The modular architecture of *Cellvibrio japonicus* mannanases in glycoside hydrolase families 5 and 26 points to differences in their role in mannan degradation

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β -1,4-Mannanases (mannanases), which hydrolyse mannans and glucomannans, are located in glycoside hydrolase families (GHs) 5 and 26. To investigate whether there are fundamental differences in the molecular architecture and biochemical properties of GH5 and GH26 mannanases, four genes encoding these enzymes were isolated from *Cellvibrio japonicus* and the encoded glycoside hydrolases were characterized. The four genes, *man5A*, *man5B*, *man5C* and *man26B*, encode the mannanases Man5A, Man5B, Man5C and Man26B, respectively. Man26B consists of an N-terminal signal peptide linked via an extended serine-rich region to a GH26 catalytic domain. Man5A, Man5B and Man5C contain GH5 catalytic domains and non-catalytic carbohydrate-binding modules (CBMs) belonging to families 2a, 5 and 10; Man5C in addition contains a module defined as X4 of unknown function. The family 10 and 2a CBMs bound to crystalline cellulose and ivory nut crystalline mannan, displaying very similar properties to the corresponding family 10 and 2a CBMs from *Cellvibrio* cellulases and xylanases. CBM5 bound weakly to these crystalline polysaccharides. The catalytic domains of Man5A, Man5B and Man26B hydrolysed galactomannan and

glucomannan, but displayed no activity against crystalline mannan or cellulosic substrates. Although Man5C was less active against glucomannan and galactomannan than the other mannanases, it did attack crystalline ivory nut mannan. All the enzymes exhibited classic endo-activity producing a mixture of oligosaccharides during the initial phase of the reaction, although their mode of action against manno-oligosaccharides and glucomannan indicated differences in the topology of the respective substrate-binding sites. This report points to a different role for GH5 and GH26 mannanases from *C. japonicus*. We propose that as the GH5 enzymes contain CBMs that bind crystalline polysaccharides, these enzymes are likely to target mannans that are integral to the plant cell wall, while GH26 mannanases, which lack CBMs and rapidly release mannose from polysaccharides and oligosaccharides, target the storage polysaccharide galactomannan and manno-oligosaccharides.

Key words: carbohydrate-binding module, *Cellvibrio*, glycoside hydrolase, mannan.

INTRODUCTION

Approx. 10¹¹ tonnes of plant biomass is recycled annually, primarily through the action of microbial enzymes. The sugars released by these enzymes represent a major energy and carbon resource within the biosphere and thus the recycling of photosynthetically fixed carbon is a key biological process. The mannose-containing polysaccharides mannan and glucomannan are important components of plant biomass. Mannan consists of a backbone of β -1,4-linked mannose residues whereas glucomannan comprises a heterogeneous polymer of β -1,4-linked glucose and mannose sugars [1]. The backbone of both mannan and glucomannan can be decorated with α -1,6-linked galactosyl residues, and thus these polysaccharides are often referred to as galactomannan and galactoglucomannan, respectively [1]. Glucomannan plays a key structural role in the plant cell walls of Angiosperms, while galactomannan is generally found in the cell

walls of seeds such as carob, where its main function is as a storage polysaccharide [1].

β -1,4-Mannanases (mannanases) are one of the key enzyme groups that attack the plant cell wall. These glycoside hydrolases cleave either mannans or glucomannans [2]. Mannanases have been grouped into two of the 85 sequence-based glycoside hydrolase families (GHs), 5 and 26 ([3]; see also see Coutinho and Henrissat's Carbohydrate-Active Enzymes server at <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>). The protein fold [$(\alpha/\beta)_8$ -barrel], catalytic machinery and mechanism of glycosidic bond cleavage (acid-base-assisted catalysis via a double-displacement mechanism) are conserved in these two enzyme families [4–6], placing both GH5 and GH26 mannanases in clan GH-A (see <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>). GH26 enzymes are exclusively prokaryotic in origin, whereas GH5 mannanases are derived from both bacteria and fungi. All the GH26 mannanases characterized to date display

Abbreviations used: AGE, affinity gel electrophoresis; CBM, carbohydrate-binding module; GH, glycoside hydrolase family; PACE, polysaccharide analysis by carbohydrate gel electrophoresis.

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very narrow substrate specificity, hydrolysing mannan and glucomannan but exhibiting no activity against other β -glycans such as barley β -glucans or soluble derivatives of cellulose [7]. In contrast, several GH5 mannanases can exhibit at least some activity for cellulosic substrates [8].

One of the distinctive features of glycoside hydrolases that attack the plant cell wall is that they generally contain non-catalytic modules that often bind to polysaccharides. These carbohydrate-binding modules (CBMs) have been classified into sequence-based families (see <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>) [9]. CBMs that bind mannan, cellulose, xylan, starch and laminarin have been identified [10–14]. The only CBMs in the 16 GH26 mannanases bind to mannan [14], whereas several GH5 mannanases are appended to CBMs that are located in families (e.g. CBMs 1, 3 and 10) which contain at least some members that bind crystalline cellulose [15–17]. It is unclear, however, whether the CBMs from families 1, 3 and 10, present in GH5 mannanases, also bind crystalline cellulose, or whether they display specificity for the mannans that are hydrolysed by the respective enzymes.

One of the most extensively characterized plant-cell-wall degrading systems is that of *Cellvibrio japonicus* (formerly *Pseudomonas fluorescens* subsp. *cellulosa*), which degrades mannan, xylan, cellulose, arabinan, galactan and pectins [18–20]. Although the *Cellvibrio* xylanases and cellulases have been extensively analysed, the consortium of *C. japonicus* glycoside hydrolases that degrade mannan and glucomannan has only been partly characterized. To date, a single non-modular *C. japonicus* GH26 mannanase (Man26A) has been reported [7]. To investigate whether the molecular architecture of *Cellvibrio* mannanases points to a difference in substrate specificity between GH5 and GH26 mannanases we have cloned and characterized the full complement of *Cellvibrio* mannanases. The data show that the two GH26 mannanases are non-modular enzymes, while the three GH5 enzymes are modular, containing multiple CBMs that recognize crystalline cellulose. Based on the differences in the molecular architecture and biochemical properties of the *Cellvibrio* mannanases, we propose that GH5 enzymes target mannans that are integral to the plant cell wall, whereas the primary substrates for GH26 mannanases are galactomannan, which is a storage polysaccharide that is more loosely associated with the cell walls of seeds, and manno-oligosaccharides.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture condition

The *Escherichia coli* strains used in this study were BL21(DE3): pLysS (Novagen) and XL1-Blue (Stratagene). The bacteriophage employed comprised a genomic library of *C. japonicus* (NCIMB 10462) constructed previously in λ ZAPII (Stratagene) [20]. The plasmid vectors used were pCR-Blunt (Invitrogen), pBluescript SK[−] (Stratagene), pET16b and pET32c (Novogen). All *E. coli* strains containing recombinant plasmids were cultured in Luria broth (LB) supplemented with 100 μ g/ml ampicillin or kanamycin, as appropriate, at 37 °C unless otherwise stated. *E. coli* cells used to propagate bacteriophage were grown on LB supplemented with 10 mM MgSO₄ and 0.2% (w/v) maltose, and were plated out on NZYM top agar (0.7%). To screen the genomic library, approx. 40 000 recombinant phage were plated out at a density of 5 plaques/cm² on an NZYM top agar overlay containing 0.5% Azo-carob galactomannan (Megazyme International, Bray, County Wicklow, Ireland). After incubation at 37 °C for 16 h mannanase-expressing phage were surrounded by a clear halo in a blue background. The *Cellvibrio* DNA in the

mannanase-positive phage was excised into pBluescript SK[−] following standard procedures [21].

Other DNA methods

Plasmid and λ phage DNA were purified using Qiagen columns. Digestion of DNA with restriction endonucleases, ligation of DNA with T4 DNA ligase, agarose gel electrophoresis, Southern hybridization and transformation of *E. coli* were carried out as described previously [21]. DNA sequencing was carried out using an Applied Biosystems Instruments (ABI) 373 automated DNA sequencer, using the PRISMTM dye-deoxy terminator sequencing kit (ABI). Custom-made primers were used where necessary to complete the sequences of both strands (Central Molecular Biology Facility, University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.).

