Applied and Environmental Microbiology, Oct. 2003, p. 5957–5967 Vol. 69, No. 10
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.10.5957–5967.2003
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Cloning, Characterization, and Functional Expression of the *Klebsiella oxytoca* Xylodextrin Utilization Operon (*xynTB*) in *Escherichia coli*†

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Received 4 June 2003/Accepted 1 August 2003

***Escherichia coli* is being developed as a biocatalyst for bulk chemical production from inexpensive carbohydrates derived from lignocellulose. Potential substrates include the soluble xylodextrins (xyloside, xylooligosaccharide) and xylobiose that are produced by treatments designed to expose cellulose for subsequent enzymatic hydrolysis. Adjacent genes encoding xylobiose uptake and hydrolysis were cloned from *Klebsiella oxytoca* M5A1 and are functionally expressed in ethanologenic *E. coli*. The xylosidase encoded by *xynB* contains the COG3507 domain characteristic of glycosyl hydrolase family 43. The *xynT* gene encodes a membrane protein containing the MelB domain (COG2211) found in Na^+/melibiose symporters and related proteins. These two genes form a bicistronic operon that appears to be regulated by xylose (XylR) and by catabolite repression in both *K. oxytoca* and recombinant *E. coli*. Homologs of this operon were found in *Klebsiella pneumoniae*, *Lactobacillus lactis*, *E. coli*, *Clostridium acetobutylicum*, and *Bacillus subtilis* based on sequence comparisons. Based on similarities in protein sequence, the *xynTB* genes in *K. oxytoca* appear to have originated from a gram-positive ancestor related to *L. lactis*. Functional expression of *xynB* allowed ethanologenic *E. coli* to metabolize xylodextrins (xylosides) containing up to six xylose residues without the addition of enzyme supplements. 4-O-methylglucuronic acid substitutions at the nonreducing termini of soluble xylodextrins blocked further degradation by the XynB xylosidase. The rate of xylodextrin utilization by recombinant *E. coli* was increased when a full-length *xynT* gene was included with *xynB*, consistent with *xynT* functioning as a symport. Hydrolysis rates were inversely related to xylodextrin chain length, with xylobiose as the preferred substrate. Xylodextrins were utilized more rapidly by recombinant *E. coli* than *K. oxytoca* M5A1 (the source of *xynT* and *xynB*). XynB exhibited weak arabinosidase activity, 3% that of xylosidase.**

Cellulose and hemicellulose (primarily methylglucuronoxylan) are the most abundant carbohydrate constituents of woody biomass and agricultural residues (2, 9). High cost associated with the depolymerization of these polymers into monomeric sugars is a primary obstacle preventing their use as a feedstock for chemicals and automotive fuels (31, 46). All native lignocellulosic materials must be pretreated to solubilize hemicellulose constituents and expose cellulose surfaces prior to enzymatic degradation. Although hemicellulose can be depolymerized by mineral acids, conditions required for complete hydrolysis generate toxins that complicate biological utilization (20, 29, 33, 49). Conditions which are less severe generate soluble xylodextrins (xylooligosaccharides) that must be further degraded prior to entering pentose metabolism.

Xylodextrin utilization has been demonstrated in a variety of bacteria (10, 15, 32, 40, 43, 44, 47). *Bacillus stearothermophilus* contains a gene cluster involved in the transport and metabolism of large soluble products from methylglucuronoxylan (40). To facilitate the bioconversion of xylodextrins into useful chemicals, such as ethanol, genes encoding xylosidase and xylanase have been expressed in *Saccharomyces cerevisiae* (23, 25, 26) but with limited success in xylan fermentation. Ethanologenic strains of *Escherichia coli* KO11 and *Klebsiella oxytoca* M5A1(pLOI555) expressing bacterial xylosidase and xylanase genes have been shown to metabolize xylan and soluble xylodextrins by using a complicated two-step process (6). None of these studies, however, have included heterologous genes encoding xylobiose uptake systems to facilitate metabolism.

Isoprimeverose, a xyloside dimer composed of xylose linked α1,6 to glucose, is transported by a proton symport in *Lactobacillus pentosus* (8, 16, 17). This gene has been cloned and expressed at high levels in *L. lactis* for detailed investigations of transport. Xylobiose uptake in *Streptomyces lividans* appears to utilize a different mechanism involving an ATP-dependent transport system (19). Fungi such as *Aureobasidium pullulans* transport xylobiose by an uncharacterized, energy-dependent permease (30).

