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A novel *Cellvibrio mixtus* family 10 xylanase that is both intracellular and expressed under non-inducing conditions

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Hydrolysis of the plant cell wall polysaccharides cellulose and xylan requires the synergistic interaction of a repertoire of extracellular enzymes. Recently, evidence has emerged that anaerobic bacteria can synthesize high levels of periplasmic xylanases which may be involved in the hydrolysis of small xylo-oligosaccharides absorbed by the micro-organism. *Cellvibrio mixtus*, a saprophytic aerobic soil bacterium that is highly active against plant cell wall polysaccharides, was shown to express internal xylanase activity when cultured on media containing xylan or glucose as sole carbon source. A genomic library of *C. mixtus* DNA, constructed in λZAPII, was screened for xylanase activity. The nucleotide sequence of the genomic insert from a xylanase-positive clone that expressed intracellular xylanase activity in *Escherichia coli* revealed an ORF of 1137 bp (*xynC*), encoding a polypeptide with a deduced M_r of 43413, defined as xylanase C (XylC). Probing a gene library of *Pseudomonas fluorescens* subsp. *cellulosa* with *C. mixtus xynC* identified a *xynC* homologue (designated *xynG*) encoding XylG; XylG and *xynG* were 67% and 63% identical to the corresponding *C. mixtus* sequences, respectively. Both XylC and XylG exhibit extensive sequence identity with family 10 xylanases, particularly with non-modular enzymes, and gene deletion studies on *xynC* supported the suggestion that they are single-domain xylanases. Purified recombinant XylC had an M_r of 41000, and displayed biochemical properties typical of family 10 polysaccharidases. However, unlike previously characterized xylanases, XylC was particularly sensitive to proteolytic inactivation by pancreatic proteinases and was thermolabile. *C. mixtus* was grown to late-exponential phase in the presence of glucose or xylan and the cytoplasmic, periplasmic and cell envelope fractions were probed with anti-XylC antibodies. The results showed that XylC was absent from the culture media but was predominantly present in the periplasm of *C. mixtus* cells grown on glucose, xylan, CM-cellulose or Avicel. These data suggest that *C. mixtus* can express non-modular internal xylanases whose potential roles in the hydrolysis of plant cell wall components are discussed.

Keywords: family 10 xylanases, *Cellvibrio mixtus*, xylanase expression

INTRODUCTION

Biological degradation of the main plant cell wall components, the polysaccharides cellulose and xylan, is a complex process that requires the synergistic interaction of a large consortium of microbial cellulases and xylanases (Tomme *et al.*, 1995). Structural polysaccharides are insoluble polymers and, consequently, micro-organisms generally secrete plant cell wall hydrolases that are remarkably stable (Fontes *et al.*, 1997). Extracellular cellulases and xylanases expressed by saprophytic aerobic soil bacteria, such as a *Pseudo-*

Abbreviation: CBMs, carbohydrate-binding modules.

The GenBank accession numbers for the sequences described in this paper are AF049493 and AF168359 for *xynC* and *xynG*, respectively.

monas sp. (Hazlewood & Gilbert, 1998), *Cellulomonas fimi* (Meinke *et al*., 1994) and *Cellvibrio mixtus* (Fontes *et al*., 1998), bind individually to the plant cell wall via the action of specific non-catalytic domains designated carbohydrate-binding modules (CBMs). Therefore, most cellulases and xylanases derived from aerobic bacteria have a modular architecture, in which the catalytic domain is linked to one or more CBMs that are known to play a crucial role in enhancing the activity of the enzymes against crystalline cellulose and insoluble xylan, respectively (Hazlewood & Gilbert, 1998). In contrast, extracellular plant cell wall hydrolases from anaerobic organisms are modular enzymes that interact to form a large M_r enzyme complex, known as the cellulosome, that is bound to the microbial cell wall. Extracellular single-domain cellulases and xylanases have, however, been identified in both aerobes and anaerobes, and are thought to be involved in the hydrolysis of the soluble components of the cell wall. Recently, the view that all plant cell wall hydrolases are extracellular has been questioned by studies on the anaerobic bacterium *Prevotella bryantii*, in which the majority of the xylanase activity was shown to be located in the periplasm and is not exposed to the extracellular environment (Miyazaki *et al*., 1997). It was argued that the internal location of these enzymes might have an important role in allowing the bacterium to sequester the products of polysaccharide hydrolysis in energy-limiting densely populated gut ecosystems. It remains to be established whether aerobic bacteria, which can generate considerably more energy from pentose metabolism than anaerobic prokaryotes, also synthesize polysaccharidases that are not exported.

