Enzyme system of *Clostridium stercorarium* for hydrolysis of arabinoxylan: reconstitution of the *in vivo* system from recombinant enzymes

Helmuth Adelsberger,[1]† Christian Hertel,[1]‡ Erich Glawischnig,[1]§ Vladimir V. Zverlov[1,2] and Wolfgang H. Schwarz[1]

[1]Research Group Microbial Biotechnology, Technische Universität München, Am Hochanger 4, D-85350 Freising-Weihenstephan, Germany

[2]Institute of Molecular Genetics, Russian Academy of Science, Kurchatov Square, 123182 Moscow, Russia

Correspondence
Wolfgang H. Schwarz
schwarz@mikro.biologie.tu-muenchen.de

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Four extracellular enzymes of the thermophilic bacterium *Clostridium stercorarium* are involved in the depolymerization of de-esterified arabinoxylan: Xyn11A, Xyn10C, Bxl3B, and Arf51B. They were identified in a collection of eight clones producing enzymes hydrolysing xylan (*xynA*, *xynB*, *xynC*), β-xyloside (*bxlA*, *bxlB*, *bglZ*) and α-arabinofuranoside (*arfA*, *arfB*). The modular enzymes Xyn11A and Xyn10C represent the major xylanases in the culture supernatant of *C. stercorarium*. Both hydrolyse arabinoxylan in an endo-type mode, but differ in the pattern of the oligosaccharides produced. Of the glycosidases, Bxl3B degrades xylobiose and xylooligosaccharides to xylose, and Arf51B is able to release arabinose residues from de-esterified arabinoxylan and from the oligosaccharides generated. The other glycosidases either did not attack or only marginally attacked these oligosaccharides. Significantly more xylanase and xylosidase activity was produced during growth on xylose and xylan. This is believed to be the first time that, in a single thermophilic micro-organism, the complete set of enzymes (as well as the respective genes) to completely hydrolyse de-esterified arabinoxylan to its monomeric sugar constituents, xylose and arabinose, has been identified and the enzymes produced *in vivo*. The active enzyme system was reconstituted *in vitro* from recombinant enzymes.

INTRODUCTION

Lignocellulosic biomass has great potential as an abundant and renewable source of fermentable sugars through enzymic saccharification. *Clostridium stercorarium* is a catabolically versatile bacterium producing a wide range of hydrolases for degradation of biomass. Together with *Clostridium thermocellum*, *Clostridium aldrichii* and other cellulose degraders, it forms group I of the clostridia. It is moderately thermophilic, with an optimum growth temperature of 65 °C, and has repeatedly been isolated from self-heated compost (Madden, 1983; Kurose *et al.*, 1988; Sakka *et al.*, 1993; Schwarz *et al.*, 1995a). The two-component cellulase system of *C. stercorarium* has been investigated thoroughly (reviewed by Schwarz *et al.*, 2004). Due to its ability to utilize the various polysaccharides present in biomass it is especially suited for the fermentation of hemicellulose to organic solvents. Some isolates have been used in Japan in a single-step ethanol-fermenting pilot-process with lignocellulosic biomass as substrate (Kurose *et al.*, 1988).

The hydrolysis of heterogeneous hemicellulose involves a set of cooperating enzymes with different modes of activity to degrade the substrate effectively and completely. The major component of hemicellulose is xylan, a linear polysaccharide of β-1,4-linked xylopyranoside residues. It may carry side chains, such as arabinofuranose in arabinoxylan or glucuronic acid in glucuronoxylan, as well as other sugars (Izydorczyk & Biliaderis, 1995). In addition, xylan is acetylated or esterified by phenolic residues, such as ferulic acid and *p*-coumaric acid. Thus it is not surprising

†Present address: II. Medical Hospital and Health Center, TU München, Klinikum rechts der Isar, Ismaninger St. 22, D-81675 München, Germany.

‡Present address: Institute of Food Technology, University of Hohenheim, Garbenstr. 28, D-70593 Stuttgart, Germany.

§Present address: Institut für Genetik, TU München, Am Hochanger 8, D-85350 Freising, Germany.

Abbreviations: CBM, carbohydrate-binding module; CMC, carboxymethylcellulose; GH, glycosyl hydrolase family; MU-, 4-methylumbelliferyl-; PASC, phosphoric-acid-swollen cellulose; pNP-, *p*-nitrophenyl-; X-, 5-bromo-4-chloro-3-indolyl-.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are: AJ508406 (Arf39A), Z94045 (Bgl3Z), AJ508404 (Bxl39A), AJ508405 (Bxl3B), AJ508407 (Xyn10B), AJ508408 (Xyn10C), and AJ508403 (Xyn11A).

that hemicellulolytic bacteria express a great number of xylanases, glycosidases and esterases.

The variability in enzymic activity is exerted either by the basic fold of the catalytic protein (the glycosyl hydrolase family, GH) or by the addition of (non-catalytic) protein modules. In bacteria, most xylanases belong to two structural families, GH10 and GH11. Often, non-catalytic cellulose-binding modules (CBMs) are attached, emphasizing the fact that xylan covers the cellulose in the primary plant-cell wall and has to be removed to allow the cellulases to access the cellulose crystals (Ali *et al.*, 2001). Hemicellulases thus play a dual role in the hydrolysis of lignocellulosic biomass: they provide soluble sugars for bacterial metabolism, and peel the surface of cellulose crystals to allow the production of glucose, the preferred substrate. Together with hemicellulose, lignin is also released.