Previous investigations in this and other laboratories developed ethanologenic strains of *E. coli* and *K. oxytoca* M5A1 that metabolize all of the monomeric sugar constituents in lignocellulose (37, 38, 45). Subsequent studies characterized genes from M5A1 encoding a cellobiose phosphotransferase system and phosphocellobiase (27) and added these genes to ethanologenic derivatives of *E. coli* (34). M5A1 was also found to hydrolyze chromogenic xylosides and to metabolize xylobiose (6), consistent with the presence of an efficient xylosidase activity and uptake system.

In this paper we report the cloning and characterization of the *xynTB* operon encoding a xylobiose/cation symport and a new xylosidase (glycosyl hydrolase family 43). Functional ex-

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† University of Florida Agricultural Experiment Station publication no. R-09658.

TABLE 1. Description of strains and plasmids used in this study

Strains and plasmids	Description	Reference or source
Strains		
E. coli KO11	Δ*frd*, Cm^R, carrying the *Zymomonas mobilis pdc adhB* cassette	38
E. coli TOP10F′	*lacI*^{q} *lacZ*ΔM15	Invitrogen
K. oxytoca M5A1	Wild type	37
Plasmids		
pCR2.1-TOPO	3.9 Kbp, Km^R Amp^R, pUC origin, TA cloning vector	Invitrogen
pNEB193	Amp^R cloning vector similar to pUC19	New England Biolabs
pLOI3701	pUC18 derivative with ~7-kbp DNA fragment, *xynT′ xynB*	This study
pLOI3702	pUC18 derivative with ~6.3-kbp DNA fragment, *xynT′ xynB*	This study
pLOI3703	pUC18 derivative with ~5.6-kbp DNA fragment, *xynT′ xynB*	This study
pLOI3704	pCR2.1-TOPO derivative with ~6.0-kbp DNA fragment, *xynT′ xynB*	This study
pLOI3705	pCR2.1-TOPO derivative with ~6.0-kbp DNA fragment, *xynTB* operon	This study
pLOI3706	derivative of pLOI3705 with all *Klebsiella* DNA removed between the two vector *Eco*RI sites	This study
pLOI3707	pNEB193 derivative carrying the 3.6-kbp *Ase*I-*Pst*I fragment with the complete *xynTB* coding regions (lacking regulatory sites); transcribed opposite to the *lac* promoter	This study
pLOI3708	pNEB193 derivative containing the 3.6-kbp *Ase*I-*Pst*I fragment with the complete *xynTB* coding regions (lacking regulatory sites); transcribed in the same direction as the *lac* promoter	This study
pLOI3709	Derivative of pLOI3708, Δ*xynT*, *xynB* (918-bp *Cla*I internal deletion of *xynT*)	This study

pression of these *K. oxytoca* M5A1 genes in ethanologenic *E. coli* KO11 enabled the metabolism of soluble β-1,4-linked xylodextrins containing up to six xylosyl residues.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are listed in Table 1. Cultures of *K. oxytoca* M5A1 and *E. coli* were grown at 37°C in Luria-Bertani (LB) medium supplemented with sugar as indicated. Ampicillin (100 μg/ml) was used for plasmid selection. Bacterial cell mass was estimated by measuring optical density at 550 nm (OD_{550}) by using a Bausch & Lomb Spectronic 70 spectrophotometer (330 mg of dry cell weight per liter at an OD_{550} of 1.0).

Isolation of clones containing *K. oxytoca* xylosidase gene. A pUC18 library containing 4- to 6-kbp *Sau*3AI fragments of *K. oxytoca* chromosomal DNA (27) was transformed into *E. coli* DH5α. A second *K. oxytoca* library was prepared from 6- to 9-kbp *Sau*3AI fragments by using vector pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.). Both libraries were screened for xylosidase activity (fluorescent colonies) by using LB plates containing ampicillin and 10 μg of 4-methylumbelliferyl 7-β-D-xylopyranoside (MUX)/ml.

Preparation of GAX1 and GAX2 standards. Aldouronic acid standards, 2′-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylose (methylglucuronoxylose [GAX1]) and 2′-O-(4-O-methyl-α-D-glucopyranosyluronic acid)-D-xylobiose (methylglucuronoxylobiose [GAX2]), were prepared from 4-O-methylglucuronoxylan using a modification of methods described by Jones et al. (21). 4-O-methylglucuronoxylan was isolated from sweet gum (*Liquidambar styraciflua*) by alkaline extraction and was structurally defined by ^{13}C nuclear magnetic resonance (NMR) spectroscopy (22). After partial acid hydrolysis in 0.1 N H_2SO_4 at 122°C (30 min), GAX1 and GAX2 were purified by using gel filtration (BioGel P2 [Bio-Rad, Hercules, Calif.] in 0.05 M formic acid). Products were identified by ^1H and ^{13}C NMR (K. Zuobi-Hasona, F. M. St. John, J. D. Rice, and J. F. Preston, unpublished data). Uncoupled NMR spectra were obtained by using a Nicollet NT-300 spectrometer at 25°C in the Fourier transform mode at 300 MHz for ^1H and 75.45 MHz for ^{13}C. Structural assignments for peaks were made on the basis of those reported for aldouronic acids ($^1H/^{13}C$ 2D-NMR) from sunflower (3) and birchwood (7) hemicellulose.

