Characteristics of a cluster of xylanase genes in *Fibrobacter succinogenes* S85

Hyun S. Jun, Jong K. Ha, Laercio M. Malburg, Jr., Ann M. Verrinder Gibbins, and Cecil W. Forsberg

Abstract: Xylanase genes *xyn10D*, *xyn10E*, and *xyn10B*, located sequentially on the *Fibrobacter succinogenes* S85 chromosome, were separately cloned and their properties characterized. Analysis of the sequences documented that xylanases Xyn10D, Xyn10E, and Xyn10B each consist of an N-terminal catalytic domain (glycosyl hydrolase family 10) and a C-terminal carbohydrate-binding module (CBM, family 6) connected by proline-rich linker sequences. The amino acid sequences exhibited similarities of between 53 and 60%. The *xyn10D*, *xyn10E*, and truncated *xyn10BΔCBM* were expressed in *Escherichia coli* and purified to homogeneity. The purified Xyn10D, Xyn10E, and Xyn10BΔCBM exhibited the same temperature optimum (40°C) and pH optimum (6.5) and the highest specific activity against arabinoxylan, oat spelt xylan, and birchwood xylan, respectively. Xyn10D exhibited an affinity for cellulose and xylan with 47 and 33% binding, respectively, while the truncated Xyn10DΔCBM did not bind to the substrates. The main hydrolysis products of the three xylanases acting on oat spelt xylan and arabinoxylan were xylose and xylobiose. RT-PCR analysis showed that the three genes were co-transcribed as a single transcript. Western immunoblot analysis revealed that the three xylanases were expressed at a very low level by *F. succinogenes* grown on either glucose or cellulose as the source of carbohydrate.

Key words: Fibrobacter succinogenes S85, xylan, xylanase, clustered genes, RT-PCR.

Résumé : Les gènes *xyn10D*, *xyn10E* et *xyn10B* de xylanases, qu'on retrouve en succession sur le chromosome de *Fibrobacter succinogenes* S85 ont été clonés séparément et leurs propriétés ont été caractérisées. Une analyse des séquences a révélé que les xylanases Xyn10D, Xyn10E et Xyn10B sont toutes composées d'un domaine catalytique N-terminal (famille 10 des glycosyle hydrolases) et d'un module C-terminal se liant aux glucides (CBM famille 6) reliés par chaînes riches en prolines. Les séquences en acides aminés étaient de 53 à 60 % semblables. Les gènes *xyn10D*, *xyn10E* et le gène tronqué *xyn10BΔCBM* ont été exprimés chez *Escherichia coli* et purifiés jusqu'à homogénéité. Les protéines purifiées Xyn10D, Xyn10E, et Xyn10BΔCBM ont présenté la même température optimale (40 °C) et le même pH optimal (6,5), ainsi qu'un activité spécifique maximale en présence d'arabinoxylane, de xylane d'épeautre d'avoine et de xylane de bouleau, respectivement. Xyn10B présentait un affinité pour la cellulose et le xylane avec des taux de liaison respectifs de 47 et 33 %, alors que le Xyn10DΔCBM tronqué ne s'est pas lié aux substrats. Les principaux produits d'hydrolyse des trois xylanases agissant sur le xylane d'épeautre d'avoine et l'arabinoxylane étaient le xylose et le xylobiose. Une analyse par RT-PCR a révélé que les trois xylanases étaient exprimées à un très faible niveau chez *F. succinogenes* cultivé avec du glucose ou du cellulose comme sources de glucides.

Mots clés : Fibrobacter succinogenes S85, xylane, xylanase, gènes regroupés, RT-PCR.

[Traduit par la Rédaction]

Introduction

Xylan is a major component of the hemicellulose fraction of plant cell walls and is a complex polysaccharide consisting of long chains of β-1,4-linked xylopyranosyl residues. Most xylans occur as heteropolysaccharides, containing *O*-acetyl, α-L-arabinofuranosyl, α-1,2-linked glucuronic, or 4-*O*-methylglucuronic acid substituents (Beg et al. 2001). The degradation of xylan, therefore, requires the cooperative actions of *endo*-β-1,4-xylanase, β-xylosidase, and a series of debranching enzymes (Beg et al. 2001) for efficient access by fibrolytic microorganisms to the intertwined cellulose fibrils in the complex plant cell wall structures.

Fibrobacter succinogenes, *Ruminococcus albus*, and *Rumino-*

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coccus flavefaciens are the major fibrolytic bacteria in the rumen. *Fibrobacter succinogenes* S85 is a predominant Gram-negative, anaerobic, rod-shaped, and fibrolytic bacterium (Ziemer et al. 2000; Michalet-Doreau et al. 2001), possessing various endoglucanases, xylanases, and other hemicellulases that enable degradation of the complex of polysaccharides constituting plant cell walls (Forsberg et al. 2000). Although at least five xylanases (Malburg, Jr. et al. 1993), an arabinofuranosidase, acetyl and feruloyl esterases (McDermid et al. 1990), and a glucuronidase (Smith and Forsberg 1991), have been identified in *F. succinogenes* S85, only one xylanase gene, *xynC*, belonging to family 11 of the glycosyl hydrolase group, has been sequenced and the enzyme product fully characterized (Paradis et al. 1993).

In this paper, we report the cloning and characterization of a cluster of three xylanase genes. In addition, the expression of these genes in *F. succinogenes* S85 has been analyzed through a combination of reverse transcription - polymerase chain reaction (RT-PCR) and Western immunoblotting. The results document the presence of bifunctional xylanases with carbohydrate-binding modules (CBMs) that are expressed at low levels in *F. succinogenes* S85 cultures.

Materials and methods

Bacterial strains, growth conditions, and plasmids

Escherichia coli HB101, DH5α, and BL21 (DE3), harboring recombinant plasmids, were grown in Luria–Bertani (LB) medium containing 100 μg of ampicillin mL^{-1} or 30 μg of kanamycin mL^{-1} at 37 or 25°C. The expression vector pET-30a (Novagen Inc., Madison, Wis.) was used throughout this study to overexpress genes of interest. Xylanase clones pBX1 and pXC30.2 were previously isolated by Sipat et al. (1987) and Malburg et al. (1993), respectively, and were composed of pBR322 and a *Sau*3A insert.

Fibrobacter succinogenes S85 was grown in a medium (Scott and Dehority 1965) with either glucose at 0.5% (w/v) or Avicel PH-105 (FMC Corporation, Philadelphia, Pa.) at 0.3% (w/v) as the sole source of carbohydrate in 9 mL of culture volume with carbon dioxide as the gas phase. Glucose cultures were grown for 24 h at 37°C and cellulose cultures for 48 h at 37°C. Cultures were transferred three times in each medium to adapt the cells. The resulting cells (18 mL) were then inoculated into 300 mL of medium, grown as described by Malburg et al. (1993), and harvested by centrifugation at 9000 × *g* at 4°C for 15 min. The cells were resuspended in 15 mL of 50 mM sodium phosphate buffer, pH 8.0, including 300 mM sodium chloride, and disrupted by three passes through a French press. The whole cell extract was separated by centrifugation at 15 000 × *g* at 4°C for 20 min. The extracellular culture fluid was concentrated to 15 mL by filtration using a PM-10 membrane (Amicon Canada, Oakville, Ont.).