Endoxylanases perform the first step in enzymic xylan hydrolysis and depolymerize the xylan backbone. β-Xylosidases degrade the resulting xylooligosaccharides. Side groups in natural xylan are split off by more-or-less specific glycosidases (e.g. β-glucuronidases, α-arabinosidases) or by acetyl xylan or phenolic acid esterases (Bronnenmeier *et al.*, 1995; Schwarz *et al.*, 1995b; Donaghy *et al.*, 2000; Kormelink *et al.*, 1993a, b; Kosugi *et al.*, 2002; Nagy *et al.*, 2002). The removal of the side chains enhances the activity of the endoxylanases towards the molecular backbone, as has been documented in synergism experiments between fungal and bacterial xylanases, β-xylosidases and α-arabinosidases from a number of biochemically purified extracellular enzyme systems (e.g. Sorensen *et al.*, 2003; Suh *et al.*, 1996a). Synergism between different xylanases, between xylanases and the associated binding modules, and between xylanases and esterases has also been reported (e.g. Suh *et al.*, 1996b; Fernandes *et al.*, 1999; de Vries *et al.*, 2000).

A number of reviews have been published on hemicellulose hydrolysis (Beg *et al.*, 2001; Shallom & Shoham, 2003; Schwarz *et al.*, 2004), but the enzymes described were from various sources: purified from crude culture supernatants, with the possibility of contamination, or purified from recombinant proteins, without the proof of expression in the bacterium. The reconstitution of a complete enzyme system of a single bacterium has not yet been accomplished.

From culture supernatants of fully grown *C. stercorarium* NCIMB 11754 cultures, Bronnenmeier *et al.* (1990) purified two cellulases, one β-glucosidase, three xylanases (two with cellobiosidase activity), one arabinosidase and one β-xylosidase. Three xylanases and one β-xylosidase were also characterized from another strain, *C. stercorarium* F-9 (Ali *et al.*, 1999, 2001; Fukumura *et al.*, 1995; Sakka *et al.*, 1993, 1994). The presence of feruloyl esterase and α-D-glucuronidase in *C. stercorarium* NCIMB 11754 has been demonstrated (Bronnenmeier *et al.*, 1995; Donaghy *et al.*, 2000).

To investigate the capacity of *C. stercorarium* NCIMB 11754 to degrade hemicellulose, and to identify the genes for the thermostable enzymes involved, an expression library was constructed from genomic DNA. It was screened for the hydrolysis of more than 20 oligo- and polysaccharide substrates (Schwarz *et al.*, 1989; W. H. Schwarz and others, unpublished results). In this study we report the function of eight *C. stercorarium* enzymes connected with the hydrolysis of de-esterified arabinoxylan: xylanases, β-xylosidases and α-arabinofuranosidases. The genes were sequenced and expressed in *Escherichia coli*, and the proteins were purified and biochemically characterized. Arabinosidase Arf51B has been published previously (Schwarz *et al.*, 1995b). Four major proteins in the bacterial culture supernatant were assigned to the genes. The combination of three of these enzymes, one of the two xylanases and one each of the β-xylosidases and arabinosidases, completely hydrolysed arabinoxylan *in vitro*.

METHODS

Bacterial strains and plasmids. *C. stercorarium* strain NCIMB 11754 was obtained from NCIMB, Aberdeen, UK, and grown in GS-2 medium (Johnson *et al.*, 1982) under strictly anaerobic conditions at 60 °C with cellobiose as carbon source. *E. coli* strain XL-1 Blue, pBTac1 (Boehringer Mannheim) and DH5α were used for cloning. Plasmids pUC18 and pUC19 were used for cloning. Cultivation of recombinant cells, media and overexpression were done as recommended in the manufacturer's handbook.

Molecular biological methods. Restriction digests of DNA were done as recommended by the manufacturer (MBI Fermentas or Boehringer Ingelheim Bioproducts). *E. coli* cells were transformed with plasmid DNA by electroporation (Gene Pulser; Bio-Rad), following the methods suggested by the supplier.

The DNA sequences were determined from both strands of supercoiled double-stranded plasmid DNA (Thermosequenase Cycle Sequencing Kit; Amersham) with a GATC 1500 Direct-Blotting Electrophoresis apparatus (GATC, Konstanz), using biotinylated oligonucleotide primers, streptavidin-conjugated alkaline phosphatase and NBT-BCIP (Promega). PCR amplification of Xyn10C-cat was carried out with the Expand High Fidelity PCR System (Boehringer Ingelheim Bioproducts). The primers pcx1 (5′-CAACTTAGGA TCCACGGGCA TGAAATC-3′) and pcx2 (5′-ATTTGTCGAC CTATTAGTCC GGATCTGCAA CAG-3′) were used. The amplified DNA was ligated into vector pUC18 and verified by sequencing. Derivation from *C. stercorarium* and physical integrity of clones was verified by Southern blot hybridization with genomic DNA digested with appropriate restriction endonucleases. DNA and RNA blotting was performed on ZETA-Probe membranes (Bio-Rad) and hybridization was done with radioactively labelled oligonucleotide probes ([γ-^{32}P]ATP or [γ-^{32}P]CTP).

Sequence data were analysed, edited and compared with the DNASIS/PROSIS for Windows package (Hitachi Software Engineering). Nucleotide and protein sequence databases were screened using the FASTA and BLAST software at the NCBI server (http://www.ncbi.nlm.nih.gov).

mRNA experiments. Total RNA was isolated from 10 ml *C. stercorarium* culture by standard techniques (Maniatis *et al.*, 1989). The size of the *xynA* transcript was determined by separating total RNA in a denaturing formaldehyde gel and hybridization with a radiolabelled *xynA*-DNA fragment. Total RNA isolated from *C. stercorarium* cultures grown on different carbon sources was spotted on

ZETA-Probe membranes (Bio-Rad) and hybridized with radio-labelled restriction fragments of cloned DNA. Autoradiographs of the membranes were evaluated densitometrically (Biomed Instruments, with Biotec Fischer densitometer software). Radioactive probes were washed off and the membranes were rehybridized with a radio-labelled oligonucleotide probe against 23S rRNA (5′-CGACAAGGAA TTTCGCTAC-3′) to allow normalization of the amount of RNA.