Plant cell wall degrading micro-organisms use a wide variety of carbohydrates as carbon and energy sources, and have therefore developed mechanisms to modulate the synthesis of polysaccharidases. Cellulases and xylanases are expressed when the organisms are grown in the presence of the structural polysaccharides and are subject to catabolite repression by readily metabolizable sugars such as glucose. However, some extracellular cellulases and xylanases have been shown to be constitutively expressed at very low levels by micro-organisms such as *Trichoderma reesei* (Zeilinger *et al*., 1996; Torigoi *et al*., 1996). In fungi, it is well established that these enzymes are crucial for triggering the expression of cellulases and xylanases; an initial attack on the cell wall by these plant cell wall hydrolases results in the absorption of the hydrolysis products by the organism and the consequent general induction of polysaccharidase expression by a mechanism which remains to be elucidated (Carle-Urioste *et al*., 1997). *Pseudomonas fluorescens* subsp. *cellulosa* was also shown to constitutively express polysaccharidases (Rixon *et al*., 1992), although their role in the regulation of gene expression in the pseudomonad is currently unknown.

Studies in our laboratories have focused on the plant cell wall degrading systems of *C. mixtus* and *Ps. fluorescens* subsp. *cellulosa*. Although both organisms have been shown to express a large number of extracellular xylanases and cellulases that are subject to catabolite repression (Hazlewood *et al*., 1992), it remains to be established whether the two aerobic prokaryotes also synthesize non-extracellular xylanases. The objective of this study was to establish whether there is evidence, in *C. mixtus*, for xylanases that are both intracellular and constitutively expressed. Data presented in this paper show that a non-modular family 10 xylanase (XylC) from *C. mixtus* is primarily secreted into the periplasm and is produced when the organism grows in the presence of various carbon sources, including glucose. A XylC homologue was also detected in *Ps. fluorescens* subsp. *cellulosa* and the roles of these enzymes in plant cell wall hydrolysis are discussed.

METHODS

Bacterial strains, vectors and culture conditions. *Cellvibrio mixtus* (NCIMB 8633) was cultured aerobically at 20 °C in Dubos mineral salts medium or on Dubos agar plates overlaid with filter paper (Millward-Sadler *et al*., 1995). Media were supplemented with sterile glucose (0·25 %), after autoclaving, or CM-cellulose (medium viscosity), Avicel or oat spelt xylan, before autoclaving (all polysaccharides were used at 0·5 % final concentration). *Pseudomonas fluorescens* subsp. *cellulosa* (NCIMB 10462) was cultured as described by Millward-Sadler *et al*. (1995). *Escherichia coli* JM83 and XL-1 Blue were cultured at 37 °C in Luria broth (LB) or on LB-agar plates. Media were supplemented with 100 mg ampicillin l^{-1} or 2 mg 5-bromo-4-chloro-3-indolyl β-D-galactoside l^{-1} to select for *E. coli* transformants and recombinants, respectively. Remazol Brilliant Blue R-xylan (Sigma) was added to solid media at a final concentration of 0·05 % (w/v) to select for recombinant *E. coli* strains expressing xylanase activity. The phages and plasmids employed in this work were λZAPII (Stratagene), pBluescript (Stratagene), pUC18 and pUC19 (Norrander *et al*., 1983).

General recombinant DNA procedures. Plasmid DNA was prepared by the method of Birnboim & Doly (1979), or by using Qiagen resin columns (Hybaid). Transformation of *E. coli*, agarose gel electrophoresis, Southern hybridization, slot blot hybridizations and the general use of nucleic acid modifying enzymes were as described by Sambrook *et al*. (1989). *C. mixtus* and *Ps. fluorescens* subsp. *cellulosa* genomic DNA was isolated as described by Berns & Thomas (1965). The genomic libraries were constructed in λZAPII using the approach described by Clarke *et al*. (1991). The libraries were screened for recombinants expressing xylanase activity by plating out the recombinant phage and host bacterium (*E. coli* XL-1 Blue) in soft agar poured onto NZY plates (NZY medium: 0·5 % NaCl; 0·2 % $MgSO_4.7H_2O$; 0·5 % yeast extract; 1 % NZ amine; pH adjusted to 7·5). The plaques generated were overlaid with agar containing 0·2 % (w/v) xylan. After incubation at 37 °C for 16 h, xylanase-producing clones were identified by the appearance of clear haloes against a red background, after staining with Congo red (Teather & Wood, 1982). Plasmids [pBluescript SK(−)] containing genomic DNA inserts from *C. mixtus* and *Ps. fluorescens* subsp. *cellulosa* were excised from xylanase-positive recombinant phage and rescued into *E. coli* XL-1 Blue, as described in the Stratagene protocol. DNA hybridizations were performed using the fluorescein system from Amersham according to the manufacturer's protocol.