Sources of carbohydrates

Konjac glucomannan (glucose/mannose, 40:60), carob galactomannan (galactose/mannose, 20:80), wheat arabinoxylan (arabinose/xylose, 41:59), rye arabinoxylan (arabinose/xylose, 49:51), β -glucan, sugar beet arabinan, potato galactan, rhamnogalacturonan, mannotriose, mannotetraose, mannopentaose and mannohexaose were from Megazyme International. Locust bean gum galactomannan, larchwood arabinogalactan, laminarin, methyl glucuronoxylan, *Saccharomyces cerevisiae* mannan and oat spelt xylan (arabinose/xylose, 10:90) were from Sigma. Hydroxyethyl cellulose (HEC) was from Aldrich. Bacterial microcrystalline cellulose (BMCC), acid swollen cellulose (ASC) were provided by the Babraham Research Institute (Cambridge, U.K.). To generate carob mannan from galactomannan, 300 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 4.5 g of carob galactomannan, was incubated with 3000 units of *C. japonicus* α -galactosidase [22] at 37 °C with gentle mixing until no further galactose could be detected using the galactose dehydrogenase kit (Roche). The resulting insoluble de-galactosylated mannan was pelleted by centrifugation, resuspended in water, boiled for 10 min and, after washing several times, was resuspended in 5 ml of the phosphate buffer. Production of sodium borohydride-reduced mannohexaose was as described by Boraston et al. [23], adapted for oligosaccharides. Briefly, 25 mg of oligosaccharide sugar was dissolved in 1 ml of 100 mM NH₄OH, pH 10.8, and 25 mg of sodium borohydride was added and left to react for 16 h at room temperature with shaking. The reaction was quenched by addition of 5 ml of water and then lyophilized. The sugar alditol was then purified by Sephadex G25F (Pharmacia) gel filtration at 1 ml/min in a mobile phase of water to remove the buffer and sodium borohydride. Its purity was then verified by HPLC.

Construction of plasmids

To produce pDH12, which encodes residues 91–486 of Man26B comprising the GH26 catalytic module (Figure 1), the region of *man26B* of nucleotides 271–1461 was amplified from pDH11 (a recombinant of pBluescript containing full-length *man26B*; Figure 1) by PCR. The two primers used contained *Nde*I and *Bgl*II restriction sites, respectively, as shown in Table 1. The amplified DNA was initially cloned into pCR-Blunt, its sequence verified, and then cloned into pET16b restricted with *Nde*I and *Bam*HI. The variants of *man5A*, *man5B* and *man5C* were cloned into expression vectors using a similar strategy. Details of the primers used to amplify appropriate regions of the four mannanase

Figure 1 Schematic of the molecular architecture of Man5A, Man5B, Man5C and Man26B

The different modules in the three enzymes are as follows: black box, signal peptide; white box, GH26; densely spotted box, GH5; thick-striped box, CBM10; checked box, CBM2a; cross-hatched box, CBM5; sparsely spotted box, X4 module; thin-striped box, sequences flanking Man5C GH5 of unknown function; black line, linker sequence. The names of the plasmids and their encoded proteins are listed. Note that the internal and C-terminal CBM10s in Man5A are defined as CBM10-1 and CBM10-2, respectively.

genes, the expression vectors used and the modules of the enzymes encoded by the recombinant plasmids are provided in Table 1.

Production and purification of recombinant proteins

E. coli BL21:pLysS harbouring recombinant plasmids was cultured in 400 ml of LB containing ampicillin (100 µg/ml) in 2 l conical flasks. Cultures were grown to mid-exponential phase at 30 °C and 200 rev./min and expression of target proteins was induced by the addition of 1 mM isopropyl β-D-thiogalactoside and incubation for a further 5 h. Under these conditions the soluble forms of the truncated derivatives of the four mannanases were evident, and these proteins were purified from cell-free extracts by immobilized metal-affinity chromatography ('IMAC') as described previously [12].

Affinity gel electrophoresis (AGE)

The capacity of the various non-catalytic modules to bind soluble polysaccharides was evaluated by AGE. Continuous native polyacrylamide gels were prepared consisting of 7.5% (w/v)

acrylamide in 25 mM Tris/250 mM glycine buffer. To one of the gels 0.1% polysaccharide was added prior to polymerization. Approx. 5 µg of each enzyme derivative and BSA (as a non-interacting negative control) was loaded on the gels and subjected to electrophoresis at 10 mA/gel for approx. 2 h at room temperature.

Qualitative binding of modules of Man5A, Man5B and Man5C to insoluble ligands

Qualitative assessment of the binding of the derivatives of Man5A, Man5B and Man5C to insoluble polysaccharides was carried out as follows. Pure protein (approx. 5 nmol) was mixed with insoluble polysaccharide (2 mg) in a final volume of 250 µl. Tubes were incubated on ice for 1 h, with regular gentle mixing before being centrifuged at 13 000 g for 1 min and the supernatant, containing unbound protein, carefully removed. The polysaccharide pellet was then washed twice in 250 µl of 50 mM sodium phosphate buffer, pH 7.0, before being re-suspended in 60 µl of 10% (w/v) SDS and boiled for 10 min to release any bound protein. Controls with protein but no polysaccharide were included to ensure that no precipitation occurred during the

Table 1 Primers and vectors used to generate truncated forms of *man5A*, *man5B*, *man5C* and *man26B*

Underlined residues in each primer indicate the restriction sites.

Plasmid	Primers	Restriction sites in primers	Vector	Gene and region	Module	His-tag position
pDH12	5'-GCGGCGCATATGGCTGCGGTGGCTACCTGTACTG-3' 5'-GGAAGATCTTCAGTCTGCCTCGTTAAAGTC-3'	<i>NdeI/BglII</i>	pET16b	<i>man26B</i> , 271–1461	GH26	N-terminus
pDH14	5'-CATGCCATGGCATCTGCGGGTTTTATGTG-3' 5'-CGCGGATCCGATGCGGCACTTGATCACAG-3'	<i>NcoI/BamHI</i>	pET32c	<i>man5A</i> , 134–1335	GH5, CBM10-1	N-terminus
pDH15	5'-CATGCCATGGCATCTGCGGGTTTTATGTG-3' 5'-CATCATGCTCGAGTGGGTACTGGTGGTGGCCAC-3'	<i>NcoI/XhoI</i>	pET32c	<i>man5A</i> , 134–1182	GH5, CBM10-1	N-terminus
pDH16	5'-CATGCCATGGCATCTGCGGGTTTTATGTG-3' 5'-CGCTCGAGACCGCTGCGGCCAAAGATGGTG-3'	<i>NcoI/XhoI</i>	pET32c	<i>man5A</i> , 134–1047	GH5	N-terminus
pDH18	5'-GCGCATATGGCATTGGCCGGCTTTAAGATTCC-3' 5'-GGATCCAGACGATGACTAATCCCCCATAATGC-3'	<i>NdeI/BamHI</i>	pET16b	<i>man5B</i> , 67–1251	GH5, CBM10	N-terminus
pDH19	5'-GCGCATATGGCAAGTTCCGCTGCACCTGCG-3' 5'-GGATCCCTAATTGCAAACCGACCCGTG-3'	<i>NdeI/HindIII</i>	pET16b	<i>man5B</i> , 1363–1693	CBM2a	N-terminus
pDH20	5'-GCGCATATGGCATTGGCCGGCTTTAAGATTCC-3' 5'-CGCGGATCTCATGCAGAACCGTAAACACTGGCC-3'	<i>NdeI/BamHI</i>	pET16b	<i>man5B</i> , 67–984	GH5	N-terminus
pDH22	5'-GCGCGCATATGTTGGGTGGCAGGCAGTTGCAG-3' 5'-CGCGCGCGGATCCTCCCTGACGAGGAAACAC-3'	<i>NdeI/BamHI</i>	pET16b	<i>man5C</i> , 51–271	CBM5	N-terminus
pDH23	5'-GCGCGCATATGTTGGGTGGCAGGCAGTTGCAG-3' 5'-CGCGCGCGGATCCTCCTGCTGCGCGGATAACAC-3'	<i>NdeI/BamHI</i>	pET16b	<i>man5C</i> , 51–450	CBM5 and CBM10	N-terminus
pDH24	5'-GGGTTTCATATGCCATTGACTATCCCACTCAG-3' 5'-CGCGCGCGGATCCTCTCCACCAATTACCCCACTC-3'	<i>NdeI/BamHI</i>	pET16b	<i>man5C</i> , 1098–2237	GH5 —	N-terminus
pDH25	5'-CGGCGTGGATCCACAGCCTGAAATTGGAAGT-3' 5'-CCCCCAAGCTTGAGAAATACTTATTCATCAG-3'	<i>BamHI/HindIII</i>	pET32c	<i>man5C</i> , 931–2490	GH5 +	N- and C-termini

assay period. Samples, which generally comprised 10 μ l of starting material, unbound protein, material in washes and 2.5 μ l (unless otherwise stated) of protein eluted from insoluble polysaccharide with SDS, were then subjected to SDS/PAGE on 10% (w/v) polyacrylamide gels.