Purification of recombinant proteins. Recombinant proteins were purified from *E. coli* cultures in LB medium (Maniatis *et al.*, 1989) containing an appropriate amount of ampicillin. The cells were harvested by centrifugation (5000 ***g***, 20 min), washed and resuspended with 50 mM phosphate buffer, pH 8·0, containing 0·3 M NaCl. Cells were lysed by three passages through a French pressure cell (AmInCo) at 110 MPa with ice cooling. The cell homogenate was centrifuged to remove intact cells and cell debris (40 000 ***g***, 30 min). Host proteins were removed by centrifugation after heat denaturation (60 °C for 30–60 min). Nucleic acids were destroyed by Benzonase (VWR).

Anion-exchange chromatography on a Q-Sepharose FF column, hydrophobic interaction chromatography on a phenyl-Sepharose HP 16/10 column and gel filtration on a Superdex 200 prep grade XK 16/60 column (Amersham Biosciences) were performed as described previously (Fuchs *et al.*, 2003).The purity of the proteins was verified by SDS-PAGE and staining with Coomassie Brilliant Blue G-250 (Serva).

Proteins from culture supernatants of *C. stercorarium*. Proteins from the cleared culture supernatant of a *C. stercorarium* culture in the exponential growth phase (in GS-2 medium with cellobiose as carbon source; Johnson *et al.*, 1982) were concentrated by tangential flow filtration.

Affinity chromatography. A slurry of HBS-cellulose (degree of polymerization, DP, 650–720, minimum fibre length 0·12 mm) was used in a 10 ml column, as described previously (Bronnenmeier *et al.*, 1996). Xylan as affinity substrate was mixed with HBS-cellulose (Serva), transferred to a 10 ml column and washed thoroughly with water. The insoluble xylan, distributed evenly in the HBS cellulose, was used to bind and elute proteins.

Denaturing gel electrophoresis (SDS-PAGE) and zymogram technique. SDS-PAGE was performed in 10 % polyacrylamide slab gels according to Laemmli (1970). For zymograms, the gels were renaturated in 50 mM phosphate/citrate buffer (pH 6·2), as described previously (Schwarz *et al.*, 1987). The gel slabs were overlaid with agarose gel containing polymeric substrates (1 %, w/v) in MES buffer (pH 6·5), incubated at 55 °C and stained with 0·1 % (w/v) Congo Red (Sigma-Aldrich). Glycosidase activity was detected by soaking the gel in an aqueous solution of *p*-nitrophenyl- (pNP-), 5-bromo-4-chloro-3-indolyl- (X-) or 4-methylumbelliferyl- (MU-) conjugated substrates (Sigma-Aldrich) in MES buffer.

Enzyme assays. Enzyme aliquots in standard assays were incubated in MES buffer (50 mM), containing 5 mM $CaCl_2$, at the optimum pH and temperature (see Table 2). The concentration was 1 % for soluble and 2 % (w/v) for insoluble polysaccharides. Reducing sugars released from polymeric substrates were quantitatively detected by the 3,5-dinitrosalicylic acid method (Wood & Bhat, 1988), assuming that one unit of enzyme liberates 1 μmol of glucose equivalent per minute and mg of protein. Specific activities were determined in the linear range of the reaction. Glucose was selectively estimated with the glucose oxidase/peroxidase assay (PGO 510A, Sigma-Aldrich). *p*-Nitrophenol liberated from pNP-glycosides was measured by A_{395} in alkaline solution (0·6 M Na_2CO_3). One Unit of activity was defined as the amount of enzyme producing 1 μmol *p*-nitrophenol min^{-1} (0·013 $\Delta A_{395}=1$ nmol). All determinations were performed in triplicate.

The optimum pH was determined by measuring the specific activity of the enzyme at a given pH (MES or citrate/phosphate buffer). The optimum temperature was the temperature with the highest activity of the enzyme during incubation for a given time. Protein concentration was determined with Coomassie Brilliant Blue (Sedmak & Grossberg, 1977).

Determination of hydrolysis products. Polymeric and oligomeric substrates were hydrolysed to completion under the conditions stated above. Hydrolysis products were separated on silica gel 60 plates, as described previously (Zverlov *et al.*, 2003) or by HPLC (Beckmann System Gold, equipped with a deashing and a HPX-42A column, Bio-Rad) and refractometric sugar detection (ERC-7512, Erma).

Substrates. Oat-spelt xylan (arabinoxylan), birch-wood xylan (glucuronoxylan), 4-*O*-methyl-D-glucurono-D-xylan, Avicel CF1, carboxymethylcellulose (CMC, low viscosity), MU- and pNP- glycosides, and X-Glc were obtained from Sigma-Aldrich, cellodextrins from Merck/VWR, and xylooligosaccharides and the mixed-linkage *β*-1,3-1,4-glucans barley *β*-glucan and lichenan from Megazym. Phosphoric-acid-swollen cellulose (PASC) was prepared from Avicel CF1 according to Wood (1988).

RESULTS

C. stercorarium actively degrades hemicellulose

The supernatant of a cellobiose-grown *C. stercorarium* NCIMB 11754 culture showed a four-magnitude-higher hemicellulase (xylanase) activity than cellulase activity towards microcrystalline cellulose (Table 1). The hydrolytic activity against CMC was two magnitudes below that against *β*-1,3-1,4-glucan (lichenan), indicating a low endo-*β*-1,4-glucanase activity. The hydrolysis of lichenan can be attributed predominantly to one type of xylanase (see below). The high hemicellulase activity is corroborated by the activities of xylosidases and arabinosidases, which degrade the products of endoxylanase activity on arabinoxylan. Thus, *C. stercorarium* is specialized in the utilization of hemicellulose and, accordingly, its hemicellulolytic degradation system was characterized, using de-esterified arabinoxylan as a paradigm.