Nucleotide sequencing. To sequence *xynC* and *xynG*, nested deletions of the *C. mixtus* and *Ps. fluorescens* subsp. *cellulosa* chromosomal DNA [in pBluescript SK(−)], respectively, were created using the Exonuclease III/S1 nuclease method (Promega). Double-stranded plasmid DNA was sequenced manually by the dideoxy-chain-termination method of Sanger *et al*. (1977), with the protocol recommended for the Sequenase DNA Sequencing kit (United States Biochemical/Amersham). Sequences were compiled and ordered using the computer software DNASIS from Hitachi. The complete sequences of *xynC* and *xynG* were determined in both strands.

Protein purification, production of antisera and Western blotting. *E. coli* JM83 harbouring full-length *xynC* in plasmid pLMA2 was cultured for 16 h in LB broth containing ampicillin (100 mg l^{-1}). A cell-free extract was prepared by sonicating the harvested cells and recovering the soluble fraction. Proteins were loaded onto a DEAE Trisacryl anion-exchange column, which was eluted in 10 mM Tris/HCl, pH 8·0, with a 0–400 mM NaCl gradient. Fractions expressing xylanase activity were further purified on a MonoQ column by anion-exchange chromatography. The purity of the xylanase fractions was confirmed by analysis through SDS-PAGE.

To produce polyclonal antisera against XylC, purified protein (approx. 500 μg) diluted to 1 ml with sterile distilled water was emulsified with Freund's complete adjuvant (1 ml) and injected into New Zealand White male rabbits by intramuscular and subcutaneous routes. Second and third injections, with half as much protein mixed with Freund's incomplete adjuvant, were made at 4-week intervals. Serum was collected 12 d after the last injection. Western blot analysis of *C. mixtus* proteins was performed essentially as described by Fontes *et al*. (1995) using the enhanced chemiluminescence system (Amersham).

Enzyme assays. Periplasmic and cell-free extracts were prepared as described by Ferreira *et al*. (1990) with a 200 ml culture. Enzyme assays were performed in 50 mM potassium phosphate/12 mM citric acid buffer, pH 6·5 (PC buffer) at 37 °C, using 0·2 % (w/v) of the appropriate plant structural polysaccharide, unless otherwise stated. Reducing sugar was measured with the dinitrosalicylic acid reagent (Miller, 1959). Insoluble xylan was prepared from oat spelt xylan as described by Fernandes *et al*. (1999). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol reducing sugar min^{-1}. HPLC analysis of hydrolysis products was performed as described by Black *et al*. (1994). Total protein was measured by the Lowry method with BSA as standard. SDS-PAGE analysis was carried out as described by Laemmli (1970).

Proteolysis, thermostability and pH optimum experiments. *E. coli* fractions containing XylC, at a concentration of approximately 10 g total protein l^{-1}, were incubated with porcine pancreatin at a final concentration of 10 g l^{-1} at 37 °C. At regular time intervals, an aliquot of the reaction mixture was removed and assayed for enzyme activity as described above. For the thermostability experiments, the xylanase-containing extracts were incubated for 15 min at temperatures ranging from 37 to 85 °C, cooled on ice for 5 min and, after centrifugation at 13000 ***g*** for 10 min, assayed for residual xylanase activity. Extracts containing XylC were assayed for xylanase activity in buffers with different pH values (pH 3–7, 50 mM phosphate citrate; pH 8–9, 50 mM sodium barbitone), as described above.

RESULTS

Production and cellular location of xylanases in *C. mixtus*

To establish the growth pattern of *C. mixtus* in the presence of various carbon sources, the bacterium was cultured at 20 °C in minimal media containing glucose (0·25 %) or oat spelt xylan (0·5 %; Sigma) as the sole carbon source, and the OD_{600} of the cultures was monitored over a 7 d period. The results (not shown) demonstrated that for the substrate concentrations used, *C. mixtus* reached stationary phase faster when grown on glucose (36 h) than on xylan (84 h). The general pattern of xylanase expression by *C. mixtus*, grown in the presence of xylan or glucose, was determined using late-exponential-phase cells. The data, presented in Table 1, demonstrate that most of the xylanase activity in xylan-grown cells was extracellular, while no apparent activity was detected in the culture supernatant of glucose-grown *C. mixtus*. However, *C. mixtus* cells grown on xylan or glucose contained considerable internal xylanase activity, which was located mainly in the periplasm. Under these experimental conditions, the majority of malate dehydrogenase activity, alkaline phosphatase and arabinase (cell membrane enzyme; unpublished data) activities were located in the cytoplasm, periplasm and cell envelope, respectively, suggesting that the cell fractions had been adequately separated. Collectively, these data suggest that *C. mixtus* expresses internal xylanase(s) which, in both glucose- and xylan-grown cells, are predominantly located in the periplasmic space.

