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*<sub>r</sub> 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*<sub>r</sub> 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

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**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 hydrodases that are remarkably stable (Fontes *et al*., 1997). Extracellular cellulases and xylanases expressed by saprophytic aerobic soil bacteria, such as a *Pseudo-
monas sp. (Hazlewood & Gilbert, 1998), Cellulomonas fimii (Meinke et al., 1994) and Cellulibrio 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 hydrolysates from anaerobic organisms are modular enzymes that interact to form a large Mₐ 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 hydrolysates 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 xylan- ases 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 microorganisms 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 hydrolysates 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-Urioiste 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 P. 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. Cellulibrio 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 (X-Gal) 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 iZAPI (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 P. fluorescens subsp. cellulosa genomic DNA was isolated as described by Berns & Thomas (1965). The genomic libraries were constructed in iZAPII 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% MgSO₄; 7H₂O; 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 P. 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 antiserum against XylIC, 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 route. 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 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). 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.

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 OD₅₆₀ 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 zZAPIII was screened for xylanase activity. Xylanase-positive plasmids 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.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Cell fraction</th>
<th>C. mixtus grown on:</th>
<th>E. coli/pLMA4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose (0.25%)</td>
<td>Oat spelt xylan (0.5%)</td>
</tr>
<tr>
<td>Xylanase [U (vol. of fraction)]⁻¹†</td>
<td>Medium</td>
<td>0 (0.00)*</td>
<td>92 (34:15)</td>
</tr>
<tr>
<td></td>
<td>Periplasm</td>
<td>94 (0.47)</td>
<td>4 (1:40)</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>0 (0.00)*</td>
<td>1 (0.26)</td>
</tr>
<tr>
<td></td>
<td>Envelope</td>
<td>6 (0.03)</td>
<td>3 (0.99)</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>Periplasm</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>76</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Envelope</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Periplasm</td>
<td>67</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Envelope</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Arabinase</td>
<td>Periplasm</td>
<td>3</td>
<td>19</td>
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<td>Cytoplasm</td>
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<td>13</td>
</tr>
<tr>
<td></td>
<td>Envelope</td>
<td>96</td>
<td>68</td>
</tr>
</tbody>
</table>

*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.

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**Fig. 1.** Restriction map of recombinant plasmids containing xynC from C. mixtus. Restriction enzyme sites are as follows: H, HindIII; C, Clal; Sp, SphI; S, SalI; E, EcoRI; P, PstI. 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 −.

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**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 \textit{Ps. fluorescens} genome exhibiting extensive homology with \textit{xynC}. The \textit{Ps. fluorescens} subsp. \textit{cellulosa} \textit{xynC} homologue, designated \textit{xynG}, was isolated from a \textit{iZAPII} genomic library and shown to encode a functional xylanase defined as XylG (data not shown).

**Nucleotide sequence of \textit{xynC} and \textit{xynG} and the primary structures of XylC and XylG**

The nucleotide sequences of the genomic fragments containing \textit{xynC} and \textit{xynG} were determined in both strands. The data revealed ORFs of 1137 and 1134 bp, for \textit{xynC} and \textit{xynG}, respectively, encoding polypeptides with predicted \(M_r\), of 43413 and 43167. The codon usage of the ORFs was very similar to other \textit{C. mixtus} and \textit{Ps. fluorescens} plant cell wall hydrolases (Fontes \textit{et al}., 1997, 1998; Millward-Sadler \textit{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 \textit{xynC} and \textit{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 \textit{Ps. fluorescens} and \textit{C. mixtus} polysaccharidases tends to be longer (Fontes \textit{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 \(\Delta G\) of \(-22.9\) kcal \((-96.18\) kJ) was noted downstream of \textit{xynC} and \textit{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 \textit{Bacteroides ovatus} (Whitehead, 1995), followed by XylA from \textit{Prev. bryantii} (39 and 40\% identity, respectively; Gasparic \textit{et al}., 1995), xylanase A from \textit{Bacillus} strain N137 (37 and 36\% identity, respectively; Tabernero \textit{et al}., 1995) and XylX from \textit{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.

**Biochemical properties of XylC**

XylC was purified from the cytoplasmic fraction of \textit{E. coli} cells harbouring pLMA2 and its biochemical properties were evaluated. The enzyme had an \(M_r\) of 41000 (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 \textit{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 \textit{et al}., 1995; Spurway \textit{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 \([443\text{ and }189\text{ U (mg protein)}^{-1}]\) and exhibited slight activity against \(\beta\)-glucan \([1.4\text{ U (mg protein)}^{-1}]\) and CM-cellulose \([0.03\text{ U (mg protein)}^{-1}]\). The xylanase was unable to hydrolyse...
Crystalline forms of cellulose such as filter paper and Avicel, even after prolonged incubation for 24 h, or 1,3-\(\beta\)-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; xylpentaoase to xylobiose and xylotriose; xylotetraose was hydrolysed exclusively to xylobiose; the enzyme did not cleave xylobiose. The relative activities of XylC against xylotriose, xylotetraose, xylpentaoase 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 \(M_r\) 41,000, 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; Fernandez et al., 1999). Despite the strong selection pressure for modular plant cell wall hydrolyses, 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 polysaccharidasises 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 thermostable (Gasparic et al., 1995). This view is supported by the observation that XynA, when expressed in Bacteroides vulgatus, 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 hydrolyase 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 thermostable 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 \( \beta \)-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 XyIC 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 XyIC in xylanase gene expression is analysed in more detail.

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