Preparation of soluble xylodextrin. A mixture of xylodextrins was prepared by partial acid hydrolysis of birchwood methylglucuronoxylan (Sigma, St. Louis, Mo.) with trifluoroacetic acid (13). Approximately 38 mg of methylglucuronoxylan was mixed with 7.5 ml of 1.6 N trifluoroacetic acid in a 15-ml screw-cap tube. Xylan was dispersed by using an ultrasonic water bath prior to hydrolysis at 100°C for 90 min (manual mixing). After cooling to room temperature, hydrolysates were neutralized over a 15-min period by adding anion exchange resin (~1.5 g of Amberlite IRA 400 in the -OH form). Neutralized hydrolysates were filtered, lyophilized, and dissolved in 150 μl of distilled water (~125 mg of total carbohydrate per ml). Based on thin-layer chromatography and densitometry, approximately half of the soluble xylodextrins contained six or fewer sugar residues: 7.4% xylose, 11.5% xylobiose (X2), 9.6% xylotriose (X3), 5.7% xylotetrose (X4), 7.4% xylopentose (X5), and 3.9% xylohexose (X6). Additional compounds were identified as methylglucuronoxylosides but were not quantified.

Fermentation of xylodextrins. Seed cultures of *K. oxytoca* M5A1- and *E. coli* KO11-harboring plasmids were grown in 250-ml flasks containing 50 ml of LB broth (5% xylose) for 12 h (37°C, 125 rpm). Sufficient culture was harvested by centrifugation to provide 0.17 mg of dry cell weight (approximately 1 ml of cells at an OD_{550} of 0.5), washed twice with 1 ml of LB lacking sugar, and resuspended in 50 μl of filter-sterilized LB containing 50% (vol/vol) soluble xylodextrins (hydrolysate). Small samples (10 μl) were removed during incubation at 37°C and were stored frozen. Xylodextrins were separated by thin-layer chromatography as described previously (50). After visualizing with *N*-(1-naphthyl)ethylenediamine reagent (4), relative amounts were estimated by densitometry using Quantity One Software and a VersaDoc Imaging System Model 1000 (Bio-Rad).

Measurement of xylosidase and arabinosidase activities. Cultures of *K. oxytoca* M5A1 and *E. coli* KO11 were grown to half maximal density (OD_{550} of ~2.0) in LB containing 5% total sugar (glucose, xylose, or a combination of both). Sufficient culture was harvested by centrifugation to provide approximately 0.33 mg of dry cell weight, washed with 50 mM sodium phosphate buffer (pH 6.8), and resuspended in 1 ml of the same buffer. Cells were permeablized by mixing with 1 drop of 0.1% sodium dodecyl sulfate (SDS) and 2 drops of chloroform for 10 s using a Vortex mixer. Dilutions of permeabilized cells were assayed at 37°C using either 1 mM *p*-nitrophenyl β-D-xylopyranoside (pNP-XP) or 1 mM *p*-nitrophenyl-α-L-arabinofuranoside (pNP-AF) as substrate (1 ml total volume). Reactions were terminated by adding 2 ml of 0.5 M sodium carbonate. Protein concentrations were measured by using the Bradford reagent (Bio-Rad). Activity is expressed as nanomoles of *p*-nitrophenol released per minute per milligram of protein.

Construction of a *xynTB* expression plasmid. The 3.6-kbp *Ase*I-*Pst*I fragment from pLOI3705 containing the ribosomal binding site and full coding region for *xynT* and *xynB* (lacking the CRP and XylR regulatory regions) was gel purified. This DNA fragment was blunt ended, ligated behind the *lac* promoter (*Pme*I site) in pNEB193 (New England Biolabs, Beverly, Mass.), and transformed into *E. coli* TOP10F′ (Invitrogen). Both orientations were recovered and were designated pLOI3708 (forward with respect to *lac* promoter) and pLOI3707 (reverse). For measurement of xylosidase activity, cultures were grown in LB broth lacking sugar. At an OD_{550} of 0.7, each culture was divided into two flasks. Isopropyl β-D-thiogalactopyranoside (IPTG; 1 mM) was added to one while the other served as a control. After 2 h of further incubation, cells were harvested by centrifugation and were assayed for β-xylosidase and α-arabinosidase activities. A portion of each culture was also harvested and washed in TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]) for protein analysis by SDS-polyacrylamide gel