Isolation and characterization of xylanase genes that encode potential intracellular enzymes

To isolate genes encoding non-extracellular xylan-degrading enzymes, a *C. mixtus* genomic library constructed in λZAPII was screened for xylanase activity. Xylanase-positive phages were isolated at a frequency of 1 in 200 clones. The *C. mixtus* genomic inserts from 12 recombinant clones were excised into pBluescript SK(−) and their similarity with the previously described *C. mixtus* xylanase genes, *xynA* and *xynB*, was determined by Southern hybridization (Millward-Sadler *et al*., 1995). The results (not shown) revealed that out of the 12 clones, six *C. mixtus* sequences cross-hybridized and did not hybridize with *xynA* or *xynB*, indicating that the six plasmids contained a novel xylanase gene. The xylanase activity expressed by *E. coli* cells harbouring one of the six recombinant plasmids (pLMA4) was found predominantly in the cytoplasm, suggesting that the encoded recombinant xylanase was not efficiently exported by *E. coli* (Table 1). A restriction map of the *C. mixtus* genomic fragment containing the new xylanase gene, designated *xynC*, is presented in Fig. 1. Deletion and subcloning experiments located the position of *xynC* within the *C. mixtus* DNA fragment.

To determine whether *xynC* was reiterated within the

Table 1. Localization of xylanase, malate dehydrogenase, alkaline phosphatase and arabinase activities in *C. mixtus* grown on different carbon sources, and in *E. coli* harbouring the plasmid pLMA4

Cultures (50 ml) of *C. mixtus* were grown in minimal media containing xylan or glucose as sole carbon source to late-exponential phase. Cells were collected by centrifugation and periplasmic (4 ml) and cytoplasmic (4 ml) fractions were prepared as described by Ferreira *et al.* (1990). The cell envelopes were resuspended in 4 ml PC buffer. *E. coli* was cultured in 50 ml LB as described in Methods and cell fractions were prepared as described for *C. mixtus* cells. Xylanase, malate dehydrogenase, alkaline phosphatase and arabinase activities were quantified as described in Methods. Values are the means of two experiments and are expressed as percentage of the total activity. ND, Not determined.

Activity	Cell fraction	*C. mixtus* grown on:		*E. coli*/pLMA4
		Glucose (0·25%)	Oat spelt xylan (0·5%)	
	Medium	0 (0·00)*	92 (34·15)	12 (85·01)
Xylanase [U (vol. of fraction)$^{-1}$]†	Periplasm	94 (0·47)	4 (1·40)	5 (35·12)
	Cytoplasm	0 (0·00)*	1 (0·26)	81 (579·87)
	Envelope	6 (0·03)	3 (0·99)	2 (14·05)
Malate dehydrogenase	Periplasm	14	8	11
	Cytoplasm	76	86	80
	Envelope	10	7	9
Alkaline phosphatase	Periplasm	67	68	ND
	Cytoplasm	12	14	ND
	Envelope	21	18	ND
Arabinase	Periplasm	3	19	ND
	Cytoplasm	1	13	ND
	Envelope	96	68	ND

* The value 0 signifies that the activity was below the level of detection.

† Percentage of enzyme activity and in parentheses total xylanase activity recovered from glucose- and xylan-grown cells, and in *E. coli* cells harbouring pLMA4.

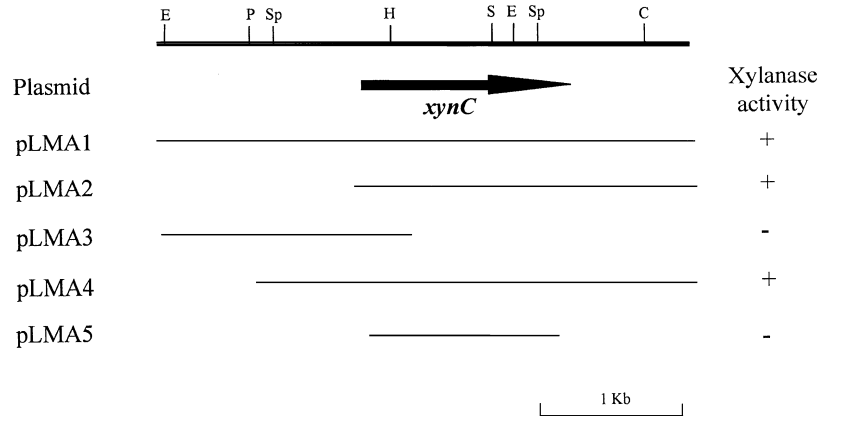

Fig. 1. Restriction map of recombinant plasmids containing *xynC* from *C. mixtus*. Restriction enzyme sites are as follows: H, *Hin*dIII; C, *Cla*I; Sp, *Sph*I; S, *Sal*I; E, *Eco*RI; P, *Pst*I. The large arrow indicates the position and orientation of *xynC* within the *C. mixtus* sequence. The fragment cloned in plasmid pLMA3 was cloned from *C. mixtus* genomic DNA by inverse PCR. The xylanase phenotype of *E. coli* harbouring the respective plasmids is indicated by + and −.