Subcloning and DNA sequencing

A 2.3-kb *Dra*I and a 2.5-kb *Xba*I–*Bgl*I fragment of pBX1 (Fig. 1) containing the coding sequences were used as templates for amplification of *xyn10D* and *xyn10E* genes, respectively. The PCR products amplified using primer sets 1 and 3 containing *Nde*I and *Xho*I (Table 1) were inserted into the *Nde*I–*Xho*I sites of the pET-30a vector to construct pETXyn10D and pETXyn10E, respectively, with 6× His tag sequences at the C-terminus. The *xyn10B* gene was amplified from a 4.0-kb *Xba*I–*Hin*dIII fragment of pXC30.2 using primer set 4 (Table 1) and inserted as described above. The truncated genes, *xyn10DΔCBM* and *xyn10BΔCBM*, were amplified from recombinant plasmids pETXyn10D and pETXyn10B using the corresponding primer sets 2 and 5 (Table 1) and inserted at *Nde*I–*Xho*I sites of pET-30a vector to construct pETXyn10DΔCBM and pETXyn10BΔCBM, respectively. All the recombinant plasmids were transformed into *E. coli* DH5α to be used for other DNA manipulations and into *E. coli* BL21 (DE3) to express the encoded proteins.

DNA sequencing was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Toronto, Ont.). The sequences were analyzed with PC/GENE software (IntelliGenetics Inc., Mountain View, Calif.). Homology searches in GenBank were carried out by using the BLAST program of National Centre for Biotechnology Information (NCBI), Bethesda, Md.

Purification of the recombinant enzymes

Each clone was expressed in *E. coli* BL21 (DE3) at 25°C. The procedure was as follows: single colonies were transferred into 40 mL of LB medium supplemented with 30 μg mL^{-1} of kanamycin and grown overnight at 37°C. Four litres of LB medium containing 30 μg mL^{-1} of kanamycin was inoculated with the 40 mL of overnight culture and grown for 24 h at 25°C with vigorous shaking. Isopropyl-β-D-thiogalactopyranoside was not added.

Cultures were centrifuged (9000 × *g*, 4°C, 10 min), and the cell pellets were washed in water at 4°C and resuspended in 40 mL of lysis buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, and 10 mM imidazole) containing phenylmethylsulfonyl fluoride and lysozyme at final concentrations of 0.5 mM and 1.0 mg mL^{-1}, respectively. The suspension was passed through the French press, centrifuged at 15 000 × *g* at 4°C for 20 min, and cell-free extract was collected. The cell-free extract (40 mL) from each batch (1 L of culture) of disrupted cells was incubated with 3 mL of Ni^{+}-nitrilotriacetic acid (Ni^{+}-NTA) resin (Qiagen Inc., Mississauga, Ont.) at 4°C for 1 h with gentle rotation. The resin was loaded into a chromatographic column and washed with 40 mL wash buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, and 20 mM imidazole). The His-tagged protein was then eluted using elution buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, and 250 mM imidazole). The solution containing the eluted protein was then exchanged with 50 mM sodium phosphate buffer, pH 6.5 by concentration of the solution, by ultrafiltration through a PM-10 membrane (Amicon Canada) and dilution in the phosphate buffer. The concentrated protein was loaded on either a G-75 or a G-150 Sephadex column pre-equilibrated with 50 mM sodium phosphate, pH 6.5, containing 100 mM NaCl and eluted with the same buffer. The fractions containing the purified enzyme were filtered through a PM-10 membrane to concentrate the protein. Sodium azide at a final concentration of 0.02% (w/v) was included, and the preparation was stored at 4°C until further use.

Fig. 1. The location of *xyn10D*, *xyn10E*, and *xyn10B* on two plasmids, pBX1 and pXC30.2, and the partial restriction map (A) and the structure of the corresponding proteins encoded by *xyn10D*, *xyn10E*, *xyn10B* and the derivative genes (B). Abbreviations: B, *Bgl*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; GH 10, glycosyl hydrolase family 10; CBM VI, carbohydrate binding module family 6.