Enzyme assays

To evaluate enzyme activity 3 ml reactions were set up comprising 50 mM sodium phosphate/12 mM citrate buffer, pH 6.5, containing 1 mg/ml BSA and soluble or insoluble substrate at 0.04–2% (w/v). The reaction, which was initiated by the addition of 200 μ l of appropriately diluted enzyme, was incubated at 37 °C for up to 30 min, and at regular time intervals 500 μ l aliquots were removed and the quantity of reducing sugar was determined [24]. The nature of the products was evaluated by HPLC (see below). The pH profile of the catalytic activity of the mannanases was determined using the following buffers: pH 3–5, 50 mM sodium acetate; pH 5–9, 50 mM sodium phosphate; pH 9–11, 50 mM CAPSO [3-(cyclohexamino)-2-hydroxy-1-propanesulphonic acid; Sigma]. The resistance of the enzymes to proteinases and their thermostability were determined as described previously [25]. To determine the reaction products released from the mannose-containing substrates, and to quantify the activity of the mannanases against manno-oligosaccharides, the enzymic incubations were subjected to HPLC and polysaccharide analysis by carbohydrate gel electrophoresis (PACE) [26]. For HPLC analysis the enzyme reactions were carried out as described above except that the 500 μ l aliquots were boiled for 10 min, filtered and then subjected to HPLC using an analytical CARBOPAC™ PA-100 anion-exchange column (Dionex) equipped with a guard column. The system had a loop size of 200 μ l and was run at a flow rate of 1 ml/min at a pressure of 2300 lbf/in² (15870 kPa). Sugars were detected by pulsed

amperometric detection ('PAD') with electrode settings of $E_1 = +0.05$, $E_2 = +0.6$ and $E_3 = -0.6$. The elution conditions were: 0–5 min, 100 mM NaOH; 5–15 min, 100 mM NaOH with a 0–75 mM sodium acetate gradient; and 15–25 min, 100 mM NaOH containing 75 mM sodium acetate. The catalytic efficiency of the mannanases against manno-oligosaccharides was determined using 2 nM–1 μ M enzyme and a substrate concentration of 10 μ M, and the data were fitted to the following equation:

$$k \cdot t = \ln([S_0]/[S_t]) \quad (1)$$

where k is $(k_{cat}/K_m) \times$ enzyme concentration, t is time, and $[S_0]$ and $[S_t]$ indicate substrate concentration at times 0 and t , respectively [27]. In the PACE method the reaction products were derivatized with a negatively charged fluorophore and their migration during PAGE was analysed [26].

RESULTS

Cloning and sequencing of *Cellvibrio* mannanase genes

Screening a gene library of *C. japonicus*, constructed in λ ZAPII, for mannanase-expressing clones, revealed 26 recombinant phage that displayed high activity against Azo-carob galactomannan, and five that hydrolysed the dyed polysaccharide very slowly. *Cellvibrio* DNA was excised from 10 of the phage (three displaying low and seven exhibiting high mannanase activities) into pBluescript SK⁺, and the DNA inserts were used as probes in plaque hybridization of all the mannanase-expressing phage. The results (not shown) revealed that the phage displaying high mannanase activity contained one of three distinct regions of *Cellvibrio* DNA, whereas the three phage that hydrolysed galactomannan slowly contained a common *Cellvibrio* genomic sequence. Recombinants of pBluescript containing the four different regions of *Cellvibrio* DNA that encoded a functional

mannanase gene were designated pDH11, pDH13, pDH17 and pDH21 respectively. Southern hybridization showed that the *C. japonicus* DNA in these four plasmids was present as a single copy within the *Cellvibrio* genome (results not shown). Thus the screening strategy employed in these experiments has identified four novel *Cellvibrio* mannanase genes, which are each present as single copies in the genome of the Gram-negative bacterium.

The nucleotide sequences of pDH11, pDH13, pDH17 and pDH21 revealed that each plasmid contained an open reading frame within the respective *Cellvibrio* inserts that probably encodes a glycoside hydrolase. The open reading frames derived from pDH11, pDH13, pDH17 and pDH21 were designated *man26B*, *man5A*, *man5B* and *man5C*, respectively. The 1293 bp *man5A* gene encodes a protein, designated Man5A, of M_r 46322; *man5B* consists of 1782 bp and directs the synthesis of Man5B, a protein of M_r 62773; *man5C* comprises 2490 bp and encodes a protein of M_r 89188, designated Man5C, while the 1459 bp *man26B* gene encodes Man26B, which has an M_r of 53230. The codon usage of the four genes is very similar to other *Cellvibrio* open reading frames encoding glycoside hydrolases, with an A + T content of approx. 45% and a preference for G or C in the wobble position of approx. 70%; little is made of the codons that modulate *E. coli* expression (results not shown).

Modular structure of *Cellvibrio* mannanases

Comparison of Man5A, Man5B and Man5C against protein databases revealed that all three enzymes have a modular structure (Figure 1). The N-terminal region of Man5A contains features typical of a bacterial signal peptide. The N-terminal 27 residues, which contains several basic residues, is followed by 14 small hydrophobic amino acids. The predicted cleavage site of the signal peptide is between Gly-53 and Lys-54 according to the A-X-B (-3 to -1) rule [28], where A and B must be small non-polar amino acids. The putative signal peptide is followed by a module comprising 292 residues that exhibits sequence similarity to proteins in GH5 (Figure 2A), displaying highest identity with β -1,4-mannanases from *Vibrio* sp. strain MA-138 (55%; SWISS-PROT accession no. 069347) and *Streptomyces coelicolor* (50%; 086599), respectively. The 6000 glycoside hydrolases currently in protein databases have been grouped into enzyme families based on sequence similarity. All proteins in the same family have a common protein fold and the catalytic mechanism and the catalytic machinery are completely conserved [3], although enzymes belonging to the same family often display differences in substrate specificity. Currently there are 85 GHs and GH5 contains, in addition to mannanases, several different enzymes that cleave β -glucan polymers. The two catalytic amino acids identified in other GH5 enzymes are also present in Man5A, with Glu-173 and Glu-269 functioning at the catalytic acid/base and nucleophile residues, respectively. The GH5 of Man5A is joined by a small serine-rich linker sequence to two modules, designated CBM10-1 and CBM10-2, comprising 30 and 35 residues, respectively, that display sequence identity to proteins in CBM family 10 (Figure 2D). Modules from this family bind to crystalline cellulose [15], and thus it is likely that the two CBM10s in Man5A will also display affinity for this polysaccharide.

Man5B also contains a typical prokaryotic signal peptide in which the two basic residues at positions 4 and 5 are followed by a stretch of 18 small hydrophobic amino acids. The signal peptide is followed by a 295-residue module that displays significant sequence identity to GH5 enzymes exhibiting greatest similarity to *C. japonicus* Man5A (Figure 2A). The catalytic

acid/base and nucleophile residues of the Man5B GH5 module are Glu-578 and Glu-672, respectively. C-terminal to the catalytic module is a 40-residue serine-rich linker sequence, which is appended to a module that displays sequence similarity to family 10 CBMs (Figure 2D). C-terminal to the CBM10 is a second 40-residue serine-rich linker that is joined to a 100-residue protein module that displays sequence identity to family 2a CBMs (Figure 2E).

Man5C contains a 20-residue N-terminal signal peptide, followed by a 57-amino-acid sequence that displays similarity to family 5 CBMs (CBM5). CBM5 from *Erwinia chrysanthemi* Cel5A (formerly known as CelZ) contains three aromatic residues (Trp-18, Trp-43 and Tyr-44) that play a pivotal role in ligand binding [29]. The CBM of Man5C lacks an aromatic residue at the equivalent position to either Trp-12 or Trp-18 in Cel5A, and the tyrosine at position 44 in the *Erwinia* module is replaced with a tryptophan in Man5C CBM5 (Figure 2F). C-terminal to CBM5 is a classic serine-rich linker sequence, which is followed by a 49-residue sequence that shows strong homology with family 10 CBMs (CBM10; Figure 2D). The CBM10 contains the three highly conserved aromatic residues (Tyr-106, Trp-120 and Trp-122) that in the equivalent module from *C. japonicus* Xyn10A CBM10 were shown to play a key role in ligand binding [15]. The CBM10 is linked by a second serine-rich linker sequence to a 104-residue protein module, designated X4, that is present in a number of different glycoside hydrolases including several mannanases. C-terminal to the X4 module is a 530-residue sequence that contains a typical GH5 catalytic module flanked by 75-amino-acid N-terminal and 85 residue C-terminal sequences that displays no significant identity with proteins in current databases. The GH5 module contains the two catalytic residues that are completely conserved in GH5 enzymes, Glu-578 (acid-base) and Glu-678 (nucleophile). As shown in Figure 2(B) the catalytic module of Man5C exhibited greatest homology (29%) to a GH5 mannanase from *Thermotoga neapolitana* (Q9RIK7).