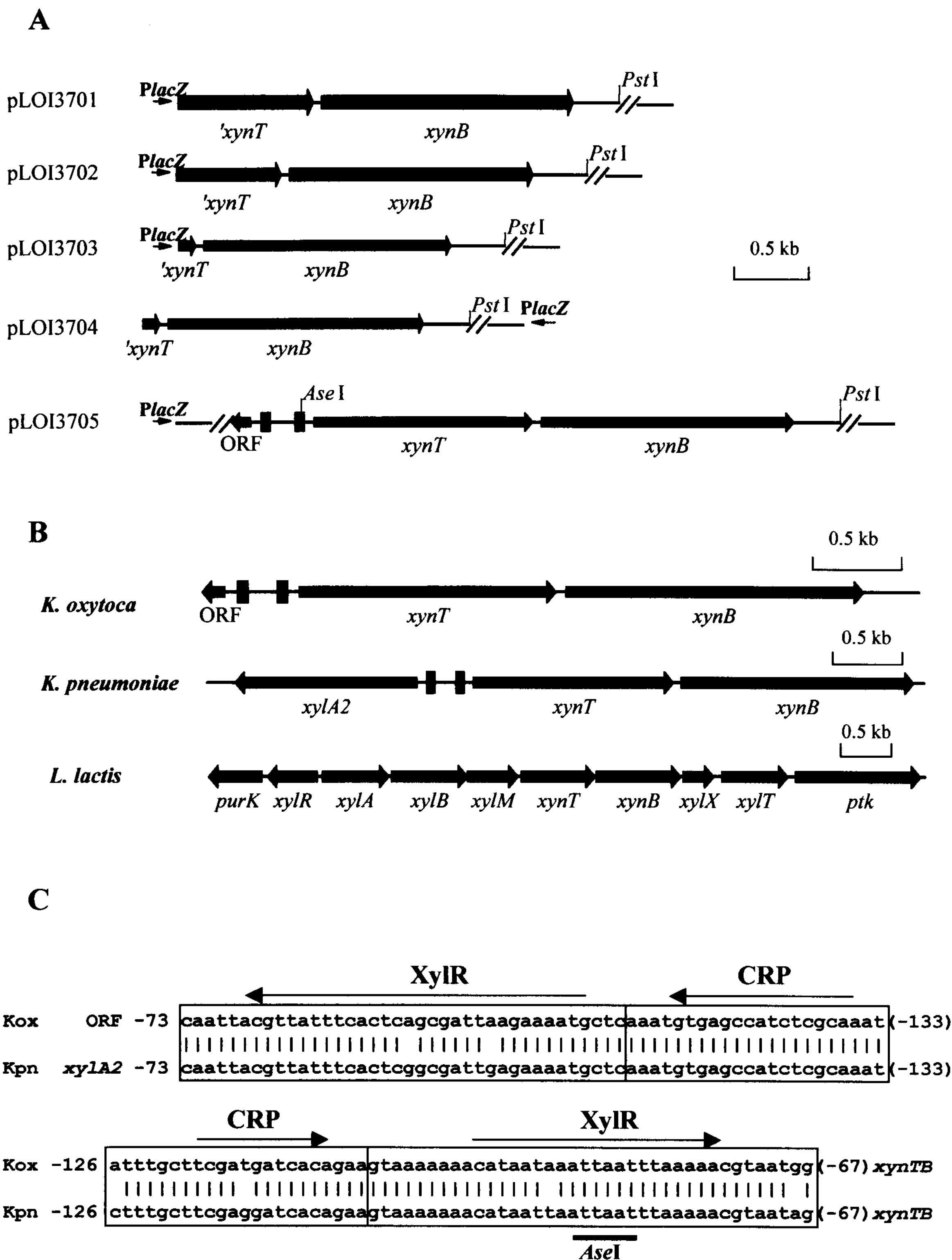
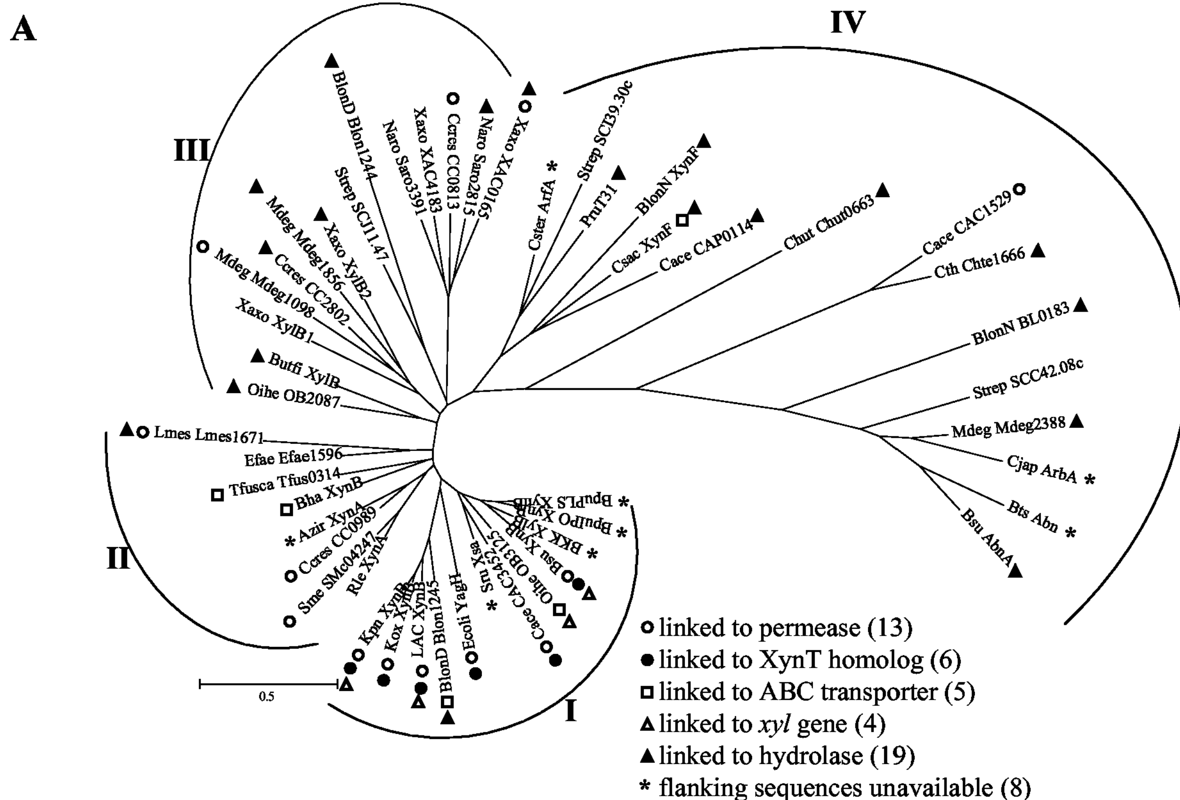