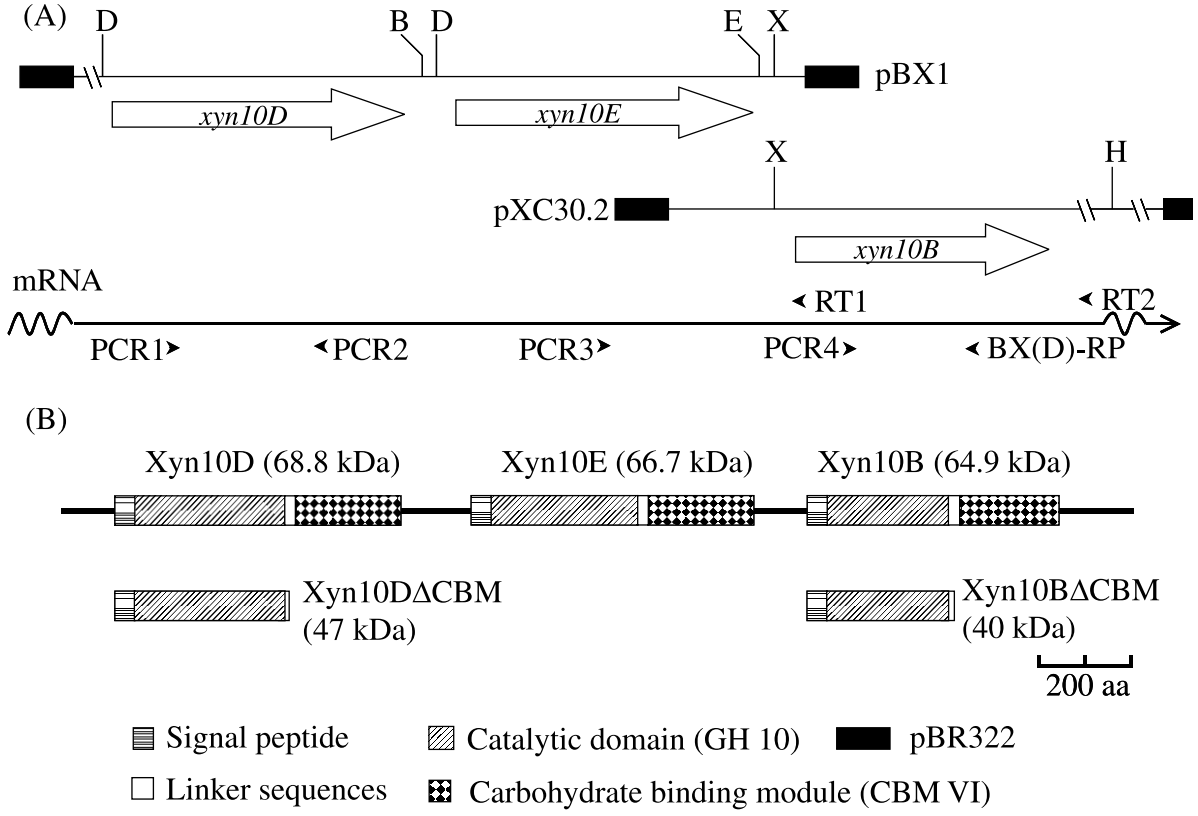

Table 1. Oligonucleotide primers used for cloning *Fibrobacter succinogenes* S85 derivative genes *xyn10D*, *xyn10E*, *xyn10B*, the truncated genes (*xyn10DΔCBM* and *xyn10DΔCBM*, and RT-PCR.

No.	Amplified DNA	Primer set	Sequence (5′→3′)
1	*xyn10D*	DX-F	ACGAGGATTAGG**CATATG**AG
		DX-R	TTAT**CTCGAG**GCGCGCAATGCGAACTG
2	*xyn10DΔCBM*	DX-F	ACGAGGATTAGG**CATATG**AG
		DX(D)-R	TATCTT**CTCGAG**GTAGTCACTGTCGCC
3	*xyn10E*	EX-F	TAACTAGA**CATATG**AAATCAAAATATTTGA
		EX-R	TATCTA**CTCGAG**GCGAGAGATACGCAC
4	*xyn10B*	BX-F	TATAGTAA**CATATG**AAATCAAATTTCATTA
		BX-R	TATCTT**CTCGAG**CACGGACCGACTTG
5	*xyn10BΔCBM*	BX-F	TATAGTAA**CATATG**AAATCAAATTTCATTA
		BX(D)-R	TATCTT**CTCGAG**AGACGCTTGAACGGCTTG
6	*xyn10D* fragment	PCR1	GTGGTGAACGAAGCTATTTG
		PCR2	CATCGAGAGAGAGTTGGAAG
7	*xyn10E* fragment	PCR3	GGCGAAGCTCTCGCTGTTCCG
		RT1	GGAGTATGGATTAGGATTCC
8	*xyn10B* fragment	PCR4	CAGTACCCGAACTGGCTCAATG
		BX(D)-R	TATCTT**CTCGAG**AGACGCTTGAACGGCTTG
9	cDNA1	RT1	GGAGTATGGATTAGGATTCC
10	cDNA2	RT2	CCTATTGGGGAGTTATGG

Note: Bold sequences are recognition sites of restriction enzymes (*Nde*I and *Xho*I).

Fig. 2. Alignment of CDs of glycosyl hydrolase family 10. FS, *Fibrobacter succinogenes* S85 (AF180368); XYLB_PSEFL, *Pseudomonas fluorescens* subsp. *cellulosa* (X54523); Xyn10A_CELFI, *Cellulomonas fimi* (M15824); Xyn10A_STRLI, *Streptomyces lividans* (M64551); XynA_BACOV, *Bacteroides ovatus* (U04957); XynA_PRERU, *Prevotella ruminicola* (Z49241). Bold letters and boxes represent the conserved regions and catalytic sites by similarity, respectively.

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Xyn10E_FS       ------GLSTDETKKAITAWFDAVKEHYP-DLEMIDVVNEAIRTGNNSYHSPYGK----- 156
Xyn10B_FS       ------GLSTDETKKAITAWFDAVAAHYP-DLEMIDVVNEAIKSGG-SYHSGYGR----- 155
Xyn10D_FS       KKK-NPGITVELTKKYITEWFDAVAAKFP-DLEYIDVVNEAIWAGN-NYHSGYGKPAAGA 173
XYLB_PSEFL      ------NLSGPEVAVEIEQWIRDYCARYP-DTAMIDVVNEAVPGHQ---PAGYAQ----- 443
Xyn10A_CELFI    N------LNGSAFESAMVNHVTKVADHFEGKVASWDVVNEAFADGDGPPQDSAFQ----- 183
Xyn10A_STRLI    S------LSGSALRQAMIDHINGVMAHYKGKIVQWDVVNEAFADGSSGARRDSNL----- 184
XynA_BACOV      VDKDGNNVSPEVLKKRMKDHITTIVKRYKGRIKGWDVVNEAIED-NGAYRKTKFY----- 174
XynA_PRERU      TDKEGKEVTREVLIDRMYHHITNVVKRYKGKIKGWDVVNEAILD-NGEYRQSPYY----- 170
                  .. .  :.       :   .     ::       ******.

Xyn10E_FS       N---KDQGIQLIQTIKKNGAPVDAYGLQAHDMMSQGGGQGGTGGGGVCLNINTLKSVLKE 258
Xyn10B_FS       Q---INEGIDLVNKLVKQGAPVDAYGQQAHDLTDMNANDFKS-------ALNKIQNSVKN 249
Xyn10D_FS       Q---INEGIELIQTIVKNGAPVDAYGQQAHDCKGMSK--------------NDFESKMTR 276
XYLB_PSEFL      Q---HN---EFIALAKAQGNYIDAVGLQAHELKGMTA--------------AQVKTAIDN 517
Xyn10A_CELFI    N-AKSNSLYDLVKDFKARGVPLDCVGFQSHLIVG-QVPGD--------------FRQNLQ 261
Xyn10A_STRLI    TWAKTQAMYNMVRDFKQRGVPIDCVGFQSHFNSGSPYNSN--------------FRTTLQ 264
XynA_BACOV      G--RREAVVKMVNDLKKRGIRIDAIGMQGHIGMDYPKISE--------------FEKSML 252
XynA_PRERU      A--KRNAVVKLVKELKAAGCRIDAVGMQSHNGFNYPNLED--------------YENSIK 248
                     :   .::      *  :*. * *.*   .