The N-terminal region of Man26B contains features typical of a prokaryotic signal peptide with a stretch of 18 residues, consisting of mainly small hydrophobic amino acids, following a single basic residue at position 5. The likely cleavage point is between residues 22 and 23. Significantly the presence of a cysteine residue at position 23 is suggestive of a sequence recognized by signal peptidase II, indicating that the mannanase may be attached to a lipid and is thus membrane bound [30]. C-terminal of the signal peptide is a 67-amino-acid serine/glycine-rich linker sequence that is appended to a 396-residue module, which exhibits sequence identity with GH26 mannanases (Figure 2C). Man26B displays greatest identity with *Caldocellum saccharolyticum* ManA (38%; SWISS-PROT accession no. P77847) and the three mannanases from *Piromyces equi* (approx. 32%; P55296, P55297 and P55298). Man26B exhibits only 20% identity with the other GH26 enzyme from *C. japonicus*, Man26A. Based on sequence identity with Man26A, where the catalytic amino acids have been experimentally determined [4], the acid/base and nucleophile residues in Man26B are predicted to be Glu-306 and Glu-410, respectively.

Biochemical properties of the non-catalytic modules of Man5A, Man5B and Man5C

To investigate the biochemical properties of the putative CBMs in the three GH5 mannanases, these modules were expressed in *E. coli* and their capacities to bind to a range of different polysaccharides were evaluated. Full-length Man5A, containing both CBM10-1 and CBM10-2 (GH5A-CBM10-1-CBM10-2)

bound to cellulose (Avicel) and crystalline mannan, while the catalytic module of this enzyme did not interact with either polysaccharide (Figure 3). Truncated Man5A comprising the catalytic module and CBM10-1 (GH5–CBM10-1), when expressed in *E. coli*, was heavily processed; however, the GH5–CBM10-1 fusion did appear to bind both Avicel and ivory nut mannan, albeit weakly as there was a significant amount of protein in the unbound fraction (Figure 3). These data suggest that CBM10-1 and CBM10-2, when present in tandem, bind to crystalline polysaccharides, while CBM10-1 alone interacts rather weakly with these ligands.

Attempts to express the GH5 of Man5B fused to CBM10 (GH5B–CBM10) generated full-length and processed forms of the protein. The full-length protein bound to Avicel and ivory nut mannan (Figure 4A). The truncated protein present in the GH5–CBM10 preparation and the GH5 catalytic module expressed as a discrete entity (Figures 4A and 4B) did not bind to either polysaccharide, indicating that CBM10 was mediating the binding of GH5–CBM10 to both cellulose and mannan. The CBM2a module of Man5B bound tightly to crystalline cellulose, but more weakly to ivory nut mannan (Figure 4C). The data presented in Figures 4(C)–4(E) showed that the ligand specificity of Man5B CBM2a was similar to the properties of CBM2a from *C. japonicus* Xyn10A and Cel5A (formerly CelE [31]).

Although the Man5C CBM5 and CBM5–CBM10 could be produced, unfortunately it was not possible to express CBM10 as

a discrete protein from this enzyme. The CBM5 bound relatively weakly to both cellulose (Avicel) and crystalline ivory nut mannan (Figure 5C), as protein was evident in the unbound fraction (Figure 5C, lanes 2 and 7). CBM5–CBM10, however, interacted more tightly with these polysaccharides as evidenced by the lack of protein in the unbound fraction (Figures 5A and 5B). The capacity of the CBMs in Man5A, Man5B and Man5C to bind the soluble polysaccharides carob galactomannan, konjac glucomannan, oat spelt and wheat xylans, hydroxyethyl cellulose, β -glucan, laminarin, sugar beet arabinan (linear and branched), β -1,4-galactan and apple pectin was evaluated by AGE. None of the CBMs displayed affinity for any of the ligands tested (results not shown). The biochemical properties of the X4 module of Man5C will be reported elsewhere.

Catalytic activity of Man5A, Man5B, Man5C and Man26B against polysaccharides

The catalytic modules of Man5A, Man5B, Man5C and Man26B were expressed and purified and their capacity to hydrolyse a range of polysaccharides was evaluated. In view of the unusual sequences flanking the GH5 of Man5C, two derivatives of the enzyme containing the presumed GH5 catalytic domain of the enzyme were expressed in soluble form. The GH5 module, without its flanking sequences (GH5–), displayed no catalytic activity, whereas the enzyme containing these additional sequen-

A									
	10	20	30	40	50				
Man5A	LGMLAAVSA	SAGFYVSGKQ	LRGNGNNFI	MKGVNLPHAW	FPDRTHQALA				
Man5B	LIGP--ATAA	LAGFYISGQ	LDGNGNNFI	MKGVNLPHAW	YTSRTQALA				
069347	LLLPWASCA	HAGFYVSGV	LYEANGSAFK	IRGINHAFTW	YFDKLSVALS				
086599	LGGLFAARAA	AGGHVSNGR	VLEGNGSVFV	MKGVNHAYTW	YPDRTG-SIA				
P51529	LGGLFAARAA	AGGHVSNGR	VLEGNGSAFV	MKGVNHAYTW	YPDRTG-SIA				
Q92F13	-----	--GLHVNGR	LYEANGQFT	IRGVSHPHNW	YQRTQ-AFA				
Man5A	LADISATGAN	SVRVVLSDGQ	QWRTTATEV	ANINWAKQN	KLIIVLEVED				
Man5B	LADISATGAN	SVRVVLSDGQ	QWRTTATEV	ANINWAKQN	KLIIVLEVED				
069347	LSGLAATGAN	TVRVVLSNGV	RWTKNDVSDV	INILNLAKEAN	NLIALEVED				
086599	LADIAAAGAN	TVRVVLSNGV	RWTKNSABEV	SALIGQCKAN	KVICVLEVED				
P51529	LADIAAAGAN	TVRVVLSNGV	RWTKNSABEV	SALIGQCKAN	KVICVLEVED				
Q92F13	FADIKSHGAN	TVRVVLSNGV	RWSKNGPSDV	ANVISLCKQN	RLICMLEVED				
Man5A	TTGYGQQTAA	TLSEAVDWTI	AERNALIGQE	DYVLINIGNE	PFNGGQSAST				
Man5B	TTGYGQQTAA	TLSEAVDWTI	AERNALIGQE	DYVLINIGNE	PFNGGQSAST				
069347	TTGYGQQTAA	TLSEAVDWTI	AERNALIGQE	DYVLINIGNE	PFNGGQSAST				
086599	TTGYGQQTAA	TLSEAVDWTI	AERNALIGQE	DYVLINIGNE	PFNGGQSAST				
P51529	TTGYGQQTAA	TLSEAVDWTI	AERNALIGQE	DYVLINIGNE	PFNGGQSAST				
Q92F13	TTGYGQQTAA	TLSEAVDWTI	AERNALIGQE	DYVLINIGNE	PFNGGQSAST				
Man5A	WLNLRHDAIN	RLRNAGFTHT	LMVDAANWQ	DWENIMRNNA	SSIFNSDPRR				
Man5B	WLNLRHDAIN	RLRNAGFTHT	LMVDAANWQ	DWENIMRNNA	SSIFNSDPRR				
069347	WLNLRHDAIN	RLRNAGFTHT	LMVDAANWQ	DWENIMRNNA	SSIFNSDPRR				
086599	WLNLRHDAIN	RLRNAGFTHT	LMVDAANWQ	DWENIMRNNA	SSIFNSDPRR				
P51529	WLNLRHDAIN	RLRNAGFTHT	LMVDAANWQ	DWENIMRNNA	SSIFNSDPRR				
Q92F13	WLNLRHDAIN	RLRNAGFTHT	LMVDAANWQ	DWENIMRNNA	SSIFNSDPRR				
Man5A	NVTFSVDMYK	VYPNDTAVIN	YNSAFNSMNL	PLVVGEPAN	HFGSYVDAGS				
Man5B	NVTFSVDMYK	VYPNDTAVIN	YNSAFNSMNL	PLVVGEPAN	HFGSYVDAGS				
069347	NVTFSVDMYK	VYPNDTAVIN	YNSAFNSMNL	PLVVGEPAN	HFGSYVDAGS				
086599	NVTFSVDMYK	VYPNDTAVIN	YNSAFNSMNL	PLVVGEPAN	HFGSYVDAGS				
P51529	NVTFSVDMYK	VYPNDTAVIN	YNSAFNSMNL	PLVVGEPAN	HFGSYVDAGS				
Q92F13	NVTFSVDMYK	VYPNDTAVIN	YNSAFNSMNL	PLVVGEPAN	HFGSYVDAGS				
Man5A	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
Man5B	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
069347	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
086599	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
P51529	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
Q92F13	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
Man5A	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
Man5B	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
069347	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
086599	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
P51529	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
Q92F13	IMARAQVGF	GYLGWSNGN	SSNLSDALDV	TNPNAGBLAT	WGNLLNINW				
Man5A	GIKNTSRKAT	IFGGSG							
Man5B	GIKNTSRKAT	IFGGSG							
069347	GIKNTSRKAT	IFGGSG							
086599	GIKNTSRKAT	IFGGSG							
P51529	GIKNTSRKAT	IFGGSG							
Q92F13	GIKNTSRKAT	IFGGSG							