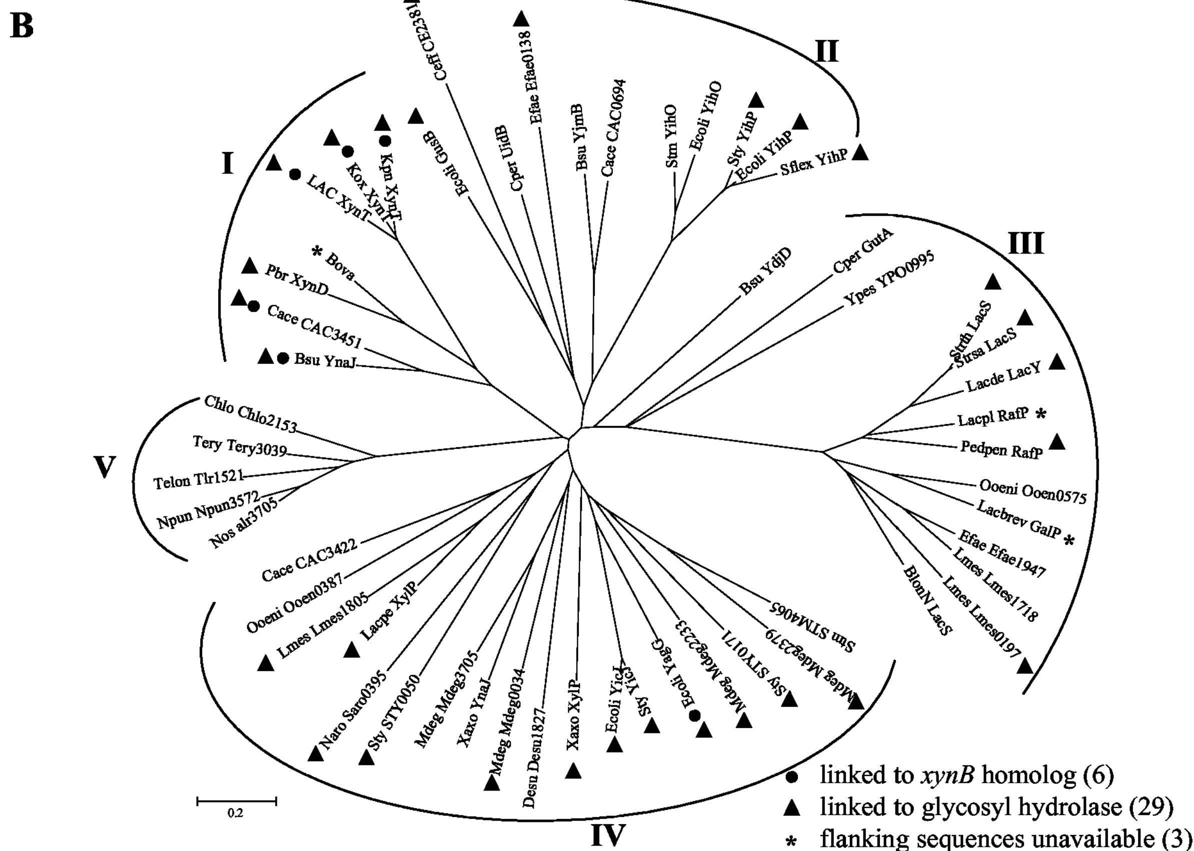