Xyn10E_FS       IWDKTQTPMFISEYDIATTDDN------IQKQCYSEQISHFMENEHIAGITIWGYIYGRT 312
Xyn10B_FS       AKGE-PMPLFITEYDIGTDNDN------QQKQRYSEQIPAFWESPQVAGITLWGYIYGAT 302
Xyn10D_FS       IHNETGLPLLVSEYDIGEADDT------KQKNDYANQIPFMWETPWVAGITIWGYINGST 330
XYLB_PSEFL      IWNQVGKPIYISEYDIGDTNDQ------VQLQNFQAHFPVFYNHPHVHGITSG------- 564
Xyn10A_CELFI    RFADLGVDVRITELDIRMRTPSDATKLATQAADYKKVVQACMQVTRCQGVTVWGITDKYS 321
Xyn10A_STRLI    NFAALGVDVAITELDIQG----------APASTYANVTNDCLAVSRCLGITVWGVRDSDS 314
XynA_BACOV      AFAGTGVKIMITELDLTVIP--------SPNPNVGAEVSASFEYKKEMNPYPDGLPEEVS 304
XynA_PRERU      AFIAAGVDVQFTELDVNMLP--------NPKSFGGAEISQNYKYNKELNPYVNGLTKAAQ 300
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Enzyme assays

Xylanase activity was determined by incubation of enzyme with presence of 1% (w/v) oat spelt xylan (Sigma–Aldrich Canada, Oakville, Ont.) in a final volume of 0.1 mL with 50 mM sodium phosphate buffer, pH 6.5, at 37°C for 15 min. Reducing sugars released from the substrate were measured with the *p*-hydroxybenzoic acid hydrazide (Sigma–Aldrich Canada) reagent, as described by Lever (1972). Oat spelt xylan (Sigma–Aldrich Canada), beechwood xylan (Sigma–Aldrich Canada), birchwood xylan (Sigma–Aldrich Canada), and arabinoxylan (Megazyme, Wicklow, Ireland) were used as other sources of xylan substrate. One unit of xylanase activity was defined as the amount of enzyme releasing 1 μmol of xylose equivalent per minute from xylan. CMCase, β-xylosidase, β-cellobiosidase, and β-glucosidase activities were assayed at 37°C with carboxymethyl cellulose (1%, CMC; Sigma–Aldrich Canada), *p*-nitrophenyl-β-D-xyloside (5 mM, Sigma–Aldrich Canada), *p*-nitrophenyl-α-D-cellobioside (5 mM, Sigma–Aldrich Canada), and *p*-nitrophenyl-α-D-glucoside (5 mM, Sigma–Aldrich Canada), respectively.

Protein was determined by bicinchoninic acid reagent, as described by Smith et al. (1985).

Binding analysis

Ni^{+}-NTA column-purified recombinant proteins Xyn10D (1.03 U) and Xyn10DΔCBM (1.38 U) were incubated with either 50 mg Avicel PH-105 or beechwood xylan by inversion for 1 h at 22°C. Prior to the binding assay, the substrates were washed three times in PBS, pH 6.5, and resuspended with added enzyme in a total volume of 1 mL using the same buffer. After centrifugation (735 × *g*, 5 min, 22°C) to remove Avicel and xylan, xylanase activity remaining in the supernatant was measured. The amounts of enzyme bound to each substrate were estimated from the difference between the amounts of enzyme in the supernatant before and after incubation. The enzymes bound to the substrates were eluted in SDS–PAGE sample buffer at 100°C for 5 min and analyzed in SDS–PAGE (Laemmli 1970).

Analysis of hydrolysis products

Oat spelt xylan (1%), arabinoxylan (1%), and 20 μmol of xylo-oligomers (xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose, obtained from Megazyme) were hydrolyzed by 0.2 U of three purified enzymes (Xyn10D, Xyn10E, and Xyn10BΔCBM) in a final volume of 1 mL using 50 mM sodium phosphate buffer, pH 6.5, for 10 h at 37°C. Controls included active enzymes replaced (*i*) by water and (*ii*) by enzyme inactivated by heating at 100°C for 15 min. The enzymatic hydrolysis products of xylans and xylo-oligomers were separated by high pressure liquid chromatography (HPLC, Waters Model 625, Canada Waters Ltd., Mississauga, Ont.) on a Dionex CarboPac PA1 ion-exchange column (4×250 mm) (Dionex, Sunneyvale, Calif.) and the products detected using a Waters 464 pulsed electrochemical detector (Canada Water Ltd.), as described by Zhu et al. (1994).

Fig. 3. Alignment of family 6 CBMs. FS, *Fibrobacter succinogenes* S85 (AF180368); CelB_CELM, *Cellvibrio mixtus* (AF003697); XynA_CLOSR, *Clostridium stercorarium* F-9 (D13325). Highly conserved residues are in boldface.

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Xyn10E_FS       IEVEDFDKPGQGKNEDGTSNESYGD-DSENHGDSDYRKDTG--ADLYKKATGVALGYNTT 437
Xyn10B_FS       IEVEDFDITGVG-ETDGVSNVSYSDGDPENHGDSDYRKSDASDVDIYKKATGNIVGYNTT 425
Xyn10D_FS       VEAEDFDIPGVGVNEDGTSNQSYGD-DSENHGDSDYRKDTG--VDLYKKATGVIVGYNSE 446
CelB_CELM       KPIDGGGGDGGGDGSGGTCTKANPG--DAIQAESWCQMSGVKTETTSDAGDGLNVGYIDG 394
XynA_CLOSR      RPPGATPKGTITQWMAGTYEIYETTRVNQPSIDGTATFQQYWSVRTSKRTSGTISVTEHF 194
                              *.              :.    .        .   *

Xyn10E_FS       GDWYEYTINIAEAGDYTAIASVATEG-TGAFTLSLDGKSLAEFEVTGTS------YDDFS 490
Xyn10B_FS       GDWLEYSVDIAEAGDYTATASVAADG-SGSFKLSIDGKSVGEFDVTGSS------WDNFI 478
Xyn10D_FS       GDWLEWTVNVKEAGDYTMFAAVAAAGSTSSFQLSLDGKALTEKITVPAAKEGEENYDDYN 506
CelB_CELM       GDWMTYSVNIPTTGTYKVSYRVAAQAGGGQLQLEKAGGSPVYSNINVPATGG---WQNWQ 451
XynA_CLOSR      KQWER--MGMRMGKMYEVALTVEGYQSSGYANVYKNEIRIGANPTPAPSQSP-IRRDAFS 251
                 :*   :::.:     *     *    . .   :              :   .   : :

Xyn10E_FS       --DVKKKVTLPAGKHVLRLDVTQQYFDIDYINFVKGEVADN-------PGGNDNPGGGDE 541
Xyn10B_FS       --DVKKKVTLPAGKHTLRMDVTAQYFDIDYINFTKGDKE---------------------- 515
Xyn10D_FS       --KVKANVTLPAGKHVLRMDVTGAWFDVDYFTFVKGKDATD-------P---------EP 548
CelB_CELM       --TISHNVVLPAGEQLIALSAITGGFNINWLKVESTGGDNSGTVIATIQAEDHSQQSGTQ 509
XynA_CLOSR      -IIEAEEYNSTNSSTLQVIGTPNNGRGIGYIENGNTVTYSN------------------- 291
                      :     ..    :       .:.::   .     .
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Table 2. Substrate specificities of Xyn10D, Xyn10E, and Xyn10BΔCBM.