Table 1. Hydrolytic activities in cell-free culture fluid of *C. stercorarium* grown on cellobiose

Substrate	Hydrolytic activity ($mU\ ml^{-1}$)
Microcrystalline cellulose	2
Phosphoric-acid-swollen cellulose	10
Carboxymethylcellulose (CMC)	120
1,3-1,4-*β*-Glucan (lichenan)	12 000
Arabinoxylan	20 000
pNP-*β*-glucopyranoside	7
pNP-*β*-cellobioside	1·7
pNP-*β*-xylopyranoside	2
pNP-*α*-arabinofuranoside	21

Table 2. Characteristics of the cloned genes of *C. stercorarium* NCIMB 11754 and their gene products

Characteristic	Gene						
	xynA	*xynB*	*xynC*	*arfA*	*bglZ*	*bxlA*	*bxlB*
Accession no.	AJ508403	AJ508407	AJ508408	AJ508406	Z94045	AJ508404	AJ508405
Length of sequence (bp)	3493	4342	4880	1842	2620	3420	3025
Location of reading frame	993–2957	2625–3788	1085–4183	66–1529	190–2454	1099–2601	333–2480
Stop codon	TAG	TAA	TAA	TAG	TAA	TAA	TAA
Putative transcription terminator (kJ mol^{-1})	2080–2109 (−52·5)				2288–2312 (−72·2)	1646–1671 (−65·9)	2169–2201 (−69·3)
No. of amino acid residues	654	387	1032	487	754	500	715
Molecular mass (kDa)	70·7	44·3	115·7	54·9	85·2	58·0	79·0

From a cosmid library of genomic DNA from the type strain NCIMB 11754, 1139 clones were tested for hydrolysis of xylan, pNP-α-arabinofuranoside and pNP-β-xylopyranoside (Schwarz *et al.*, 1990). Thirty-seven clones active towards xylan, forty-five active towards pNP-β-xyloside (most of which were also active towards pNP-arabinofuranoside) and six clones active towards pNP-arabinofuranoside were identified. Eight different groups were identified by activity pattern, restriction fragment analysis or DNA–DNA hybridization. One clone of each group was sequenced. Eight genes were identified, which coded for three xylanases (*xynA*, *xynB*, *xynC*), two α-arabinofuranosidases (*arfA*, *arfB*), two β-xylosidases (*bxlA*, *bxlB*), and one β-glucosidase (*bglZ*) active on β-xyloside (Table 2). The characteristics of the *arfB* gene and its product have been described previously (Zverlov *et al.*, 1998).

Endoxylanase Xyn11A

The *xynA* gene encodes a modular protein consisting of a 30 aa signal peptide, a catalytic module of GH11 (234 aa) and three consecutive C-terminal CBM6 of 124 aa residues which are highly conserved (80–91 % identity; Coutinho & Henrissat, 1999a) (Fig. 1). In Southern blots, a 2·3 kb mRNA band hybridized to a labelled *xynA* probe (data not shown). This size corresponded well with the distance between a putative promoter detected 200 bp upstream of the translation start and a palindromic sequence 115 bp downstream of the stop codon. *xynA* appears therefore to be transcribed as a monocistronic gene.

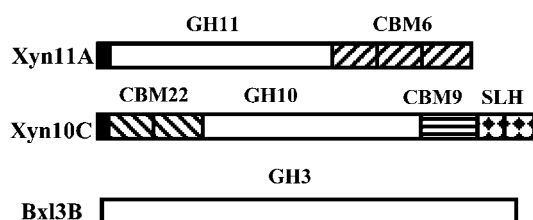

Fig. 1. Structure of the glycosyl hydrolases. Protein modules are drawn as boxes (only approximately to scale); the module family is indicated above the boxes. GH, glycosyl hydrolase family; CBM, carbohydrate-binding module; SLH, S-layer homologous module. Black boxes indicate a leader peptide.

xynA is apparently the gene for the major xylanase in *C. stercorarium* culture supernatants, xylanase A, which has an identical N-terminus. The recombinant protein bound to crystalline cellulose (Bronnenmeier *et al.*, 1996). Its molecular mass was 62 kDa, lower than the calculated mass from the sequence (Table 2), probably due to irregular electrophoretic behaviour or C-terminal proteolysis.

The purified recombinant enzyme was as active towards glucuronoxylan as it was towards arabinoxylan (258 U mg^{-1}). Initially, large oligosaccharides were liberated (data not shown). Their successive degradation to xylotriose, xylobiose and traces of xylose indicated an endo- mode of action (Fig. 2). Arabinose residues were found within the larger oligosaccharides (see analysis below). The enzyme hydrolysed mixed-linkage β-1,3-1,4-glucan only marginally (Table 3). The low activity towards CMC was confirmed by a viscosimetric assay upon addition of excess enzyme. A comparison of the increase in reducing sugars with the reduction in viscosity also indicated an endo- mode of hydrolysis on CMC (data not shown).

Endoxylanase Xyn10B

Xyn10B consists of a catalytic module without a single cysteine codon, and has a leader peptide of 29 aa residues with a positively charged N-terminus, as well as a stretch of hydrophobic amino acids. *xynB* was very well expressed from its own promoter in *E. coli*, forming up to 12 % of total cell protein without inclusion body formation. It was highly active towards soluble arabinoxylan (1080 U mg^{-1}). In addition, glucuronoxylan (455 U mg^{-1}), *O*-Me-glucuronoxylan (280 U mg^{-1}) and lichenan (80 U mg^{-1}) were hydrolysed.

Endoxylanase Xyn10C

Xyn10C is a modular xylanase with a central catalytic module. Two copies of CBM22 modules are present N-terminally, and a CBM9 module and two S-layer homologous modules are located C-terminally (Fig. 1).