Figure 2 For legend, see facing page

C									
	10	20	30	40		370			
Man26B	SWGFENNDYC	ISHSFPCANR	TSLPEVATRE	LPVNDQASAK	Man26B	LVITLDKLPD	FNEAD		
X82179	ICLIALLCN	HAANKSSASR	ADVKPVTIKL	--VDSQATME	X82179	RPENINNGTL	EDFQA		
P77847	GGYTIAIDYF	TIEELVLANK	NKIS-VDIKL	VTNPNHPNAQ	P77847	RVLTRDELDP	WRSYV		
P55298	SWGNIWIDAL	VVNDTPNVAK	DVSPHINPTL	--VNPKAIPA	P55298	TVMNMDELPS	FNVNS		
P55297	SWGMMWVDAF	VINDAPNAAK	----DVTSKL	NPINPKAIPA	P55297	TVMNMDELPS	FNVNS		
P55296	SWGMMWVDAF	VINDAPNAAK	----DVSSKL	NPVNPKAIPA	P55296	TVMNMDELPS	FNVDS		
	50	60	70	80					
Man26B	TRRVYSYLSK	IWGSKMIAGQ	QDLTWKDAID	MYQRVFDG					
X82179	TRSLFAFMQE	QRRHSIMFGH	QHETTOGLTD	GTQSTFNAV					
P77847	--KLKMYLAS	IYGEKILSGQ	Q--SSGDGKE	I-QMIFDVTK					
P55298	AKKLYDFLRS	NYGKRILSGQ	VGGAGQDEGQ	EQRIQKATG					
P55297	AKKLYDFLKT	NYGKRILSGQ	VGAAGQDEGQ	EQRIQKATG					
P55296	AKKLYDFLKT	NYGKRILSGQ	VGAAGQDEGQ	EQRIQKATG					
	90	100	110	120					
Man26B	KYPALMGYDF	MNYGMTANWV	EGLGQTEEAI	AHAQRGGIVT					
X82179	DFAAVYQWDT	LS--IVAPKA	EGVAQVKKAY	A--RGGLIT					
P77847	RYPAVRGDFD	MDY--SPSRV	EGTKGTETAI	KWWKSGGIVA					
P55298	KLPAVWNMDF	IFESNDCTWR	ENPDITETAI	NKYQKGIMS					
P55297	KLPAVWNMDF	IFESNDCTWR	QNPDIETAI	NKYQKGIMS					
P55296	KLPAVWNMDF	IFESNDCTWR	QNPDIETAI	NWKKKGIMA					
	130	140	150	160					
Man26B	FAWHWRDPA	LNTANVNSAE	FYSEKTDFTI	PLANCALDTS					
X82179	VSSHFDNPKT	DTQKGVWVPG	TSWDQTPAVV	DLPGCAYN--					
P77847	FCWHWNAPTQ	L--IDQPGKE	FYTEATTFDI	---KKAMDNN					
P55298	AQWHWNIAAG	----TGDFPA	FYKKTTFESI	---DNAVTEG					
P55297	AQWHWNIAAG	----TGDFPA	FYKKTTFESI	---DNAVTEG					
P55296	AQWHWNIAAG	----TGDFPA	FYKKTTFESI	---DNAVTEG					
	170	180	190	200					
Man26B	SPAYAQINAG	IDVIAAELKK	LGDADVPVLW	RPLHEASGYN					
X82179	--PV---LNGY	LDQVVAEWANN	LKDEQIPVIF	RLYHEHTG--					
P77847	STYEKLILRD	IDAIAEQLKR	LQKEGVVPLF	RPLHEASG--					
P55298	TWYEKILKD	IDRVAGHIKK	LQAVNMPLIW	RPLHENDG--					
P55297	TWYEKILKD	IDRVAGHIKK	LQAVNMPLIW	RPLHENDG--					
P55296	TWYEKILKD	IDRVAGHIKK	LQAVNMPLIW	RPLHENDG--					
	210	220	230	240					
Man26B	GNGWFWGARA	RTDGAPQAY	NILLWRHLYD	RLVNYHQLDN					
X82179	--SWFWWGDK	Q--LFYYSVE	YLRDVKGVNR	FLYAYSPNN					
P77847	--GFWWG-A	K--GPEPY-	-IKLWLKMF	RLVNYHGLNN					
P55298	--DWFWWG--	---NNPKSCA	K--LWKILYE	RMVNYHGLNN					
P55297	--DWFWWG--	---NNPKSCA	K--LWKILYE	RMVNYHGLNN					
P55296	--DWFWWG--	---NNPKACA	K--LWKILYE	RMVNYHGLNN					
	250	260	270	280					
Man26B	LIWVWNGQNP	AWFPGNDVAD	IMSFDIYDNT	DNKTYKSQLA					
X82179	FLYAYSNN--	FWYPGDEWVD	VLGFDIYDNA	D--WFRNVVA					
P77847	LIWVWNGQDA	AWYPGDNVVD	IIGEDIYE--	EKAQYSPYAD					
P55298	LIWLWNGKND	ANTP-VDYID	IIGVDIYAND	----HGPOTT					
P55297	LIWLWNGKND	ANTP-VDYID	IIGVDIYAND	----HGPOTT					
P55296	LIWLWNGKND	ANTP-VDYID	IIGVDIYAND	----HGPOTT					
	290	300	310	320					
Man26B	TYTQVRQYSA	ENKMIALSEN	SYIPDPDKIQ	ADNANWLFWM					
X82179	NAALVRMAEA	RKIPVISEI	GIAPDIEAGL	YDNQWYRRLI					
P77847	RFAKALKYTN	ARKMIALTEC	GIPTDPAVLK	QEGISWSWFS					
P55298	AYNTHDFDYG	GKKMVVLSEN	GRIPDIQQCV	DQNAWVWFYQ					
P55297	AYNTHDFDYG	GKKMVVLSEN	GRIPDIQQCV	DQNAWVWFYQ					
P55296	AYNTHDFDYG	GKKMVVLSEN	GRIPDIQQCV	DQNAWVWFYQ					
	330	340	350	360					
Man26B	TWNDCGNFAT	TLASGTHQDN	FWSGDYNYNTS	AHKTHVYNDH					
X82179	SGLKADPDAR	EIAFLLVWRN	PQGVPGPNGT	QVPHYWVPAN					
P77847	VWA-GNF---	-VWGTSGKYND	EWNDNH---	--LRKIYNSD					
P55298	TWNS-----	-----E	FILQDSYHTD	AQLKEYFTHK					
P55297	TWNS-----	-----E	FILQDSYHTD	AQLKEYFTHK					
P55296	TWNS-----	-----E	FILQDSYHTD	AQLKEYFTHK					

Figure 2 Alignment of *C. japonicus* Man5A, Man5B, Man5C and Man26B with protein modules