	% of maximum activity		
Substrate	Xyn10D	Xyn10E	Xyn10BΔCBM
Oat spelt xylan	65.0	100.0*	88.1
Arabinoxylan	100.0*	70.4	96.1
Birchwood xylan	16.0	47.1	100.0*
Beechwood xylan	13.0	8.3	51.1
Starch	1.0	0.2	1.5
Arabinan	0.3	0.2	1.0
CMC	1.5	3.1	1.7
p-Nitrophenyl-α-D-glucoside	0.0	0.0	0.0
p-Nitrophenyl-β-D-xyloside	0.0	0.0	0.0
p-Nitrophenyl-α-D-cellobioside	0.0	0.0	0.0

*One hundred percent activity of Xyn10D, Xyn10E, and Xyn10BΔCBM was 101.15, 9.45, and 25.54 U mg^{-1}, respectively.

Transcript analysis

Total cellular RNA was isolated from cells grown with either glucose for 24 h or cellulose for 48 h at 37°C, using TriPure isolation reagent according to the manufacturer's protocol (Boehringer Mannheim Canada, Laval, Que.). The RNA was treated with RNase-free DNase I (Invitrogen Canada Inc., Burlington, Ont.). For reverse transcription (RT)-PCR, 2 μg of RNA was used for first-strand cDNA synthesis with Superscript II RT according to the manufacturer's protocol (Invitrogen Canada Inc.). RT reactions were done as described by Gupta (1999). PCR was performed using Plati-*Taq* polymerase (Invitrogen Canada Inc.), and amplification products were analyzed by 0.8% agarose gel electrophoresis and stained with ethidium bromide. The RT and PCR primers are described in Table 1 and Fig. 1.

Purification of anti-Xyn10B antibodies, SDS–PAGE, and Western immunoblotting

Preparation of monospecific antibody and SDS–PAGE were performed as previously described (McGavin et al. 1989). Western immunoblotting was done using a 1:20 dilution of monospecific anti-Xyn10B antibodies as the primary antibodies. Goat anti-rabbit IgG alkaline phosphatase-conjugated antibodies (Sigma–Aldrich Canada) were used as the secondary antibody. Detection was performed as described by McGavin et al. (1989).

Nucleotide sequence accession No.

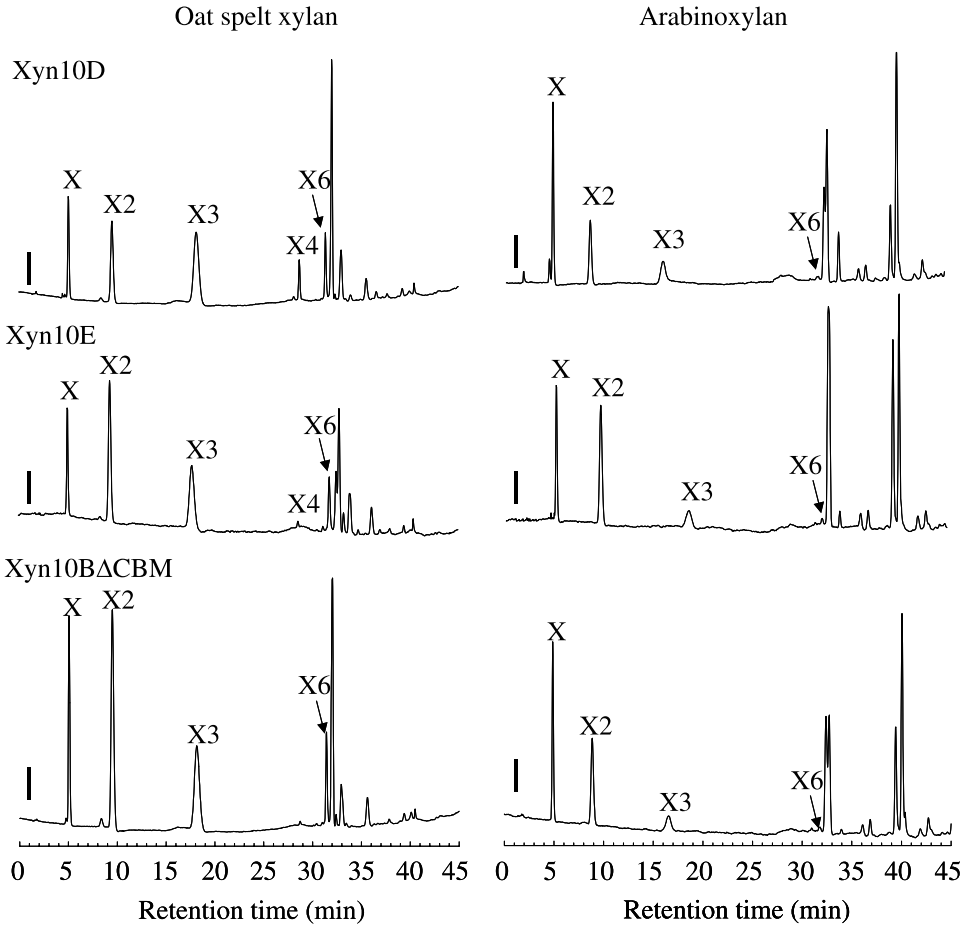
The nucleotide sequences reported in this paper were previously submitted to GenBank nucleotide sequence databases under the acc. No. AF180368.

Results

Subcloning and nucleotide sequence analysis

The nucleotide sequence analysis of the DNA insert in BX1 and XC30.2 plasmids revealed three adjacent genes, *xyn10D* and *xyn10E*, and *xyn10B*, respectively. The location and transcription direction of these genes are shown in Fig. 1.

Fig. 4. HPLC analysis of products of hydrolysis by Xyn10D (upper), Xyn10E (middle), and Xyn10BΔCBM (lower). Oat spelt xylan (1% (w/v), left column) and arabinoxylan (1% (w/v), right column) were used as a substrate. Xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose were designated as X, X2, X3, X4, X5, and X6, respectively. The vertical solid bars to the left of each graph represent a pulsed-amperometric response of 2×10^5 V.



The upstream regions of *xyn10D*, *xyn10E*, and *xyn10B* genes were AT-rich (70%), and a putative ribosome binding site of sequence GAGGA was found eight or nine nucleotides upstream from each initiation codon. The –10 and –35 sequences were highly similar to each other, but no hairpin structures were found in the intergenic regions.

The open reading frames (ORFs) of *xyn10D* and *xyn10E* were amplified by PCR from a 2.5-kb *Dra*I fragment and a 2.3-kb *Bgl*I–*Xba*I fragment, respectively, of plasmid pBX1. Likewise, *xyn10B* was amplified by PCR from a 4.0-kb *Xba*I–*Hin*dIII fragment isolated from plasmid pXC30.2. All three genes were subcloned into the expression vector pET-30a and sequenced in both orientations.