Cellvibrio genome, genomic DNA was subjected to Southern hybridization using the DNA insert from the recombinant plasmid containing *xynC* (pLMA2) as the probe. The data, not shown, indicate that *xynC* is present as a single copy in the *C. mixtus* genome. It was previously demonstrated that *xynA* and *xynB* from *C. mixtus* share considerable sequence identity with the xylanase genes *xynE* and *xynF* from *Ps. fluorescens* subsp. *cellulosa* (Millward-Sadler *et al.*, 1995). To assess whether a homologue to *xynC* was present in the pseudomonad genome, *xynC* derived from pLMA2 was used to probe *Ps. fluorescens* subsp. *cellulosa* DNA. The

results (not shown) revealed the presence of a single locus in the *Ps. fluorescens* genome exhibiting extensive homology with *xynC*. The *Ps. fluorescens* subsp. *cellulosa xynC* homologue, designated *xynG*, was isolated from a λZAPII genomic library and shown to encode a functional xylanase defined as XylG (data not shown).

Nucleotide sequence of *xynC* and *xynG* and the primary structures of XylC and XylG

The nucleotide sequences of the genomic fragments containing *xynC* and *xynG* were determined in both strands. The data revealed ORFs of 1137 and 1134 bp, for *xynC* and *xynG*, respectively, encoding polypeptides with predicted M_r of 43 413 and 43 167. The codon usage of the ORFs was very similar to other C. *mixtus* and *Ps. fluorescens* plant cell wall hydrolases (Fontes *et al.*, 1997, 1998; Millward-Sadler *et al.*, 1994, 1995). The proposed ATG translational start codons were preceded (7 bp) by the sequence GAGGA, which exhibits strong similarity to the ribosome-binding motif most frequently found in genes from Gram-negative bacteria. The presence of translational stop codons in all three reading frames of the 5′ flanking sequence of *xynC* and *xynG* provides further support for the validity of the putative translational start. The N-terminal sequences of XylC and XylG contain a basic N-terminus followed by 12 small hydrophobic residues. These sequence motifs exhibit similarities to bacterial signal peptides, although the hydrophobic region of the corresponding secretion signals from other *Ps. fluorescens* and C. *mixtus* polysaccharidases tends to be longer (Fontes *et al.*, 1998). The two genes terminated at a TAA codon followed by translational stop codons in all three reading frames. A DNA palindromic sequence capable of forming a stem–loop with a ΔG of $-22{\cdot}9$ kcal ($-96{\cdot}18$ kJ) was noted downstream of *xynC* and *xynG*. This structure was followed by an A + T-rich region, which is characteristic of a rho-independent transcription termination sequence.

Deletion of the 45 N-terminal or 35 C-terminal amino acids, respectively, from XylC resulted in the complete loss of xylanase activity, suggesting that the protein is a single-domain enzyme. This was supported by homology studies, which revealed extensive sequence identity between both XylC and XylG and the catalytic domains of family 10 glycosyl hydrolases, as defined by Henrissat & Bairoch (1993). The sequence with the highest level of homology to XylC and XylG was XylA (43 and 45% identity, respectively) from *Bacteroides ovatus* (Whitehead, 1995), followed by XylA from *Prev. bryantii* (39 and 40% identity, respectively; Gasparic *et al.*, 1995), xylanase A from *Bacillus* strain N137 (37 and 36% identity, respectively; Tabernero *et al.*, 1995) and XylX from *Aeromonas caviae* (36 and 36% identity, respectively; accession no. 3299808). Interestingly, all these enzymes are non-modular xylanases, suggesting that this subset of family 10 enzymes (Henrissat & Bairoch, 1993) may share a common ancestral origin. Together, these results suggest that XylC and XylG are family 10 single-domain xylanases.

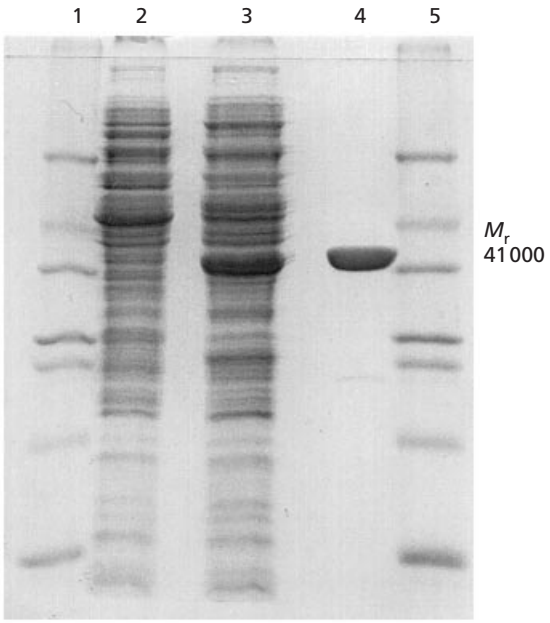

Fig. 2. Purification of XylC from *C. mixtus*. SDS-PAGE analysis using a 10% (w/v) polyacrylamide gel of cell-free extract from *E. coli* JM83 (lane 2) and recombinant *E. coli* harbouring pLMA2 (lane 3). Lane 4 contains XylC purified from *E. coli* harbouring pLMA2. Lanes 1 and 5 contain Sigma low-M_r markers. The M_r of purified XylC is shown.