FIG. 1. Genes encoding β-xylosidase and xylobiose uptake. (A) *K. oxytoca* library clones. *Ase*I and *Pst*I restriction sites were used to subclone the full-length *xynT* and *xynB* genes lacking CRP and XylR regulatory sequences (solid bars). The directions of the vector *lacZ* promoters are also shown here. A single scale bar denotes size for all plasmids. (B) Comparison of gene organization in the *xynTB* region. Each solid rectangle represents a contiguous set of CRP and XylR regulatory sequences. Individual scale bars are included for each plasmid. (C) Comparison of CRP and XylR regulatory sequences associated with the *xynTB* operon and respective upstream genes in *K. oxytoca* M5A1 and *K. pneumoniae*. Sequences are numbered relative to start codons for the unidentified ORF (*K. oxytoca* M5A1), *xylA2* (*K. pneumoniae*), and *xynT* (both organisms). Thin arrows indicate direction of transcription for the respective genes.


A
I
II
III
IV
BlonD Blon1244
Naro Saro3391
Xaxo XAC4183
Ccres CC0813
Naro Saro2815
Xaxo XAC0165
Strep SCI11.47
Cster ArfA
Strep SCI39.30c
PruT31
BlonN XynF
Csac XynF
Cace CAP0114
Chut Chut0663
Cace CAC1529
Cth Chte1666
BlonN BL0183
Strep SCC42.08c
Mdeg Mdeg2388
Cjap ArbA
Bts Abn
Bsu AbnA
Mdeg Mdeg1856
Xaxo XylB2
Ccres CC2802
Mdeg Mdeg1098
Xaxo XylB1
Butfi XylB
Oihe OB2087
Lmes Lmes1671
Efae Efae1596
Tfusca Tfus0314
Bha XynB
Azir XynA
Ccres CC0989
Sme SMc04247
Rle XynA
Kpn XynB
Kox XynB
LAC XynB
BlonD Blon1245
Ecoli YagH
Sru Xsa
Cace CAC3452
Oihe OB3125
Bsu XynB
BKK XynB
BpuIPO XynB
BpuPLS XynB
0.5
linked to permease (13)
linked to XynT homolog (6)
linked to ABC transporter (5)
linked to xyl gene (4)
linked to hydrolase (19)
flanking sequences unavailable (8)


B
I
II
III
IV
V
Ceff CE2381
Efae Efae0138
Ecoli GusB
Cper UidB
Bsu YjmB
Cace CAC0694
Stm YihO
Ecoli YihO
Sty YihP
Ecoli YihP
Sflex YihP
Kpn XynT
Kox XynT
LAC XynT
Bova
Pbr XynD
Cace CAC3451
Bsu YnaJ
Bsu YdjD
Cper GutA
Ypes YPO0995
Stth LacS
Strsa LacS
Lacde LacY
Lacpl RafP
Pedpen RafP
Ooeni Ooen0575
Lacbrev GalP
Efae Efae1947
Lmes Lmes1718
Lmes Lmes0197
BlonN LacS
Chlo Chlo2153
Tery Tery3039
Telon Tlr1521
Npun Npun3572
Nos alr3705
Cace CAC3422
Ooeni Ooen0387
Lmes Lmes1805
Lacpe XylP
Naro Saro0395
Sty STY0050
Mdeg Mdeg3705
Xaxo YnaJ
Mdeg Mdeg0034
Desu Desu1827
Xaxo XylP
Ecoli YicJ
Sty YicJ
Ecoli YagG
Mdeg Mdeg2233
Sty STY0171
Mdeg Mdeg2379
Stm STM4065
0.2
linked to xynB homolog (6)
linked to glycosyl hydrolase (29)
flanking sequences unavailable (3)

electrophoresis (PAGE) (10 to 15% acrylamide gradient) using a PhastGel system (Amersham, Piscataway, N.J.).