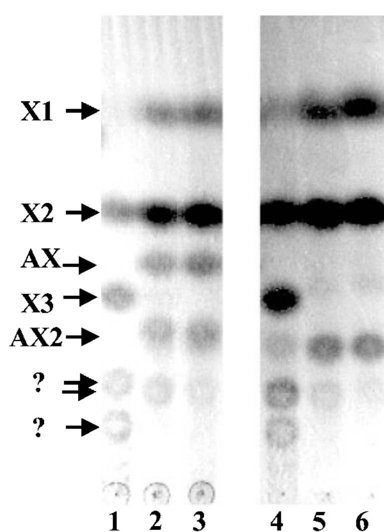

Fig. 2. TLC of reaction products from complete enzymic hydrolysis of xylan with excess enzyme and incubation time. Lanes 1–3, arabinoxylan; lanes 4–6, glucuronoxylan; lanes 1 and 4, Xyn11A; lanes 2 and 5, Xyn10C; lanes 3 and 6, Xyn10B. The positions of marker sugars are indicated: X1, xylose; X2, xylobiose; X3, xylotriose; AX, arabinosyl xylose; AX2, arabinosyl xylobiose.

Expression of Xyn10C in various *E. coli* hosts and in *Bacillus subtilis* yielded a heavily degraded protein with several bands which were active in zymograms towards MU-cellobioside or xylan. Whereas the size of the largest protein band (110 kDa) corresponded well with the 112 kDa calculated from the sequence (without leader peptide), the majority of the protein was found to have a smaller molecular mass.

To characterize the activity of a homogeneous enzyme preparation, the isolated catalytic module Xyn10C-cat was prepared. It had a broad substrate activity on arabinoxylan, glucuronoxylan, 4-*O*-methyl-glucuronoxylan, β-1,3-1,4-glucan and pNP-cellobioside. Specific activity on arabinoxylan in the presence of 20 mM NaCl was 550 U mg^{-1}. On pNP-cellobioside, the K_m and V_{max} were 1·82 mM and 14·3 μmol min^{-1} mg^{-1}, respectively. Arabinoxylan was degraded in an endo- mode, mainly to xylose and xylobiose (Fig. 2). TLC analysis revealed that xylooligosaccharides were more easily hydrolysed with growing chain length, with a very slow and incomplete degradation of xylobiose. Cellodextrins longer than cellotriose were degraded mainly to cellobiose, and resulted in the formation of β-1,4-transglucosylation products (data not shown).

Optima for pH and temperature were different on arabinoxylan and pNP-cellobioside (pH 6·5 and 65 °C, pH 5·0 and 55 °C, respectively). SDS (>1 %) and bivalent heavy metal ions (1–10 mM) had a strong inhibitory effect, but not Triton X-100, DTT or EDTA (10 mM). NaCl stimulated the enzyme activity more than threefold, with a maximum at 20 mM at 65 °C. The stimulation was lower in the presence of Na_2SO_4 or at 55 °C, indicating an effect of chloride anions on protein stability.

Table 3. Temperature and pH optima and substrate specificity of the recombinant proteins

Enzyme activity is given as a percentage of the maximum activity for the enzyme. The value of activity taken as 100 % corresponds to 258 (Xyn11A), 1080 (Xyn10B), 549 (Xyn10C-cat), 0·15 (Arf43A), 0·8 (Arf51B), 0·4 (Bgl3Z), 0·4 (Bxl39A) and 0·75 (Bxl3B) U mg^{-1} protein, respectively. Data for Arf51B obtained from Schwarz *et al.* (1990). ND, Not detected; −, not active; +, detectable; ++, highly active.

Characteristic	Recombinant protein							
	Xyn11A	Xyn10B	Xyn10C-cat	Arf43A	Arf51B	Bgl3Z	Bxl39A	Bxl3B
Temp. optimum (°C)	75	75	65	55	70	60	60	50–55
pH optimum	6·5–7·0	6·0–7·0	6·5	6·5	5·0	5·5–5·8	5·5–6·5	6·0
Substrate specificity (% activity) for:								
pNP-β-glucoside	ND	ND	ND	ND	0·2	100	ND	0·9
pNP-β-cellobioside	ND	2·6	0·2	ND		++	3	ND
pNP-β-xyloside	ND	0·2	ND	65	0·4	7·3	100	100
pNP-α-arabinofuranoside	ND	ND	ND	100	100	ND	ND	16
pNP-β-fucoside	ND	0·1				19·8	3	
pNP-β-galactoside	ND	ND			0·3	ND	ND	
pNP-α-mannoside							22	ND
Xylobiose		+		−	−	−	+	++
Avicel	1·5	ND	ND			ND		
PASC	1·0	0·7				ND		
CMC	0·5	ND	ND	ND	ND		ND	ND
Arabinoxylan	100	100	100	ND	1·2	ND	ND	ND
Barley β-glucan	0·8	16·1	1·7	ND		0·5	ND	ND

β-Xylosidase Bxl3B

β-Xylosidase B of *C. stercorarium* comprises a single catalytic module of family GH3 (Fig. 1). The sequence with the greatest homology was that of the *β*-glucosidase Bgl3Z of the same organism (55 % identity), indicating a gene-duplication event. Recombinant Bxl3B was isolated as two co-purifying bands of 70 and 80 kDa, with an activity of 440 U mg^{-1} towards pNP-*β*-xyloside. Protein of both bands had an identical N-terminus in agreement with the DNA sequence and were therefore C-terminally processed forms of Bxl3B.

Despite the sequence similarity to *β*-glucosidase Bgl3Z, Bxl3B showed very low activity towards pNP-*β*-glucoside (Table 3). In contrast to Bxl39A (see below), Bxl3B hydrolysed xylobiose and *β*-xyloside end groups of oligosaccharides from the non-reducing end quickly and completely (Figs 3 and 5) and released limited amounts of xylose from xylan. It was not active towards pNP-*α*-mannoside. The enzyme was stimulated twofold by 10 mM chloride, similar to the chloride stimulation described elsewhere for other glycosidases (Huang *et al.*, 1988). This is at least in part due to protein stabilization: at 50 °C the half-life increased from 75 min to 100 h if 10 mM NaCl was added.

