose (0.9–50 litres/g). These data show that the internal CBD homologue of XYLD is a discrete XBD that is capable of binding to xylan and does not require interactions with other components of the xylanase to elicit polysaccharide binding.

In a previous study we reported that *E. coli* harbouring pJS7, which encodes GST fused to the C-terminal CBD of XYLD (GST–CBD), when cultured at 37 °C produced very low levels of a soluble GST–CBD hybrid that bound to cellulose, but binding of the hybrid protein to xylan was not detected. However, in view of the observation that the GST–XBD hybrid is insoluble when synthesized by *E. coli* grown at 37 °C, it could be argued that, at this temperature, the bulk of GST–CBD was also insoluble and that the small amount of soluble GST–CBD had not folded correctly and thus displayed aberrant polysaccharide-binding properties. To investigate this possibility we cultured *E. coli* containing pJS7 at 30 °C and observed a 20-fold increase in soluble GST activity compared with the bacterium grown at

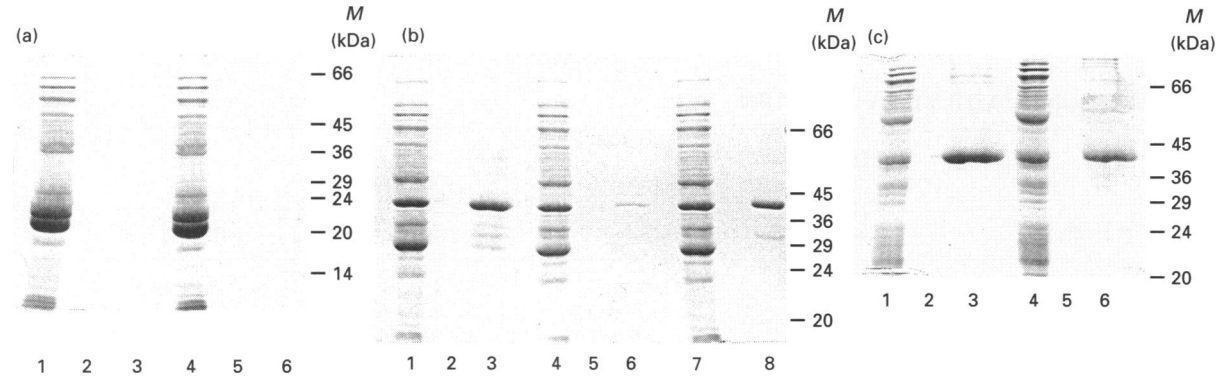

Figure 3 SDS/PAGE of polysaccharide-bound GST–XBD, GST–CBD and GST

Cell-free extracts from *E. coli* JM83 expressing GST (**a**), the GST–XBD hybrid (**b**) and the GST–CBD fusion protein (**c**) were incubated with cellulose or xylan and the material, which remained bound after repeated washing, was eluted from the polysaccharides with 10% SDS and analysed by SDS/PAGE using a 10% polyacrylamide gel. Lanes 1, 4 and 7 contained cell-free extract from the appropriate strains; lanes 2 and 5 contained material in the final wash of xylan or cellulose respectively; lanes 3 and 6 contained the bound polypeptides recovered from xylan and cellulose in SDS respectively. The GST–XBD hybrid protein purified by GSH–Sepharose affinity chromatography is displayed in lane 8.

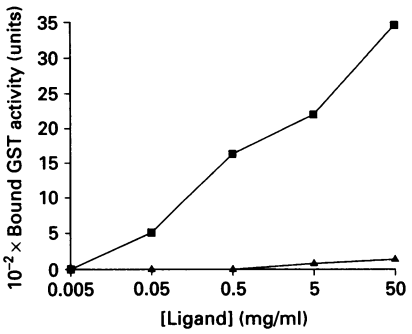

Figure 4 Binding of GST–XBD to different concentrations of cellulose and xylan

Equal amounts of GST–XBD were incubated with various concentrations of insoluble xylan (■) or cellulose (▲) and the quantity of enzyme that bound to the polysaccharides, was evaluated by assaying GST activity remaining in the supernatant after recovering the polysaccharide by centrifugation.

37 °C. Polysaccharide-binding studies showed that the GST–CBD derived from *E. coli* cultured at 30 °C binds to both cellulose and xylan (Figure 3; Table 2) . These data suggest that the C-terminal CBD homologue of XYLD constitutes a CBD/XBD.

Effect of the XBD on XYLD catalytic activity

To evaluate the effect of the internal XBD on the catalytic activity of XYLD, full-length XYLD, XYLDcd and XYLDcbd were purified and the specific activities and K_m values of the three enzymes against soluble and insoluble xylan were determined. The data, presented in Table 3, show that all three enzymes had similar catalytic activities against soluble and insoluble xylan, with the enzymes displaying higher activity against the soluble substrate. Confirmation of the similar activities of the three enzymes was obtained by treatment of the full-length XYLD with proteinase K for 3 h, which did not influence the catalytic activity of the enzyme, although the enzyme's size was reduced to that of the catalytic domain. Thus the proteinase-treated and untreated samples contained identical amounts (in mol) of full-length XYLD and XYLDcd respectively. As the proteinase treatment did not affect the catalytic activity of the enzyme (Table 3), these data support the view that XYLD and XYLDcd exhibit very similar catalytic activities. The K_m values of the three forms of XYLD were also very similar when soluble xylan was used as the substrate. However, full-length XYLD and XYLDcbd had significantly lower K_m values than XYLDcd when insoluble xylan was used as the substrate. These data indicate that the XBD does increase the affinity of XYLD for insoluble xylan, but does not influence the enzyme's turnover rate.