Phylogenetic analyses. Homologs of XynB and XynT were identified by BLASTP search (1). Protein sequences were aligned using ClustalX version 1.81 (42). Phylogenetic trees were constructed using MEGA (v.2.1) (http://www.megasoftware.net) (24). Phylogenetic relationships were inferred by using the neighbor-joining algorithm and were tested by bootstrap analysis with 1,000 repetitions.

Nucleotide sequence accession number. The sequence for the *K. oxytoca xynTB* operon and upstream region was submitted to GenBank and was assigned accession number AY297960.

RESULTS AND DISCUSSION

Isolation of the *K. oxytoca xynTB* operon. Five unique clones exhibiting β-xylosidase activity were initially isolated as fluorescent colonies (MUX positive) from the 4- to 6-kbp *K. oxytoca* library. Two were weakly fluorescent and contained the previously characterized *K. oxytoca casB* gene encoding phosphocellobiase (27). The three remaining clones (Fig. 1A) contained an identical open reading frame (ORF) encoding a 559-amino-acid product (64 kDa) that shared homology with a putative xylosidase (XynB) from *Lactococcus lactis* (accession number NP_267661). This new *K. oxytoca* gene was also designated *xynB*. Based on a SignalP analysis (http://www.cbs.dtu.dk/services/SignalP/) (35), *K. oxytoca xynB* was predicted to encode a cytoplasmic protein that lacks a signal peptide. All three MUX-positive clones (pLOI3701, pLOI3702, and pLOI3703) also included an upstream incomplete ORF that resembled the carboxy terminus of a transport protein in *L. lactis*.

A second *K. oxytoca* gene library was constructed to facilitate isolation of the complete sequence for the putative transport protein. One MUX-positive clone was recovered that contained both genes (pLOI3705). An additional clone was recovered that contained a truncated transport gene (pLOI3704). Using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html), the putative transporter gene was predicted to encode a membrane protein (484 amino acids) containing at least 10 transmembrane helices. This translated sequence was very similar to XynT in *L. lactis* (accession number NP_267662; 83% identity). Based on the proximity of the two *K. oxytoca* genes and their concordant direction of transcription, both genes are presumed to form a single transcriptional unit, the *xynTB* operon (Fig. 1B).

A divergently transcribed, incomplete ORF was also identified that began 425 bp upstream from the start codon for *K. oxytoca xynT* (Fig. 1B). This ORF exhibited no significant homology to other sequences in the database. Comparison of *K. oxytoca xynT* and *xynB* to the unannotated sequence for *K. pneumoniae* (http://genome.wustl.edu) readily identified corresponding genes. In *K. pneumoniae*, however, a *xylA* homolog denoted *xylA2* resides upstream from the *xynTB* region (Fig. 1B) (28). In *L. lactis*, this upstream region contains a xylose mutarotase (*xylM*) required for the efficient metabolism of xylan (14). Based on an in silico analysis of the *K. pneumoniae* genome (28), potential regulatory sites for catabolite repression (CRP) and for xylose induction by XylR were predicted to be within the ORF-*xynTB* intergenic region. These sequences were very similar in *K. oxytoca* M5A1 and *K. pneumoniae* (Fig. 1C) despite the difference in upstream genes (unidentified ORF and *xylA2*, respectively). In contrast, the gram-positive