In (A) the primary sequences of the catalytic domains of Man5A (SWISS-PROT accession no. AY187031) and Man5B (AY187032) from *C. japonicus* are aligned with the following GH5 enzymes ManA from *Vibrio* sp. strain MA-138 (069347), ManA2 from *S. coelicolor* (086599), ManA from *Streptomyces lividians* 66 (P51529) and ManA from *Thermospora fusca* (Q92F13). The alignments, which do not include signal peptides, begin at residues 35 and 16 in Man5A and Man5B, respectively. In (B) the catalytic domain of Man5C (AY187033) is aligned with GH5 enzymes including ManB from *Thermotoga neapolitana* (Q9RIK7), ManF from *Bacillus stearothermophilus* (Q92I87), ManB from *Thermotoga maritima* (Q9RIK9) and a second endo- β -1,4-mannanase from *Thermotoga maritima* (Q9X0VA). Alignments begin at residue 376 of Man5C. (C) Alignments of *C. japonicus* Man26B (AY187034) with GH26 enzymes including *C. japonicus* Man26A (X82179), ManA from *Caldocellum saccharolyticum* (P77847), and ManA, ManB and ManC from *Piromyces* sp. (P55296, P55297, P55298). Alignments do not include the N-terminal signal peptide and linker sequence of Man26B and thus begin at residue 112. In (A)–(C) the catalytic acid/base (*AB) and nucleophile (*) residues are depicted. (D) Alignment of the CBM10 modules in Man5A and Man5B with other CBM10 modules in *C. japonicus* enzymes, which are Xyn11A (Q59674), Xyn10A (P14768), CelA (P10476), CelB (P18126) and Cel5A (Q59665). (E) Alignment between the CBM2a module in Man5B with other CBM2a in *C. japonicus* enzymes, which are CelA (P10476), CelB (P18126), CelC (P27033), CelE (Q59665), XylA (P14768), XylB (P23030), XylC (P23031) and XylD (Q51815). (F) The CBM5 from Man5C aligned with the corresponding module from *E. chrysanthemi* CelZ (Y00540). Conserved residues are displayed in bold. Aromatic residues in the CBMs in (D)–(F) that bind directly to crystalline cellulose are indicated by +.

Figure 3 The binding of Man5A CBMs to insoluble polysaccharides

GH5-CBM10-1-CBM10-2 (**A**), GH5-CBM10-1 (**B**) and GH5 (**C**) were subjected to insoluble Avicel- and ivory nut mannan-binding assays (see the Materials and methods section), and the various fractions were analysed by SDS/PAGE. Lanes 1–5 show results from Avicel-binding assays, whereas lanes 6–10 show ivory nut mannan-binding data. Lanes 1 and 6, original protein; lanes 2 and 7, supernatant after pelleting polysaccharide; lanes 3, 4, 8 and 9, washes; lanes 5 and 10, material eluted from polysaccharides that were boiled for 5 min in 10% (w/v) SDS. High- (H) and low- (L) M_r markers (Sigma) are shown and the 66 and 45 kDa bands are identified in (**A**).

ces (GH5+) displayed glycoside hydrolase activity. The four enzymes displayed no activity against oat spelt, rye or wheat xylan, hydroxyethyl and carboxymethyl cellulose, barley β -glucan, laminarin, sugar beet arabinan (linear and branched), β -1,4-galactan, apple pectin and 4-nitrophenyl- β -D-mannopyranoside (results not shown). Man5A, Man5B, Man5C and Man26B, however, exhibited activity against carob galactomannan, locust bean galactomannan and konjac glucomannan. The kinetic constants displayed by the four enzymes against these substrates are presented in Table 2. Against carob galactomannan Man5A was the most active enzyme at saturating substrate concentrations (k_{cat}), while Man5B displayed the highest catalytic efficiency. Man5A, Man5B and Man26A were significantly more active than Man26B against konjac glucomannan, whereas Man5C was considerably less active than the other three enzymes against the galactomannans and glucomannan, although it was the only mannanase that attacked ivory nut crystalline mannan. HPLC analysis of the reaction products generated when Man26B, Man5A, Man5C and Man5B were incubated with carob galactomannan (Figure 6) revealed an

extensive range of oligosaccharides. These data indicate that the four mannanases display a classical endo-mode of action. Examples of PACE analysis of the reaction products generated when the mannanases were incubated with glucomannan, mannan or galactomannan are displayed in Figure 7. The fingerprint of oligosaccharides produced was characteristic of the enzyme and the substrate. To determine whether the mannanases can accommodate glucose at the +1 subsite, after glucomannan digestion by the mannanases, the enzyme was inactivated by heat treatment, and a β -glucosidase, which removes glucose from the non-reducing end of oligosaccharides, was added. Several oligosaccharides were sensitive to the glucosidase treatment (Figure 7A, *), indicating that all the mannanases can accommodate glucose at the +1 subsite. Furthermore, subtle differences in the oligosaccharides produced by the different enzymes were observed, particularly during the initial stages of hydrolysis. The most notable difference was that Man5A and Man26B, but not Man26A, appeared to produce an oligosaccharide that migrates between mannose and mannobiose. Given that cello-oligosaccharides migrate quicker than the equivalent manno-oligosaccharide, it is likely that this product is a disaccharide of glucose and mannose (Glc-Man). As β -glucosidase attacked the presumptive Glc-Man product, glucose is likely to be at the non-reducing end. It is probable, therefore, that glucose is not easily accommodated in the -2 subsite of Man26A, while Man5A and Man26B both appear to readily bind glucose at the -2 subsite. Man5B also produces a product that co-migrates with the presumptive Glc-Man disaccharide; however, the oligosaccharide is not attacked by β -glucosidase, suggesting that it comprises glucose at the reducing end, i.e. Man-Glc, which points to the accommodation of glucose at the -1 subsite of this enzyme.

Catalytic activity of Man5A, Man5B, Man5C and Man26B against manno-oligosaccharides

The capacity of the four enzymes to hydrolyse a range of manno-oligosaccharides was evaluated by HPLC. The catalytic efficiency (k_{cat}/K_m) of these enzymes was determined by plotting a decrease in substrate concentration against time using eqn (1). The data (Table 3) show that Man5B displayed maximal activity against mannopentaose, indicating that this enzyme contains five mannose-binding subsites. Man5A and Man26B hydrolysed mannohexaose more efficiently than mannopentaose, suggesting that both these enzymes contain a minimum of six mannose-binding sites. Man5C displayed similar catalytic efficiency against mannopentaose and mannohexaose but hydrolysed mannotetraose extremely slowly, indicating that this mannanase contains five sugar-binding sites. The four mannanases displayed no activity against 3,4-digalactosyl- α 1,6-mannopentaose, indicating that the galactose side chains cannot be accommodated in adjacent glycone subsites.

Topology of the subsites in Man5A, Man5B, Man5C and Man26B

The nature of the reaction products released from manno-oligosaccharides by the four mannanases were also analysed by HPLC. The data presented in Figure 8 show that Man26B generated the corresponding $n-1$ oligosaccharide and mannose from mannohexaose, mannopentaose, mannotetraose and mannotriose, respectively. These data suggest that the mannanase

Figure 4 The binding of Man5B CBMs to insoluble polysaccharides

GH5-CBM10 (A), GH5 (B), CBM2a (C), *C. japonicus* Xyn10A CBM2a (D) and *C. japonicus* Cel5A CBM2a (E) were subjected to insoluble cellulose- and ivory nut-binding assays as described in the Materials and methods section. Lanes 1–5 and 6–10 depict Avicel- and mannan-binding results, respectively. Details of lane contents are as for Figure 3. The amount of material loaded on the gels was as described in the Materials and methods section except for (C)–(E) lane 5, where 10 μ l of protein eluted with SDS from Avicel was applied instead of the normal 2.5 μ l.

contains at least five subsites glycone and one subsite aglycone of the site of bond cleavage. Man5A hydrolysed mannohexaose equally into two molecules of mannotriose or mannobiose and mannotetraose. Mannopentaose was cleaved mainly into mannobiose and mannotriose, although significant amounts of mannose and mannotetraose were also evident. Mannotetraose was hydrolysed exclusively into mannotriose and mannose. These data indicate that Man5A contains at least seven binding sites, with four subsites glycone and three aglycone of the site of bond cleavage. Man5B hydrolysed mannohexaose into either two molecules of mannotriose or mannobiose and mannotetraose; mannopentaose was cleaved exclusively into mannobiose and mannotriose, while mainly mannose and mannotriose were generated from mannotetraose, although mannobiose was also evident. Man5B is thus likely to contain two subsites aglycone and three subsites glycone of bond cleavage. Against mannopentaose Man5C released exclusively mannotetraose and mannose, while hydrolysis of mannohexaose yielded only mannobiose and mannotetraose (Figure 8). These data indicate that Man5C contains five mannose-binding sites. As the enzyme acts via a double-displacement mechanism (all enzymes in the same gly-

coside hydrolase family have a common mechanism [32]), four subsites are likely to be in the glycone region of the substrate-binding site, as the binding energy released when substrate binds at these locations can be harnessed in both the glycosylation and deglycosylation reactions.