Other glycosidase genes

Arf51B has been described earlier (Schwarz *et al.*, 1995b; Zverlov *et al.*, 1998). Arf43A consists of a catalytic module with greatest homology to the GH43 xylosidase XylB of *Butyrivibrio fibrisolvens* (35·8 % similarity). Both enzymes are active towards pNP-*α*-L-arabinofuranoside and pNP-*β*-D-xyloside (Utt *et al.*, 1991). Arf43A had no activity on arabinoxylan or xylobiose (Table 3, Fig. 3). Bxl39A had a specific activity of 398 U mg^{-1} on pNP-*β*-xyloside. Degradation of xylobiose was slow and incomplete. From arabinoxylan, detectable amounts of xylose or arabinose were not released (HPLC). However, the enzyme showed affinity to xylan and could be purified by affinity chromatography using a xylan affinity column. The family GH39 has so far only a few biochemically characterized members.

Another clone hydrolysing aryl *β*-xyloside contained the gene *bglZ*. A zymogram staining with X-glucoside showed that the *β*-glucosidase activities of the cloned enzyme, of *C. stercorarium* cell extract and of the major *β*-glucosidase purified from the supernatant of a *C. stercorarium* culture ran with identical speed in native gel electrophoresis. These data suggest that Bgl3Z constitutes the major intra- and extracellular *β*-glucosidase of *C. stercorarium*. However, Bgl3Z was not active towards xylobiose.

Hydrolysis of xylan

Arabinoxylan as well as glucuronoxylan were depolymerized by all three xylanases, but the product pattern differed (Fig. 2): with Xyn11A, the main products were xylobiose and xylotriose; with Xyn10B and Xyn10C, they were xylose and xylobiose. In all digests, longer oligosaccharides remained, which probably consisted of arabinosylated or glucuronylated xylooligosaccharides for which no reference compounds were available. These oligosaccharides were not further degraded by the xylanases alone, even after prolonged incubation with excess enzyme.

The specificity of the glycosidases ArfB (described previously by Zverlov *et al.*, 1998) and BxlB allowed the structural identification of some of the arabinoxylan degradation products by single or co-digestion. The enzymes split sugar residues (arabinose or xylose, respectively) off the non-reducing end of the oligosaccharides. This made it possible to identify some of the products of xylanase hydrolysis unequivocally by TLC (see Fig. 4).

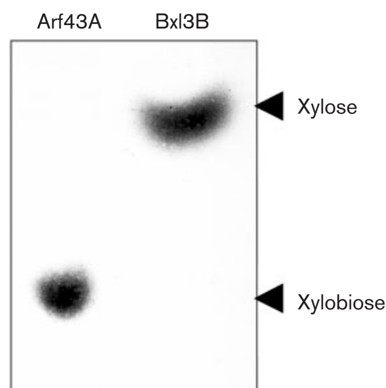

Fig. 3. Hydrolysis of xylobiose. A solution of 1 % (w/v) xylobiose was incubated overnight with 5 mU of purified enzymes Arf43A and Bxl3B. Samples of 1 µl were applied to TLC. The positions of xylose and xylobiose markers are indicated.

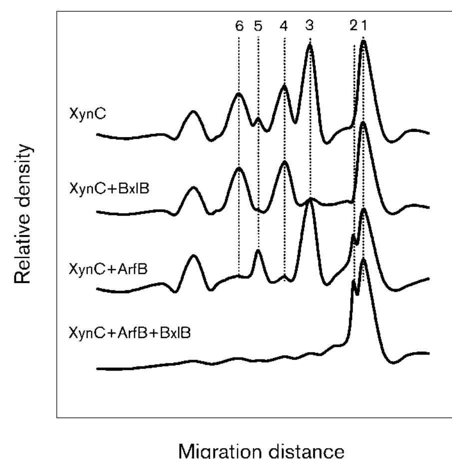

Fig. 4. TLC densitogram of the reaction products from enzymic hydrolysis of arabinoxylan. XynC, Xyn10C; XynC+BxlB, Xyn10C and Bxl3B; XynC+ArfB, Xyn10C and Arf51B; XynC+ArfB+BxlB, Xyn10C and Bxl3B and Arf51B. Products are: 1, xylose (X); 2, arabinose (A); 3, X2; 4, AX; 5, X3; 6, AX2.

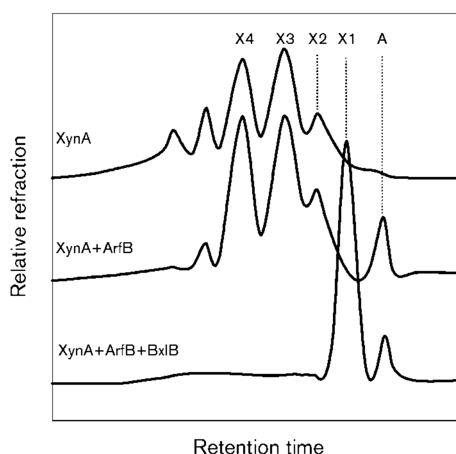

Fig. 5. HPLC of degradation products from enzymic hydrolysis of arabinoxylan by digestion with Xyn11A, Arf51B and Bxl3B in the presence of different combinations of the enzymes, as indicated. A, arabinose; X1, xylose; X2, xylobiose, X3, xylotriose; X4, xylotetraose.

The combination of xylanase Xyn10C, arabinofuranosidase Arf51B and β-xylosidase Bxl3B yielded peaks corresponding to the two monomeric components of arabinoxylan, namely xylose and arabinose (Fig. 4, peaks 1 and 2, respectively). Arabinose was present only when Arf51B was added. Peaks 4 and 6 disappeared after the addition of Arf51B, hence they must have represented arabinosylated oligosaccharides. Since they were not hydrolysed on addition of Bxl3B, they seemed to be blocked (arabinosylated) at the non-reducing end: AX and AX2, respectively. From those products, the enzyme mechanism can be deduced: Xyn10C seems to split the arabinoxylan backbone at the non-reducing end of a derivatized xylose residue. Peaks 3 and 5 are hydrolysed by the addition of Bxl3B, accumulate in higher amounts upon treatment with Arf51B, and hence represent the unsubstituted xylooligosaccharides X2 and X3, which is corroborated by identical R_F values for the reference sugars xylobiose and xylotriose.