Analysis of the nucleotide sequences of the three adjacent genes, *xyn10D*, *xyn10E*, and *xyn10B*, revealed three ORFs of 1869, 1866, and 1767 bp, encoding polypeptides of 623, 622, and 589 amino acids with calculated molecular masses of 68.8, 66.7, and 64.9 kDa, respectively. The first 22, 28, and 23 amino acids, respectively, of each protein corresponded to a signal peptide. In addition, all had the same structural organization that consisted of an N-terminal catalytic domain (CD) and a C-terminal CBM connected by proline-rich linker sequences (Fig. 1).

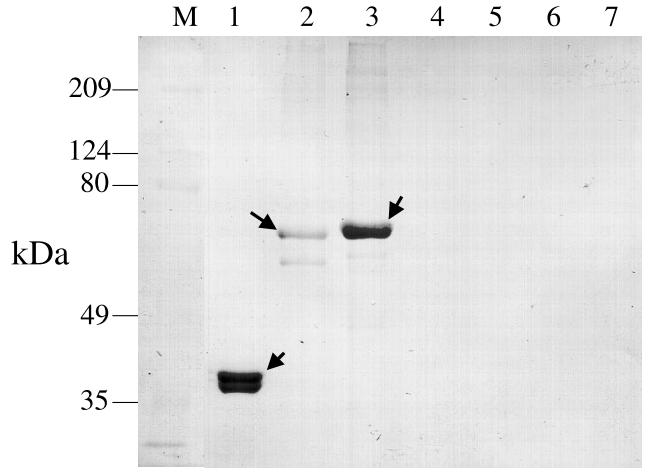
Truncated versions of genes *xyn10D* and *xyn10B*, *xyn10DΔCBM*, and *xyn10BΔCBM*, lacking the CBM, were amplified by PCR and cloned into pET-30a. The deduced Xyn10DΔCBM and Xyn10BΔCBM comprised 425 and 359 amino acids with calculated molecular masses of 47 and 40 kDa, respectively.

Comparisons of the sequences

The deduced N-terminal CDs of Xyn10D (Asp_{23} to Phe_{368}), Xyn10E (Asp_{29} to Leu_{359}), and Xyn10B (Gly_{24} to Phe_{342}) each showed a high degree of homology, but the identity scores of the CDs of Xyn10D, Xyn10E, and Xyn10B differed with 49% identity between Xyn10D and Xyn10E, 55% identity between Xyn10D and Xyn10B, and 66% identity between Xyn10E and Xyn10B. The catalytic domains of all three enzymes displayed sequence homology with those of other xylanases belonging to glycosyl hydrolase family 10 (Fig. 2). Xyn10B of *Pseudomonas fluorescens* subsp. *cellulosa* (Kellett et al. 1990) showed the highest identity to Xyn10D, Xyn10E, and Xyn10B with 37, 38, and 42% identity, respectively (Fig. 2). In addition, the amino acid alignment of Xyn10D, Xyn10E, and Xyn10B with family 10 xylanases revealed a high degree of homology in highly conserved regions including the catalytic sites (boldface, Fig. 2).

The amino acid alignment of the C-terminal CBMs of the three enzymes showed a high degree of homology and dis-

Fig. 5. Immunoblot of purified Xyn10D, Xyn10E, and Xyn10BΔCBM proteins and of the extracellular culture fluid and cell extract from cells of *Fibrobacter succinogenes* grown on glucose and cellulose. Lane M, prestained molecular mass markers (Bio-Rad Laboratories, Mississauga, Ont.); lanes 1, 2, and 3, 3.2 μg of purified Xyn10BΔCBM, Xyn10E, and Xyn10D proteins, respectively; lane 4, 40 μg of extracellular protein from cells grown on glucose; lane 5, 40 μg of cell extract protein from cells grown on glucose; lane 6, 40 μg of extracellular protein from cells grown on cellulose; and lane 7, 40 μg of cell extract protein from cells grown on cellulose.



played similar identity scores (60%, Fig. 3). Alignment of the C-terminal CBMs of the three enzymes with other microorganisms' CBMs belonging to family 6 showed highly conserved identical residues (Trp, Tyr, Gly) (Fig. 3).

Purification of recombinant xylanases

The gene products of *xyn10D*, *xyn10E*, and *xyn10BΔCBM* were purified from the cytoplasmic fraction of *E. coli* BL21 (DE3) by selective binding to Ni^+-NTA, followed by either G-75 or G-150 Sephadex column chromatography. The purified enzymes analyzed by SDS–PAGE had molecular masses of about 68, 66, and 40 kDa (data not shown). The N-terminal amino acid sequence of purified Xyn10D was identified as Ala-Leu-Ala-Asp-Gly-Gly-Ala-Lys-Phe-Leu-Gln-Asn, indicating that the signal peptide cleavage site of Xyn10D is Ala_{22}–Ala_{23}. The encoded Xyn10E and Xyn10B enzymes had typical putative signal peptide cleavage sites, Ala_{28}–Asp_{29} and Ala_{23}–Gly_{24}, respectively. The signal peptides of the three enzymes presumably mediate secretion of the proteins to the periplasmic region of *F. succinogenes*; however, as shown in *E. coli*, xylanase in the periplasmic region accounted for less than 8% of the total xylanase, suggesting that the efficiency of the signal peptides was very low in *E. coli*.

In the case of Xyn10B, recovery of enzyme activity from whole cell extracts was less than 1% due to apparent aggregation of the protein, even though the eluates from the Ni^+-NTA column contained a protein with a molecular mass estimated by SDS–PAGE to be similar to that of the deduced amino acids. From several attempts to purify the intact enzyme, our experience has been that it is stable in very dilute solution, but as soon as it is concentrated it loses activity, presumably because of aggregation. The derivative *xyn10BΔCBM* gene, which lacked a CBM, was therefore cloned, expressed, and purified by affinity chromatography and gel filtration chromatography using Sephadex G-75. It was fully stable and retained its activity during purification and concentration.

Table 3. Binding of Xyn10D and Xyn10DΔCBM to insoluble substrates.

	Activity bound			
	Xyn10D		Xyn10DΔCBM	
Substrate	Units	%	Units	%
Avicel	0.51	47.3	0.04	2.8
Beechwood xylan	0.35	32.5	0.02	1.6
Control*	0.05	5.1	0.00	0.0

Note: The following units of enzyme were included in the binding assay: Xyn10D, 1.08 U; Xyn10DΔCBM, 1.38 U. Values are the means of triplicate experiments and are expressed in total units by subtracting residual xylanase activity present in the supernatant after binding to the substrates from total units of xylanase activity before binding.