To determine conclusively the mode of action of Man5C, its activity against sodium borohydride-reduced mannohexaose was assessed. In this substrate the reducing end has been chemically modified to a sugar alditol. HPLC analysis showed that the sugar alditols were eluted at different times from the corresponding non-reduced sugar, allowing the determination of the cleavage position within the oligosaccharide. The data presented in Figure 9 show that reduced mannohexaose was cleaved exclusively into mannotetraose and mannobitol (the reduced form of mannobiose). Spiking the completed reactions with appropriate standard sugars and sugar alditols unambiguously identified the products. Mannotetraose contained the new reducing end formed by the cleavage event, and this product must therefore bind to the glycone region of the Man5C active site, indicating that the topology of the substrate binding cleft of this enzyme extends from -4 to $+1$.

Figure 5 The binding of Man5C CBMs to insoluble polysaccharides

CBM5-10 versus Avicel (A) and ivory nut mannan (B). Lanes 1, original protein; lanes 2, supernatant after pelleting polysaccharide; lanes 3–5, washes; lanes 6, material eluted from polysaccharides that were boiled for 5 min in 10% (w/v) SDS. (C) CBM5 versus Avicel (lanes 1–5) and ivory nut mannan (lanes 6–10); lane details are as for Figure 3. The amount of material loaded on the gels was as described in the Materials and methods section except for (A) and (B) where 15 μ l of protein eluted with SDS from the polysaccharide was applied instead of the normal 2.5 μ l.

Biophysical properties of Man5A, Man5B, Man5C and Man26B

The pH optima of Man5A and Man26B were between 7.5 and 8.0, typical of the majority of *C. japonicus* plant-cell-wall hydrolases [7,19,33]. In contrast, Man26B had a much broader pH optimum ranging from 5.0 to 8.5, while Man5C had a pH optimum extending from pH 5.0 to 6.5 (results not shown). Man26B and Man5C were particularly thermolabile, being rapidly inactivated at temperatures above 35 and 40 °C, respectively, whereas Man5A and Man5B were stable up to 50 °C. In common with the majority of extracellular plant-cell-wall-degrading microbial glycoside hydrolases, Man5A, Man5B, Man5C and Man26B were all completely resistant to proteolytic attack (results not shown).

DISCUSSION

This report describes the modular structure and catalytic activity of four mannanases from *C. japonicus*. It is interesting to note that the three GH5 mannanases described in this paper have a modular structure in which the CBMs, appended to the catalytic modules, display affinity for crystalline polysaccharides. The presence of a CBM5 in Man5C and a CBM2a in Man5B extends the range of enzymes that contain these 'crystalline CBMs'. CBM5s have previously been found in chitinases, endo- β -1,4-glucanases and a xylanase, while CBM2as have been described in a range of enzymes that attack cellulose, the backbone and side chains of xylan and pectins [18,29,33,34]; however, prior to this report neither module has been shown to be a component of mannan-degrading enzymes. It is also interesting to note that, in addition to Man5B, several other *C. japonicus* glycoside hydrolases including three endo- β -1,4-glucanases, a cellodextrinase and a xylanase contain a CBM10 adjacent to a CBM2a [33,35,36]. Given that these modules recognize similar ligands it is possible that their presence in the same enzyme increases cellulose affinity via co-operative effects, similar to the effect of tandem copies of CBM2b, CBM6 and CBM29 in other proteins [13,37,38]. Studies by Gill et al. [39], however, have shown that CBM10 and CBM2a do not act co-operatively to bind cellulose. Thus although the common occurrence of both CBM10 and CBM2a modules in the same *Cellvibrio* glycoside hydrolases points to a strong evolutionary selection pressure for this type of molecular architecture, it is currently unclear how these modular combinations increase the catalytic efficiency of plant cell wall hydrolases.

Comparison of the binding properties of CBM2a from Man5B with the corresponding module from a *Cellvibrio* cellulase and xylanase revealed very similar ligand specificities [31]. Similarly, CBM10s from *Cellvibrio* GH5 mannanases, GH10 xylanases and GH5 cellulases all bind to crystalline cellulose [39]. Thus, it would appear that members of CBM families that bind crystalline polysaccharides recognize very similar ligands irrespective of the substrate specificity of the enzyme from which the module is derived, a view that is supported by the observation that the CBM2a in the *Cellvibrio* rhamnogalacturonan lyase Rgl11A also binds to crystalline cellulose [18]. The specificity of family 10 and 2a CBMs for crystalline β -linked polysaccharides probably reflects the topology of their respective binding sites, which comprise a planar hydrophobic surface and are thus only able to bind crystalline ligands [40]. This is consistent with the mechanism of binding which is primarily via hydrophobic stacking interactions; there is a scarcity of hydrogen bonds between these CBMs and their crystalline ligands [41]. Furthermore, the similarity of the structure of crystalline cellulose and mannan [42] explains why these CBMs are able to bind both these polysaccharides. This is in sharp contrast with CBMs from families such as 4 and 6, which recognize single polysaccharide chains, where different members of the same family can bind very different ligands [11]. For example, CBM6 modules from the *Clostridium thermocellum* xylanase Xyn11A and a *Cellvibrio mixtus* cellulase bind preferentially to xylan [43] and cellulose (H. J. Gilbert, unpublished work), respectively, consistent with the substrate specificity of the appended enzymes.

In contrast with CBMs in families 2a and 10, the general specificity of CBM5s is a little less certain. While the Man5C CBM5 did exhibit some affinity for crystalline cellulose and mannan, qualitatively the binding was weaker than the CBM5 from the *Erwinia* cellulase Cel5A [29]. Furthermore, the capacity of the *Erwinia* enzyme to bind crystalline mannan has not been reported. CBM5 also appeared to bind more weakly to cellulose and mannan than the Man5C CBM5–CBM10 fusion protein. It

Table 2 Catalytic activity of *Cellvibrio* mannanases against polysaccharides

ND, no catalytic activity was detected.

Enzyme	Substrate	k_{cat} (s ⁻¹)	K_{m} (mg · ml ⁻¹)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ · mg · ml ⁻¹)
Man26A	Carob galactomannan*	2904 ± 32	8.5 ± 1.2	342
Man26B	Carob galactomannan	1031 ± 176	12.7 ± 0.23	91.5
Man26B	Locust bean galactomannan	259 ± 29	3.3 ± 0.42	80.6
Man26B	Konjac glucomannan	271 ± 16	1.7 ± 0.3	134.2
Man26B	Ivory nut mannan	ND	ND	ND
Man5A	Carob galactomannan	2381 ± 66	8.5 ± 1.5	246.0
Man5A	Locust bean galactomannan	2549 ± 161	13.9 ± 1.4	215.6
Man5A	Konjac glucomannan	1025 ± 35	6.2 ± 1.2	165.4
Man5A	Ivory nut mannan	ND	ND	ND
Man5B	Carob galactomannan	1075 ± 27	2.2 ± 0.3	446
Man5B	Locust bean galactomannan	125 ± 38	3.0 ± 0.4	38.2
Man5B	Konjac glucomannan	951 ± 32	1.4 ± 0.12	750
Man5B	Ivory nut mannan	ND	ND	ND
Man5C	Carob galactomannan	3.8 ± 0.5	4.7 ± 0.8	0.74
Man5C	Locust bean galactomannan	4.1 ± 0.3	8.4 ± 0.5	0.53
Man5C	Konjac glucomannan	4.7 ± 0.6	9.0 ± 0.8	0.48
Man5C	Ivory nut mannan	1.2 ± 0.3	7.7 ± 1.1	0.26

* Data from Hogg et al. [6].

is possible that the binding to these polysaccharides by CBM5–CBM10 is mediated through the CBM10 module; however, this view must be treated with some caution as it was not possible to assess the affinity of just the CBM10 module as it could not be expressed as a discrete entity.

It is somewhat surprising that the three GH5 mannanases contain different combinations of CBMs that bind crystalline polysaccharides. It is possible that these modules recognize distinct regions of crystalline cellulose and thus the three mannanases are targeted to different locations within the plant cell wall. This view is supported by the study of Carrard et al. [44] which showed that CBMs belonging to families 1, 2a and 3a, respectively, do indeed bind to different regions of highly crystalline cellulose. Alternatively, it is possible that the selection pressure driving the evolution of these modular mannanases is simply a requirement to bind to the crystalline polysaccharides within the plant cell wall, and this has occurred by fusion of DNA sequences encoding the GH5 modules with a range of DNA sequences that direct the synthesis of CBMs that bind these ligands.