By the same token, the left-most peak in the Xyn11A digest (Fig. 5) must be arabinosylated, since it disappeared in the presence of Arf51B and arabinose accumulated. The X1, X2, X3 and X4 peaks were not affected by Arf51B; thus, none of them was arabinosylated. These xylooligosaccharides were completely hydrolysed by the addition of Bxl3B. From this product pattern, it is clear that the difference in product formation (in comparison to Xyn10B and Xyn10C; Fig. 2) is due to the fact that Xyn11A is unable to accommodate substituted substrates in its active site. Similar products were produced by the *Aspergillus awamori* xylanase from wheat-flour arabinoxylan (Kormelink *et al.*, 1993a).

Arabinoxylan was completely digested by recombinant enzymes encoded by the genes which produce the major components of the culture supernatant: Xyn11A and Xyn10C degraded arabinoxylan to oligosaccharides; in combination with Arf51B, arabinose was split off the xylooligosaccharides, which were degraded further by the xylanases (Figs 4 and 5). The addition of Bxl3B finally hydrolysed the resulting xylooligosaccharides completely to xylose. The ratio of the integrated sugar peak areas was 10 : 1 (xylose : arabinose).

Induction by growth on xylan

Compared to glucose-grown cultures, total β-xylosidase and xylanase activities in the cultures were enhanced up to 9·5-fold if *C. stercorarium* was grown on xylose or arabinoxylan. A large part of the xylosidases, which have no obvious leader peptide, was found within the cells (around 20 % of the total activity), whereas the xylanases were predominantly secreted (0·1–0·5 % cell bound; Table 4). In order to differentiate between single xylanases and β-xylosidases, the result was verified with quantitative dot-blot hybridization of total RNA prepared from the cultures, which was hybridized with labelled nucleotide

Table 4. Induction of xylan-hydrolysing enzymes and their genes

Cultures of *C. stercorarium* were grown to the same density ($OD_{600}=0{\cdot}6$). Enzymic activity was determined for xylanase (Xyn) using the DNSA method, and for β-xylosidase (Xyl) using pNP-β-xyloside as substrate. Identical amounts of total RNA were hybridized with radiolabelled oligonucleotides derived from cloned genes. The intensity of the autoradiography spot of the RNA from the glucose-grown culture was taken as 100 %.

Carbon source	Activity (U mg^{-1})				Amount (%) of specific mRNA of:		
	Culture supernatant		Cell extract		*xynA*	*bxlA*	*bxlB*
	Xyn	Xyl	Xyn	Xyl			
Glucose	5	72	0·02	17	100	100	100
Xylose	19	169	0·03	39	100	170	80
Xylan	36	330	0·19	84	190	160	180

probes derived from the genes *xynA*, *bxlA* and *bxlB*. Compared to growth on glucose, on xylose only *bxlA* mRNA was increased, whereas the transcription of all three genes was stimulated by xylan (Table 4).

DISCUSSION

The thermophilic, saccharolytic bacterium *C. stercorarium* is well suited for the degradation of cellulose and hemicellulose in lignocellulosic biomass. It grows on (for example) cellulose or xylan as sole carbon source, but its extracellular enzyme system is optimized for the substrate xylan, which also is utilized as a carbon source. This is obvious not only from the intrinsically higher enzyme activity towards the soluble substrate xylan, but also from the low activity towards soluble CMC. Thus *C. stercorarium* is a promising candidate for an industrial process employing direct hydrolysis for the bioconversion of cellulose as well as hemicellulose in biomass (Kurose *et al.*, 1988).

A genomic library, screened for enzyme activities involved in arabinoxylan hydrolysis, revealed eight different genes for endoxylanases, α-arabinofuranosidases and β-xylosidases, some of them having previously been shown to be randomly distributed over the genome (Schwarz *et al.*, 1995a). Three genes, *xynA*, *xynB* and *xynC*, encoded xylanases. The amino-acid sequence of Xyn11A and Xyn10C showed an unusually complex module architecture, similar to that present in the homologous genes of *C. stercorarium* strain F-9 (Ali *et al.*, 1999; Fukumura *et al.*, 1995; Sakka *et al.*, 1993). Their catalytic activity followed the usual pattern of GH11 and GH10 endoxylanases, including the high mixed-linkage β-glucanase activity associated with GH10 enzymes. The identified low activity on CMC and cellodextrins might not be of physiological relevance, but could help to degrade β-glucans associated with hemicellulose in plant cell walls.

From the culture supernatant of *C. stercorarium*, several xylanases were purified by Berenger *et al.* (1985). The immunological cross-reactivity and similarity of their biochemical reactions suggested that the three proteins identified were derived from a single polypeptide. In two independent studies, the substrate specificity of the major xylanases isolated from culture supernatants was identical to that of the recombinant Xyn11A enzyme, as confirmed by N-terminal sequencing (Berenger *et al.*, 1985; Bronnenmeier *et al.*, 1996). Furthermore, Sakka *et al.* (1994) identified the major extracellular xylanase of *C. stercorarium* strain F-9 as Xyn11A, which has in the overlapping areas a 99 % identical sequence with the *xynA* sequence from the type strain. However, the first CBM is missing in strain F-9. Xyn11A thus represents one of the major extracellular xylanases in *C. stercorarium*.