Biochemical properties of XylC

XylC was purified from the cytoplasmic fraction of *E. coli* cells harbouring pLMA2 and its biochemical properties were evaluated. The enzyme had an M_r of 41 000 (Fig. 2) and displayed activity over a limited pH range, with a maximum at pH 7·5 (not shown). XylC was thermolabile, displaying considerable loss in activity at temperatures in excess of 40 °C and with a half-life of less than 10 min at 50 °C. XylC was also rapidly inactivated when incubated with pancreatic proteinases, demonstrating very high susceptibility to proteolysis (only less than 10% of XylC residual activity was recovered after a 3 min incubation with proteinases). The addition or removal of 5 mM $CaCl_2$, which has been shown to stabilize at least one family 10 xylanase (Spurway *et al.*, 1997), did not affect the thermal stability or the proteolytic sensitivity of XylC. Collectively, these results suggest that XylC is particularly sensitive to proteinase inactivation, which is in sharp contrast to previously characterized extracellular xylanases, from both mesophilic and thermophilic organisms, which are completely resistant to proteolytic inactivation over a 3 h incubation period with pancreatic proteinases (Fontes *et al.*, 1995; Spurway *et al.*, 1997). Analysis of the substrate specificities of XylC revealed that the enzyme hydrolysed both the soluble and the insoluble fractions of oat spelt xylan [443 and 189 U (mg protein)$^{-1}$] and exhibited slight activity against β-glucan [1·4 U (mg protein)$^{-1}$] and CM-cellulose [0·03 U (mg protein)$^{-1}$]. The xylanase was unable to hydrolyse

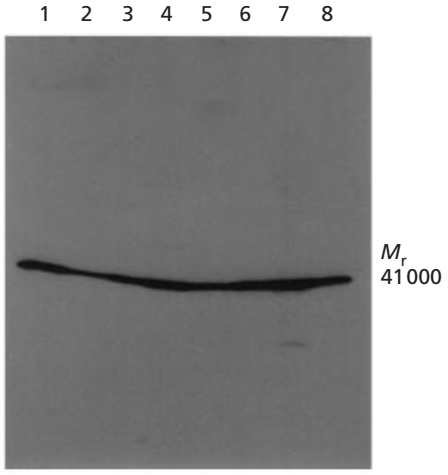

Fig. 3. Production of XylC by *C. mixtus*. The bacterium was grown in LB (lane 1) or in minimal media containing Avicel (lane 2), glucose (lane 3), oat spelt xylan (lane 4), CMC (lane 5), CMC/xylan (lane 6) and Avicel/xylan (lane 7), as described in Methods, until stationary phase. Total cellular proteins were fractionated by SDS-PAGE. Lane 8 contains total proteins from *E. coli* harbouring pLMA4. Western blotting was carried out using antisera raised against purified recombinant XylC as described in Methods. The M_r of the immunoreactive polypeptides is indicated.

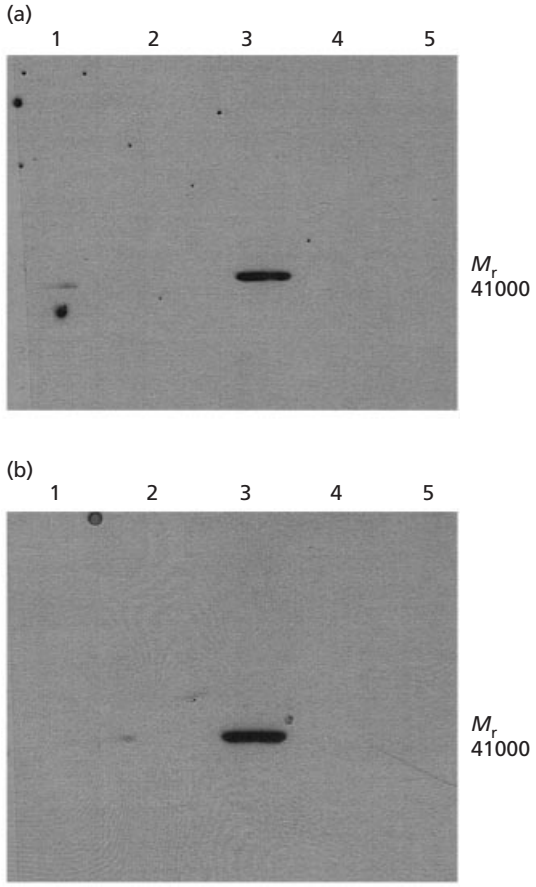

Fig. 4. Cellular localization of XylC from *C. mixtus*. Cells grown in the presence of glucose (a) and xylan (b) were grown to late-exponential phase (for 30 and 60 h, respectively) and treated as described in Methods for isolation of cell-bound (lane 1), cytoplasmic (lane 2) and periplasmic (lane 3) proteins. Lane 4 contains extracellular polypeptides and lane 5 the sucrose released protein. SDS-PAGE, performed using 10 μg protein, and Western analysis were performed according to the legend of Fig. 3. The M_r of the immunoreactive polypeptide is indicated.