It is interesting to note that in contrast with Man5A, Man5B and Man5C the two GH26 *C. japonicus* mannanases do not contain CBMs (this study and [7]). This may indicate that the target substrates for GH26 mannanases are mannans that are not tightly associated with the plant cell wall. Potential targets for Man26A and Man26B are galactomannan, which is loosely associated with the plant cell wall and represents a major storage polysaccharide in a variety of seeds [1], and/or manno-oligosaccharides released by the action of mannanases that target the plant cell wall. In view of the presence of CBMs that bind crystalline polysaccharides in all the GH5 mannanases, the likely target for these enzymes is the major hemicellulose in Angiosperms, glucomannan, which is integral to the plant cell wall, forming extensive hydrogen bonds with cellulose microfibrils [1]. It should also be noted that the two GH26 enzymes contain signal peptidase II cleavage sequences, and are thus likely to be anchored to the membrane of *C. japonicus*, whereas Man5A, Man5B and Man5C lack these membrane-attachment sequences and are thus probably secreted into the culture medium. Con-

sistent with this proposal is the observation that approximately one-third of the mannanase activity displayed by *C. japonicus* is membrane-associated (results not shown). This spatial organization of mannanase activity is consistent with the concept that the extracellular mannanases release manno-oligosaccharides from insoluble mannans, which are then hydrolysed at the surface of the bacterium so that the organism can preferentially utilize the mannose and mannobiose produced. There is a similar spatial organization of *Cellvibrio* xylanases where the GH10 enzymes are generally membrane-associated, while the GH11 xylanases are secreted into the culture medium [20].

The evolutionary rationale for more than one type of GH5 and GH26 mannanase is not immediately apparent, although it may reflect the variety of mannans presented to the bacterium. Among the GH5 enzymes the biochemical properties of Man5C are quite different from Man5A and Man5B. Although all three enzymes display similar activities towards manno-oligosaccharides, Man5A and Man5B are much more active than Man5C against galactomannan and glucomannan, whereas Man5C is the only enzyme that attacks crystalline mannan. It is possible that the primary function of Man5C is to hydrolyse crystalline mannan, while the other two GH5 enzymes target glucomannan, which is an integral component of the cell wall of Angiosperms, but are also able to degrade galactomannan. It should be noted, however, that while mannanases generally display no or very little activity against ivory nut mannan, Man26A from *C. thermocellum* is considerably more active against ivory nut mannan [45] than Man5C and thus the suggested role of this GH5 enzyme in the degradation of the crystalline polysaccharide must be viewed with some caution. The GH5 enzymes also produce different reaction products from glucomannan. While Man5A and Man5C generate Glc-Man, Man5B appears to produce Man-Glc. This probable difference between the GH5 mannanases increases the range of glycosidic bonds that these enzymes can hydrolyse, and thus provides a further evolutionary rationale for mannanase isoenzymes. The primary substrates for the two GH26 mannanases are soluble mannans or mannans that are loosely associated with the plant cell wall, such

Figure 6 HPLC analysis of carob galactomannan hydrolysis by Man5A, Man5B, Man5C and Man26B

Carob galactomannan was incubated with Man5A (**A**), Man5B (**B**), Man5C (**C**) and Man26B (**D**) and the products generated were analysed by HPLC. Chromatograms 1 depict the products generated at 10 min, whereas chromatograms 2 show the products produced at 60 min. The manno-oligosaccharides with a degree of polymerization of 1–4 are also indicated.

Figure 7 PACE of reaction products generated by Man5A, Man5B, Man26A and Man26B

Initial hydrolysis patterns of the mannanases are shown. Enzymes were incubated with glucomannan (GlcM), solubilized mannan (Mannan) and carob galactomannan (GalM) for 1 h and then inactivated by boiling. The reaction products were incubated with (+) or without (–) β -glucosidase (GlcDase) or α -galactosidase (GalDase), and the products analysed by PACE. The bands corresponding to glucose (G1), mannose (M1), mannotriose (M2), mannotetraose (M3), mannotetraose (M4) and cellopentaose (G5) are indicated. Galactose co-migrates with M1. Examples of bands that are lost on treatment with the β -glucosidase are indicated by *.

as galactomannans and manno-oligosaccharides. It is apparent, however, that differences in the topology of the substrate-binding cleft of Man26A and Man26B may point to subtle differences in the function of these two enzymes. Thus Man26A has four subsites, two on either side of the active centre of the enzyme [6], while Man26B has a minimum of six mannose-binding sites, with at least five glycone and one aglycone of the site of glycosidic bond cleavage. Thus Man26B rapidly generates large amounts of mannose even in the early stages of galactomannan or manno-oligosaccharide hydrolysis. Man26A is an atypical endo- β -1,4-glycanase in that it displays remarkably high activity against

Table 3 Catalytic activity of Man5A, Man5B, Man5C and Man26B against manno-oligosaccharides

Enzyme	Substrate	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{M}^{-1}$)
Man26A	Mannotriose	1.8×10^6 *
Man26A	Mannotetraose	1.4×10^7 *
Man26A	Mannohexaose	2.8×10^7 *
Man26B	Mannotriose	2.6×10^2
Man26B	Mannotetraose	4.8×10^4
Man26B	Mannopentaose	4.4×10^6
Man26B	Mannohexaose	1.8×10^7
Man5A	Mannotriose	1.5×10^1
Man5A	Mannotetraose	3.9×10^3
Man5A	Mannopentaose	1.4×10^5
Man5A	Mannohexaose	2.8×10^6
Man5B	Mannotriose	4.8×10^2
Man5B	Mannotetraose	2.7×10^5
Man5B	Mannopentaose	5.2×10^7
Man5B	Mannohexaose	5.6×10^7
Man5C	Mannotetraose	1.8×10^2
Man5C	Mannopentaose	3.2×10^6
Man5C	Mannohexaose	7.6×10^6

* Data from Hogg et al. [6].

small manno-oligosaccharides, hydrolysing mannotriose approx. 10000 times more efficiently than Man26B and the other *Cellvibrio* mannanases. Man26A and Man26B also display differences in their capacity to hydrolyse glucomannan. Man26B appears to generate a product that is likely to be Glc-Man during the early stages of hydrolysis, indicating that glucose can easily be accommodated in the –2 and +1 subsites. Whereas Man26A also appears to accommodate glucose in the +1 subsite, as evidenced by the appearance of oligosaccharides with glucose at the non-reducing end during the early stages of glucomannan hydrolysis, the presumptive Glc-Man product is not evident, suggesting that the –2 subsite has low affinity for glucose. In summary, the combined biochemical properties of the two GH26 mannanases indicate that these enzymes are able to rapidly generate large quantities of mannose, an important energy and carbon source for *C. japonicus*, from both polysaccharides (Man26B) and small oligosaccharides (Man26A), and thus provides a clear rationale for the evolution of multiple GH26 mannanases. Furthermore, the likely location of the GH26 enzymes on the cell surface of the bacterium would also confer a selection advantage as the generation of mannose by cell-associated mannanases would enable the products of mannan degradation to be utilized primarily by *C. japonicus*, rather than competing micro-organisms which inhabit these complex ecosystems.

Conclusions

This report, in conjunction with the study of Braithwaite et al. [7], shows that *C. japonicus* expresses five distinct mannanases. Biochemical analysis of the different modules in these enzymes indicates that the GH5 enzymes are targeted to the plant cell wall as they contain several different cellulose-binding CBMs, while the GH26 mannanases lack non-catalytic modules. We propose that the primary targets for GH26 enzymes are the storage polysaccharide galactomannan and/or manno-oligosaccharides.

Figure 8 For legend, see facing page

Figure 8 Reaction products generated from the hydrolysis of manno-oligosaccharides by Man5A, Man5B, Man5C and Man26B

Products generated when Man26B (A), Man5A (B), Man5B (C) and Man5C (D) were incubated with mannotriose (1), mannotetraose (2), mannopentose (3) and mannohexaose (4). The percentage of substrate remaining (■) and the amounts of mannose (▲), mannobiose (●), mannotriose (▼), mannotetraose (◆) and mannopentose (□) generated are shown.

Figure 9 Reaction products generated by the hydrolysis of reduced mannohexaose by Man5C

(A) Shows time 0 min, 6 μ g/ml mannohexitol; (B) after 10 min incubation with 50 nM Man5C; and (C) after 30 min incubation. The peak elution times corresponding to mannobitol (1), mannohexitol (2), mannobiose (3) and mannotetraose (5) are shown on each chromatogram.

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