*Enzyme incubated without substrates and analyzed before and after incubation and centrifugation.

General characterization of Xyn10D, Xyn10E, and Xyn10BΔCBM proteins

The substrate specificity of the Xyn10D, Xyn10E, and Xyn10BΔCBM enzymes is shown in Table 2. The Xyn10D, Xyn10E, and Xyn10BΔCBM enzymes exhibited the highest specific activity toward arabinoxylan, oat spelt xylan, and birchwood xylan, respectively; practically no activity on starch, arabinan, and CMC; and no activity against *p*-nitrophenyl-α-D-glucoside, *p*-nitrophenyl-α-D-cellobioside, or *p*-nitrophenyl-β-D-xyloside.

The binding ability of Xyn10D and Xyn10DΔCBM was determined by incubation of the different recombinant enzymes with Avicel and insoluble beechwood xylan and measuring the enzyme activity remaining in solution after sedimenting the substrate by centrifugation ($735 \times g$, 5 min, 22°C) (Table 3). Xyn10D bound to Avicel cellulose and xylan with efficiencies of 47.3 and 32.5%, respectively, while the truncated derivative of Xyn10D (i.e. Xyn10DΔCBM) did not show detectable binding activity with either Avicel cellulose or beechwood xylan (Table 3), nor was Xyn10BΔCBM bound to the substrates detected on SDS–PAGE (not shown).

The K_m and V_{max} of the recombinant enzymes are shown in Table 4. It is notable that the K_m and V_{max} values of the three enzymes are in the same range. Whether the values for XynBΔCBM reflect that of the intact enzyme is unknown. The pH optima for the Xyn10D and Xyn10E were similar, and they retained over 70% of their maximum activity between pH 5.5 and 7.0. Maximum activity of both enzymes was observed at 40°C, and 70% of their activities were retained between 30 and 50°C.

HPLC analysis showed that Xyn10D, Xyn10E, and Xyn10BΔCBM digested both oat spelt xylan and arabinoxylan, releasing xylose and xylobiose as the main end products after 10 h of hydrolysis (Fig. 4). Since no arabinose was detected by HPLC using arabinoxylan as a substrate, these xylanases apparently lack arabinofuranosidase activity. By using xylo-oligosaccharide substrates, we analyzed the degree of hydrolysis and product distribution by Xyn-

Table 4. Kinetic parameters, pH optimum, maximum temperature, and pI of purified Xyn10D, Xyn10E, and Xyn10BΔCBM.

Enzyme	pH of maximum activity	Temperature (°C) of maximum activity	pI*	K_m (%)	V_{max} (U mg^{-1})
Xyn10D	6.5	40	5.12	0.803	23.51
Xyn10E	6.5	40	5.25	0.798	35.69
Xyn10BΔCBM	—	—	5.34†	0.500	34.81

Note: Oat spelt xylan was used as substrate and incubations were for 15 min at 37°C. Assay results are the mean values of triplicate determinations.

*pI values were calculated by computational methods (http://www.mbshortcuts.com).

† pI value of intact Xyn10B protein.

Table 5. Distribution of products from hydrolysis of xylo-oligosaccharides by Xyn10BΔCBM.

	Product distribution (%, peak area)					
Substrate	X	X2	X3	X4	X5	X6
X2	0.0	100.0	—	—	—	—
X3	48.5	35.1	16.4	—	—	—
X4	40.6	40.2	19.2	0.0	—	—
X5	28.7	42.8	28.5	0.0	0.0	—
X6	24.1	48.3	27.6	0.0	0.0	0.0

10BΔCBM by HPLC (Table 5). Xyn10BΔCBM hydrolyzed all of the xylo-oligosaccharides except xylobiose, and the main end products were xylose and xylobiose. Both of the recombinant Xyn10D and Xyn10E lacked activity against xylobiose (data not shown).

Reverse transcription - polymerase chain reaction (RT-PCR) transcript analysis

RT-PCR was performed to analyze the transcripts of the adjacent xylanase genes in *F. succinogenes* S85. To compare the expression level of the xylanase genes under different growth conditions, we isolated total RNA from S85 cells grown on Avicel cellulose and glucose, and the RNA was used as the template for RT reactions. Because the contiguous xylanase genes might be expected to synthesize single or multiple transcripts, RT oligonucleotide primers RT1 and RT2 (primers 9 and 10, Table 1), located at the 3′ ends of the *xyn10E* and *xyn10B* genes, respectively, were used for cDNA synthesis. The products of each RT reaction were used as templates for amplification of unique segments of individual genes by PCR using primer sets 6, 7, and 8 (Table 1).

With RNA isolated from cellulose-grown cells and cDNA synthesized using the RT2 primer, *xyn10D*, *xyn10E*, and *xyn10B* genes were detected by PCR using primers specific for each gene, indicating that all three xylanase genes were synthesized as one transcript. The *xyn10B* fragment was not amplified by PCR from the template cDNA synthesized by the RT1 primer (data not shown). With equivalent amounts of RNA isolated from cells grown on glucose and cDNA synthesized from the RT2 primer, the amounts of PCR product from each of the three genes were less than that from cells grown on cellulose. As a control, RT reactions lacking reverse transcriptase were used and no PCR product was detected. Contamination of genomic DNA was eliminated by treating RNA with RNase-free DNase I before performing RT reactions to ensure that no PCR amplification products arose from the contaminated DNA (results not shown).

Expression of *xyn10D*, *xyn10E*, and *xyn10B* genes in *F. succinogenes* S85

Upon performing Western immunoblotting with monospecific anti-Xyn10B antibody, we showed that the purified recombinant Xyn10D, Xyn10E, and Xyn10BΔCBM had major immunoreactive bands of 68, 66, and 40 kDa, respectively, while no band was detected in extracellular culture fluid or whole cell extract fractions from *F. succinogenes* S85 cells grown on either Avicel cellulose or glucose (Fig. 5). It has been reported that antibodies prepared against His-tagged proteins have in some cases exhibited a primary specificity for the His-tag. However, the XynB antibodies did not react with His-tagged, purified UDP-galacturonic acid epimerase from *Klebsiella pneumoniae*, suggesting that the Xyn10B antibodies were specific for the protein rather than for the tag. The proteins of less than 68, 66, and 40 kDa appeared to be degraded proteins of Xyn10D, Xyn10E, and Xyn10BΔCBM, respectively.

Discussion

A previous study of *F. succinogenes* S85 showed that the bacterium produces high levels of xylanase activity whether grown on cellulose or ball-milled barley straw (Smith and Forsberg 1991). The xylanases exist as both acidic and basic proteins (Matte and Forsberg 1992). Matte and Forsberg (1992) purified from this mixture two basic proteins, endoxylanases 1 and 2, that in addition to xylanase activity, possessed arabinofuranosidase and endoglucanase activities, respectively. From the studies by Sipat et al. (1987) and Malburg et al. (1993), it was concluded that at least five xylanase genes were located on the *F. succinogenes* S85 chromosome. In this work, we have located *xyn10D* and *xyn10E* from clone BX1 and *xyn10B* from clone XC30. They are different from endoxylanases 1 and 2 as demonstrated by the absence of arabinofuranosidase and endoglucanase activities.