DISCUSSION

There have been several reports of the location of CBDs in xylanases [1]. However, the identification in such an enzyme of a non-catalytic XBD that exhibits negligible affinity for other polysaccharides is novel. The only XBD previously identified was in xylanase A from *Thermomonospora fusca* [4]. However, this XBD exhibited significant affinity for cellulose, and it is unclear whether the domain interacted with other components of the enzyme to elicit xylan binding, since the catalytic domain also exhibited significant binding to xylan. The results of the present study clearly show that the XYLD XBD exhibits specificity for xylan and only negligible affinity for cellulose and can function independently of the rest of the xylanase. The major effect the XBD has on the catalytic properties of XYLD is to increase the enzyme's affinity for insoluble xylan, as reflected in a diminished K_m. The presence of a non-catalytic substrate-binding domain did not alter the activity of the xylanase against the soluble substrate or modulate the specific activity of the enzyme against either soluble or insoluble xylan. The mechanism by which the XBD influences the K_m for XYLD against the insoluble substrate could be as follows. The diffusion rate of insoluble xylan in the aqueous phase of the reaction mixture will be low compared with the soluble substrate, as reflected in the different K_m exhibited by XYLD for the two polysaccharides. However, by mediating the binding of the xylanase to insoluble xylan, the XBD effectively raises the local concentration of substrate in the vicinity of the enzyme, and thus, at non-saturating substrate concentrations, the xylanase will exhibit enhanced catalytic activity. Although the XBD should also mediate an elevated concentration of the soluble substrate around XYLD, because the diffusion of soluble xylan within the mobile phase of the reaction mixture is already rapid, the XBD has less effect on the K_m of the enzyme. Interestingly, full-length XYLD appears to exhibit a lower K_m for the insoluble substrate when compared with XYLDcbd. This could reflect the capacity of the C-terminal CBD to bind xylan.

The rationale for the evolution of an enzyme that hydrolyses only xylan, but contains a discrete and specific XBD and

Table 3 Catalytic properties of XYLD, XYLDcbd and XYLDcd

Enzyme	Specific activity* (units/mg of catalytic domain)		K_m (mg/ml)	
	Soluble xylan	Insoluble xylan	Soluble xylan†	Insoluble xylan†
XYLD	678 ± 6.9‡	312 ± 34	2.13 ± 0.102	12.4 ± 0.6
XYLDcbd	652 ± 3.2	278 ± 48	3.10 ± 0.059	30.7 ± 5.5
XYLDcd	693 ± 8.9	339 ± 27	3.50 ± 0.004	98.6 ± 9.9
XYLD + proteinase K	685 ± 12.4	325 ± 27	ND§	ND

* The specific activities of the enzymes using soluble xylan as the substrate were determined at saturating substrate concentration (20 mg/ml). The specific activities of the xylanases using insoluble xylan as the substrate were determined from the $V_{max.}$ value derived from a Lineweaver–Burk plot of the reciprocal of catalytic activity against the reciprocal of substrate concentration.

† The K_m values were determined from Lineweaver–Burk plots; the concentration of soluble xylan employed ranged from 0.15 mg/ml to 20 mg/ml; the concentration of insoluble xylan used was 2–200 mg/ml.

‡ Results are means ± S.E.M.

§ ND, not determined.

CBD/XBD respectively is intriguing. In previous studies a CBD from a *C. fimi* endoglucanase appeared to increase the enzyme's activity against the crystalline substrate by disrupting the ordered structure of cellulose, making the recalcitrant substrate more accessible to the enzyme [18]. It could be argued that the C-terminal CBD of XYLD fulfills a similar role in disrupting the crystalline structure of cellulose. This could increase the accessibility of native substrates to a range of plant cell-wall hydrolases, including xylanases. However, the role of the XBD in hydrolysing a cellulose–xylan complex is not so readily apparent. Given the heterogeneous nature of the substrate, which varies depending on its origin, it is possible that the XBD plays an important role when the enzyme is presented with a substrate, such as cereal husks, that contain significant quantities of arabinoxylan, but little cellulose. In this situation the XBD would facilitate hydrolysis by increasing the enzyme's affinity for the substrate.

Inspection of the primary structure of the XYLD XBD and CBD/XBD reveals a low sequence similarity to Type I CBDs [19]. In contrast, the two domains exhibit 65% sequence identity with each other [5]. Thus both domains clearly evolved by duplication of either the CBD or the XBD, or possibly a progenitor sequence that exhibited different polysaccharide-binding properties. Future work will focus on the subtle differences between the structures of the two domains that contribute to their different polysaccharide-binding properties.

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