crystalline forms of cellulose such as filter paper and Avicel, even after prolonged incubation for 24 h, or 1,3-*β*-glucans such as laminarin. Very low activity against soluble cellulosic substrates has been reported for other family 10 xylanases, exemplified by Cex from *Cellulomonas fimi* (Gilkes *et al*., 1984). To evaluate whether full-length XylC was able to bind insoluble polysaccharides, the recombinant enzyme was mixed with Avicel and insoluble oat spelt xylan and the retention of xylanase activity in the pellets was assessed. The data indicated that XylC is unable to bind significantly to either cellulose or xylan (not shown).

To evaluate the mode of action of XylC, the products generated by the action of the enzyme against oligosaccharides were analysed by HPLC. The data revealed that XylC displayed a typical endo-mode of activity against xylo-oligosaccharides. For example, xylohexose was cleaved to mainly xylotriose and small amounts of xylobiose and xylotetraose; xylopentaose to xylobiose and xylotriose; xylotetraose was hydrolysed exclusively to xylobiose; the enzyme did not cleave xylobiose. The relative activities of XylC against xylotriose, xylotetraose, xylopentaose and xylohexaose were 1:603:3038:3050, respectively. The activity of XylC and that reported for *C. fimi* Cex by Charnock *et al*. (1998) against these xylo-oligosaccharides were very similar. These data suggest that XylC is not an exo-acting enzyme and that the enzyme has a substrate-binding site that accommodates five xylose units. The products of xylopentaose hydrolysis showed that two and three xylose-binding sites must be located on either site of the nucleophile and the acid–base residues. From the above discussion, it is apparent that the biochemical properties of XylC are very similar to other family 10 xylanases, which have been shown to have substrate-binding clefts ranging from five (*C. fimi* Cex) to seven (*Pseudomonas* Xyn10A; Charnock *et al*., 1998) sugar-binding subsites.

Cellular location of XylC from *C. mixtus*

To determine the cellular location of XylC in its original host, *C. mixtus* was grown to stationary phase in minimal medium containing either glucose (0·25 %), oat spelt xylan (0·5 %), CM-cellulose (0·5 %), Avicel (0·5 %), CMC/xylan (0·25 % each) or Avicel/xylan (0·25 % each) as sole carbon source, and the proteins present in the culture supernatant (not shown) and bacterial cell pellet were probed with anti-XylC polyclonal antibodies by Western blot analysis. The results, presented in Fig. 3, showed that a protein of M_r 41000, which was immunoreactive with anti-XylC antiserum, was present exclusively in the cell pellet (negative Western blot results

with the culture media are not shown). To test the possibility of XylC being inactivated by proteinases potentially present in the culture media, the enzyme was incubated with media collected from stationary phase cells over a 3 h period. The data (not shown) demonstrated that XylC was completely resistant to inactivation when incubated under the conditions described. The similar size of XylC produced by *C. mixtus* and the recombinant form of the enzyme suggested that the xylanase was not subject to post-translational modifications in its endogenous host (Fig. 3). In addition, the results showed that XylC is not subject to catabolite repression, but is expressed when *C. mixtus* grows in the presence of glucose. To evaluate the localization of XylC in *C. mixtus* cells, the bacterium was grown to late-exponential phase in minimal media supplemented with glucose (0·25 %) or oat spelt xylan (0·5 %) and periplasmic, cytoplasmic and membrane envelope fractions were prepared as described by Ferreira *et al.* (1990). To verify that fractions were correctly prepared, samples were assayed for periplasmic, cytoplasmic and cell envelope enzymes (data not shown). The data, in Fig. 4, show that XylC was present predominantly in the periplasm of *C. mixtus* and is expressed when the organism is cultured on glucose or xylan. Although purely qualitative, the Western blot data of Fig. 4 confirm that significant amounts of XylC are expressed by glucose-grown cells.