Xyn10D, Xyn10E, and Xyn10B proteins each consist of an N-terminal CD belonging to glycosyl hydrolase family 10 and a C-terminal family 6 CBM linked by proline-rich linker sequences. The catalytic domains of these enzymes were homologous to XYLB of *P. fluorescens* subsp. *cellulosa* (Kellett et al. 1990), Xyn10A of *Cellulomonas fimi* (O'Neill et al. 1986), XynA of *Bacteroides ovatus* (Whitehead 1995), Xyn10A of *Streptomyces lividans* (Shareck et al. 1991), and XynA of *Prevotella ruminicola* (Gasparic et al. 1995), with

five regions that are highly conserved and presumably serve to form the overall structures of these enzymes (Fig. 2), suggesting that these xylanases have diverged from a common evolutionary ancestor. In addition, two glutamic acid residues (shown in Fig. 2 as boldface), which have been confirmed as the catalytic amino acids by site-directed mutagenesis and crystallography in *Cellulomonas fimi* and *S. lividans* (Derewenda et al. 1994; MacLeod et al. 1994; Tull et al. 1991), were conserved in two identical regions, and it is assumed that the highly conserved His-259, 227, and 225 of Xyn10D, Xyn10E, and Xyn10B, respectively, presumably serve to stabilize the transition state with oxocarbonium ion character (Sakka et al. 1993).

Xyn10D bound to insoluble cellulose or xylan when incubated with these polymers in solution and consequently exhibited characteristics similar to the CBM complex of the XynA of *Clostridium stercorarium* F-9 that was shown to bind to cellulose and to xylan (Sakka et al. 1993). The binding of the *Fibrobacter* Xyn10D due to the CBM was confirmed by the absence of binding of the truncated enzyme Xyn10DΔCBM. Amino acid sequence alignment of the C-terminal region of Xyn10D, Xyn10E, and Xyn10B with the CBM of the CelB from *Cellvibrio mixtus* (Fontes et al. 1998) and XynA from *Clostridium stercorarium* (Sakka et al. 1993) showed highly conserved tryptophan and tyrosine residues, corresponding to Trp_{449} and Tyr_{462} of Xyn10D, Trp_{440} and Tyr_{454} of Xyn10E, and Trp_{428} and Try_{441} of Xyn10B. These highly conserved aromatic amino acids presumably play a pivotal role in the interaction of the three xylanases with the substrates, as has been shown for tryptophan and tyrosine residues of CBM2a and CBM10 in *P. fluorescens* subsp. *cellulosa* (Ponyi et al. 2000; Nagy et al. 1998).

The general characteristics of Xyn10D, Xyn10E, and Xyn10B, including temperature and pH for maximum activity and pI, are very similar to each other, and although the maximum activities against several xylans are different from each other, the enzymes have narrow substrate specificities. Previous study showed that the extracellular xylanases of *F. succinogenes* S85 displayed a broad range of pIs from 4.0 to 8.7 (Malburg, Jr. et al. 1993) and the computed pIs of Xyn10D, Xyn10E, and Xyn10B are similar to the somewhat acidic xylanase profile of the S85 enzymes. This contrasts with pI of XynC, which is 8.0 (Paradis et al. 1993). Xyn10D, Xyn10E, and Xyn10B of *F. succinogenes* S85 hydrolyze xylan substrates, releasing xylose and xylobiose as the major products (Fig. 4). HPLC analysis with purified xylo-oligosaccharides showed that all the xylo-oligosaccharides (xylotriose, xylotetraose, xylopentaose, and xylohexaose) were hydrolyzed except for xylobiose (data not shown), suggesting that all three enzymes hydrolyze xylan in a manner typical of many *endo*-β-1,4-xylanases.

To study expression of the three xylanases, we isolated RNA from cells grown with either cellulose or glucose as the carbon source, and reverse transcription (RT) was performed with a single 3′ flanking primer, RT2, whereas the subsequent PCR was performed with specific primers for *xyn10D*, *xyn10E*, and *xyn10B* (Table 1 and data not shown). The presence of fragments of all three genes on the one reverse transcriptase product showed that the three genes are co-transcribed as a single transcript. Furthermore, the amount of PCR products from cells grown on cellulose was more than that from cells grown on glucose for all three genes, suggesting that *xyn10D*, *xyn10E*, and *xyn10B* genes can be induced by growth on cellulose. A modest transcriptional gradient in the three genes was observed with the *xyn10D* product exceeding that of *xyn10E* and *xyn10B*, which might be a result of differences in the primer binding efficiency.

Extracellular culture fluid and whole cell extracts of *F. succinogenes* S85 grown with either cellulose or glucose as a carbon source were probed with affinity-purified anti-Xyn10B antibody. Although the results of Western immunoblot analysis showed that Xyn10D, Xyn10E, and Xyn10B shared antigenic epitopes, there was no detectable immunoreactive protein band in any of the fractions from the *F. succinogenes* cultures. Despite the enrichment of the xylanases by binding to Avicel cellulose and eluting bound xylanases from the substrate, no immunoreactive protein band was shown, suggesting that expression levels of the three genes were very low. It is possible that the presence of xylan in the culture medium may lead to greater induction of the cluster of genes; however, previous results by Smith and Forsberg (1991) showed that the bacterium produced nearly as much xylanase when grown on cellulose as on barley straw, an interesting substrate that would be expected to contain a variety of natural inducers.

Clustered genes encoding fibrolytic enzymes have been reported previously, for example, endoglucanases D and E of *F. succinogenes* (Malburg, Jr. et al. 1996) and two endoxylanases of *Clostridium thermocellum* (Hayashi et al. 1999) with high similarity (70 and 96.9% identities, respectively), suggesting that the adjacent genes resulted from an ancient gene duplication occurrence and subsequent evolution based on criteria similar to those described by Jordan et al. (2002) and Kondrashov et al. (2002). *Fibrobacter succinogenes* gene cluster of *xyn10D*, *xyn10E*, and *xyn10B* are comparatively homologous with the proteins exhibiting identities of nearly 60%. Based on the relatively high sequence identity and related overall organization of the catalytic domains and cellulose-binding modules, these three genes also appear to have resulted from gene duplication. Assuming this was an ancient duplication, other evolutionary events probably have occurred, including limited domain shuffling, subsequent gene modifications, and gene divergence (Kondrashov et al. 2002). It will be fascinating to see if these three genes occur in other species of *Fibrobacter* in the same order.

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