$Xyn11A_{NCIMB11754}$ is the first example of a xylanase containing three consecutive modules of one CBM family (Coutinho & Henrissat, 1999b). These are more closely related to each other than to their next closest relative, xylanase XynZ of *C. thermocellum*, and thus seem to have originated from module shuffling by duplication. The binding modules are functionally active, with a specificity for crystalline cellulose, as was shown for both strains (Bronnenmeier *et al.*, 1996; Sakka *et al.*, 1997; Sun *et al.*, 1998). This is also supported by the unusually high number of aromatic phenylalanine and tyrosine residues, which are known to be involved in hydrophobic binding to the crystalline cellulose surface. The binding through the type-strain CBMs was corroborated by a deletion clone containing only the N-terminal 300 aa of the catalytic module and less than half of the first CBM: this protein did not bind to the cellulose column (assay as in Bronnenmeier *et al.*, 1996; data not shown), indicating that the CBMs are indeed responsible for cellulose binding.

The binding activity of the CBMs to cellulose, combined with the activity of the enzyme towards β-glucans (albeit very low), suggests that Xyn11A uses cellulose for anchoring and hydrolyses hemicelluloses in the vicinity or on the surface of the cellulose microfibrils in plant cell walls. Moreover, the CBMs may play a role in the hydrolysis of insoluble xylan, as was shown by Sun *et al.* (1998).

The other two xylanases, Xyn10B and Xyn10C, had been initially designated celloxylanases CelW and CelX, respectively, due to their residual activity on mixed-linkage β-glucan and pNP-β-cellobioside, a typical trait of GH10 xylanases. Xylanases Xyn10B and Xyn10C have an identical mode of action on arabino- and glucuronoxylan, which is different to that of xylanase Xyn11A. 'Celloxylanases' I and II, isolated by Bronnenmeier *et al.* (1990) as the major xylanases in the culture supernatant of *C. stercorarium* besides XynA, are both encoded by the gene *xynC*, the smaller protein being a processing product (N-terminal sequence). Ali *et al.* (2001) ascribed to the homologous Xyn10C of strain F-9 a role in cell attachment to acid-swollen (amorphic) cellulose, due to the presence of two CBMs of family 22 and family 9 and two copies of an S-layer homologous module (SLH). This underlines the possible role of Xyn10C in lignocellulose degradation.

Xyn10C is, after Xyn11A, the second major extracellular xylanase produced by *C. stercorarium*. Xyn10B was not detected by purification from culture supernatant or cells, neither in our nor in Sakka's group. Accordingly, it was not investigated further. Thus, the genes for the two major xylanases in *C. stercorarium* culture supernatant were identified as *xynA* and *xynC*.

Five clones encoded glycosidases, all of which were more-or-less active on β-xyloside as substrate. Despite their differences in structure (families GH3, GH39, GH43 and GH51), they had a rather similar aryl-glycosidase specificity: they more or less degraded both types of substrate, varying from a pure β-xylosidase with no arabinosidase activity (Bxl39A) to an almost pure arabinosidase with only 0·4 % activity towards β-xyloside (Arf51B) (Table 3). However, this continuum of activities is superficial, as α-L-arabinofuranose and β-D-xylopyranose have

identical configurations in positions C1 to C4, and both accommodate the binding pocket of the enzyme (Hövel *et al.*, 2003). The activity towards both substrates is therefore not the result of dual specificity, but of imprecise substrate recognition.

All glycosidases described here consisted of a single catalytic module and were most active towards aryl glycosides. Only Arf51B hydrolysed arabinose residues from arabinoxylan and from arabinoxylan-derived oligosaccharides, while Bxl3B degraded xylooligosaccharides. Therefore only Arf51B and Bxl3B can be ascribed a direct role in the degradation of arabinoxylan. A similar role in arabinoxylan hydrolysis was found for the arabinofuranosidase B of *Aspergillus niger*, which also removed all arabinose residues from arabinoxylan, both α-1,2- and α-1,3-linked (Kormelink *et al.*, 1993a). Support for involvement in the xylan-degradation pathway *in vivo* is found in the higher activity of xylosidase and xylanase in the cultures after induction with xylan. This is paralleled by an increase in *bxlB* and *xynA* mRNA (Table 4). Furthermore, in an earlier study, proteins corresponding to Arf51B and Bxl3B could be isolated from the culture supernatant and were the only α-arabinosidase and β-xylosidase present (Bronnenmeier *et al.*, 1990), although the genes lacked obvious leader peptides.

Despite its lack of activity towards the polymer, Bxl39A had a high affinity for xylan. The strong binding suggests some role in xylan hydrolysis. But apart from a barely measurable activity towards xylobiose, no evidence for a role in arabinoxylan hydrolysis could be identified by *in vitro* methods.

To reconstitute the enzyme system for the hydrolysis of arabinoxylan, the recombinant enzymes Xyn11A or Xyn10C, Bxl3B and Arf51B from *C. stercorarium* were combined. Simultaneous addition of the three components hydrolysed de-esterified arabinoxylan completely to its monomers, xylose and arabinose. Using quantitative HPLC analysis of the sugars formed, the products from completely hydrolysed oat-spelt arabinoxylan were found to be xylose and arabinose, in the ratio 10 : 1, with no other sugars in detectable amounts. This ratio is in agreement with the specification obtained from the supplier (Sigma-Aldrich).

Of the eight genes cloned, four could be identified as encoding the major extracellular hydrolases in culture supernatants that are possibly involved in the *in vivo* hydrolysis of arabinoxylan to its sugar monomers. The corresponding proteins constitute an enzyme system for the catabolism of arabinoxylan, and have considerable potential for application in biotechnological processes, due to their high activity and intrinsic stability. In addition, the enzymes can be used for an easily performed analysis of oligosaccharide structure or of the enzyme mechanism. For example, structural analysis of arabinoxylan, by determination of the composition of intermediate products and quantitative determination of the composition, is achievable with enzymic degradation. For the hydrolysis of natural xylans, additional glycosidases and esterases will be necessary, and the role of these enzymes in the hydrolysis of natural hemicellulose by *C. stercorarium* remains to be elucidated.

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