DISCUSSION

It is well-established that plant cell wall degrading organisms secrete extensive consortia of modular cellulases and hemicellulases containing non-catalytic CBMs, suggesting the existence of a strong selective pressure for the retention of these modules. Cellulose, and more recently, xylan binding domains have been shown to play a pivotal role in the hydrolysis of cell wall polysaccharides, both by promoting the interactions between the enzymes and the substrates and, in some instances, by contributing to the physical disruption of the substrates (Din *et al.*, 1991, 1994; Millward-Sadler *et al.*, 1994; Bolam *et al.*, 1998; Sun *et al.*, 1998; Fernandes *et al.*, 1999). Despite the strong selection pressure for modular plant cell wall hydrolases, single-domain cellulases and xylanases are expressed by micro-organisms, suggesting that these enzymes also play an important role in plant cell wall hydrolysis. It could be argued that the anchoring of enzymes to the polysaccharides would limit the hydrolysis of soluble oligo- and polysaccharides released from the cell wall and thus non-modular enzymes would primarily be involved in the hydrolysis of soluble substrates (Fontes *et al.*, 1998). Evidence presented in this study shows that *C. mixtus* expresses a protease-sensitive xylanase, XylC, when grown in the presence of various carbon sources and directs the secretion of the enzyme into the periplasm. XylC and its homologue in *Ps. fluorescens* subsp. *cellulosa*, XylG, exhibited highest identities with non-modular family 10 xylanases, particularly those from *Prev. bryantii* and *B. ovatus*. Furthermore, studies on the biophysical properties of XylC showed that the enzyme was unusually sensitive to proteolytic inactivation, while, to our knowledge, extracellular xylanases are resistant to proteinase attack (Fontes *et al.*, 1995).

In this report, we show that *C. mixtus* expresses low levels of periplasmic xylanase activity when grown on glucose, but on xylan produces high levels of extracellular xylanase activity, and also increased internal xylanase expression. One of the components responsible for the periplasmic xylanase activity seems to be XylC, although it remains to be elucidated if it is unique. The presence of an N-terminal sequence that resembles a signal peptide in both XylC and XylG suggests that the enzymes are subject to a protein sorting mechanism. However, the size of the hydrophobic domain is smaller than that of signal peptides from other previously characterized extracellular polysaccharidases of both *C. mixtus* and *Ps. fluorescens* subsp. *cellulosa*. The periplasmic location of XylC suggests that the enzyme's primary targets are xylo-oligosaccharides that have been transported into the bacterium. XylC preferentially hydrolyses oligosaccharides with DP >5 and therefore *C. mixtus* can either import relatively large xylo-oligosaccharides (xylopentaose or larger) or the enzyme is hydrolysing xylotriose and xylotetraose relatively slowly. The concept that microbial xylanases can be intracellular is supported by a recent study which showed that approximately 80 % of xylanase activity expressed by *Prev. bryantii* is located in the periplasm (Miyazaki *et al.*, 1997).

We propose that the protected periplasmic environment of XylC has removed the selective pressures for the enzyme to become highly stable – a general feature of extracellular xylanases is resistance to proteinases and thermal denaturation, for example. As the majority of *Prev. bryantii* xylanase activity was found in the periplasm, it is likely that XynA from this bacterium is also periplasmic, which would be consistent with the enzyme's designation as highly thermolabile (Gasparic *et al.*, 1995). This view is supported by the observation that XynA, when expressed in *Bacteroides vulgartus*, is intracellular (H. J. Flint, personal communication). Based on the data presented in this paper, and results reported by Gasparic *et al.* (1995), it is likely that within glycosyl hydrolase family 10, there is a subset of non-extracellular xylanases that includes XylC (*C. mixtus*), XylG (*Ps. fluorescens*), XynA (*Prev. bryantii*) and XylI (*B. ovatus*), which are particularly labile. Whether these enzymes have all evolved from an ancestral family 10 enzyme that was particularly thermolabile and sensitive to proteolytic attack, or exhibit these properties because of their intracellular location remains to be elucidated.

In contrast with the general pattern of microbial cellulase and hemicellulase synthesis, XylC was shown to be expressed when *C. mixtus* was grown on glucose. In fungi, constitutively expressed extracellular xylanases play an important role in regulating polysaccharidase gene expression (Zeilinger *et al.*, 1996). Furthermore, the production of the general cellulase inducer, sophorose, was shown to be mediated by a constitutive

intracellular β-glucosidase which generated the inducer by catalysing the transglycosylation of cellobiose. Constitutive expression of a bacterial xylanase has also been reported in *Streptomyces cyaneus* (Wang *et al*., 1992): one of that organism's three xylanases (xylanase III) was expressed in the presence of glucose, and it was suggested that this enzyme acts as a 'xylan' sensor probably involved in the regulation of other xylanase genes. In view of the expression of XylC when the organism grows on different carbon sources, it is tempting to speculate that this enzyme plays a key role in generating signals, from absorbed xylo-oligosaccharides, that induce xylanase expression in *C. mixtus*. Clearly this hypothesis can only be viewed as tentative until the role of XylC in xylanase gene expression is analysed in more detail.

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