FIG. 2. Unrooted phylogenetic trees of XynB (A) and XynT (B) homologs. Trees have been provisionally assigned into different groups based on similarities in primary structure of XynB (groups I to IV) and XynT (groups I to V), respectively, and the functions of neighboring genes. Abbreviations for the organisms and their XynB and XynT homologs are listed in alphabetical order with accession numbers in parentheses: Azir, *Azospirillum irakense*, XynA (AAF66622); Bha, *Bacillus halodurans*, XynB (BAB07402); BKK, *Bacillus* sp. strain KK-1, XylB (AAC27699); BlonD, *B. longum* DJO10A, Blon1245 (ZP_00121429), Blon1244 (ZP_00121428); BlonN, *B. longum* NCC2705, XynF (AAN25335), BL0183 (AAN24037), LacS (NP_696148); *Bpu*PLS, *B. pumilus* strain PLS, XynB (AAC97375); *Bpu*IPO, *B. pumilus* strain IPO, XynB (S19729); Bsu, *B. subtilis*, XynB (AAB41091), AbnA (CAA99586), YnaJ (NP_389639), YjmB (NP_389113), YdjD (NP_388497); Bts, *Bacillus thermodenitrificans* TS-3, Abn (BAB64339); Butfi, *B. fibrisolvens*, XylB (A49776); Cace, *C. acetobutylicum*, CAC3452 (AAK81382), CAC1529 (NP_348156), CAP0115 (NP_149278), CAC3451 (NP_350041), CAC0694 (NP_347331), CAC3422 (NP_350012); Ccres, *C. crescentus* CB15, CC0989 (AAK22973), CC2802 (AAK24766), CC0813 (AAK22798); Ceff, *Corynebacterium efficiens* YS-314, CE2381 (NP_738991); Chlo, *Chloroflexus aurantiacus*, Chlo2153 (ZP_00019154); Chut, *Cytophaga hutchinsonii* Chut0663 (ZP_00117295); Cjap, *C. japonicus*, ArbA (CAA71485); Cper, *C. perfringens*, UidB (NP_561069), GutA (NP_561685); Csac, *Caldicellulosiruptor saccharolyticus*, XynF (AAB87371); Cster, *Caldicellulosiruptor stercorarium*, ArfA (CAD48310); Cth, *Caldicellulosiruptor thermocellum* ATCC 27405, Chte1666 (ZP_00061257); Desu, *Desulfitobacterium hafniense*, Desu1827 (ZP_00098712); Ecoli, *E. coli* K12, YagH (P77713), YagG (NP_414804), GusB (AAA68924), YihO (NP_418312), YihP (AAC76874), YicJ (AAC76681); Efae, *Enterococcus faecium*, Efae1596 (ZP_00036717), Efae0138 (ZP_00035300), Efae1947 (ZP_00037059); Kpn, *K. pneumoniae* unfinished genome sequence (NC_002941; Washington University, http://genome.wustl.edu); LAC, *L. lactis* subsp. *lactis* IL-1403, XynB (AAK05603), XynT (NP_267662); Lacpe, *L. pentosus*, XylP (P96792); Lacbrev, *L. brevis*, GalP (AAK54067); Lacpl, *L. plantarum*, RafP (AAL09166); Lacde, *L delbrueckii* subsp. *bulgaricus*, LacY (P22733); Lmes, *L. mesenteroides* subsp. *mesenteroides* ATCC 8293, Lmes1671 (ZP_00064177), Lmes1718 (ZP_00064224), Lmes0197 (ZP_00062720), Lmes1805 (ZP_00064310); Mdeg, *M. degradans* 2-40, Mdeg1098 (ZP_00065724), Mdeg2388 (ZP_00066989), Mdeg1856 (ZP_00066469), Mdeg2233 (ZP_00066837), Mdeg0034 (ZP_00064673), Mdeg3705 (ZP_00068288), Mdeg2379 (ZP_00066980); Naro, *Novosphingobium aromaticivorans*, Saro3391 (ZP_00096353), Saro2815 (ZP_00095782), Saro0395 (ZP_00093393); Nos, *Nostoc* sp. strain PCC 7120, alr3705 (NP_487745); Npun, *N. punctiforme*, Npun3572 (ZP_00109128); Oihe, *Oceanobacillus iheyensis*, OB3125 (BAC15081), OB2087 (BAC14043); Ooeni, *Oenococcus oeni* MCW, Ooen0387 (ZP_00069379), Ooen0575 (ZP_00069564); Pbr, *P. bryantii*, XynD (CAD21012); Pedpen: *Pediococcus pentosaceus* RafP (P43466); Pru T31, *Prevotella ruminicola* strain T31 (BAA78558); Rle, *Rhizobium leguminosarum* bv. *trifolii*, XynA (AAL14914); Sflex, *Shigella flexneri* 2a strain 301, YihP (NP_709677); Sme, *Sinorhizobium meliloti*, SMc04247 (CAC46496); Sru, *Selenomonas ruminantium*, Xsa (AAB97967); Stm, *Salmonella enterica serotype* Typhimurium LT2, STM4065 (NP_462946), and YihO (NP_462897); Strep, *Streptomyces coelicolor* A3 (2), SCJ11.47 (CAB52932), SCC42.08c (CAB92901), and SCI39.30c (CAD55182); Strsa, *Streptococcus salivarius*, LacS (AAL67293); Strth, *S. thermophilus* A147, LacS (P23936); Sty, *S. enterica* subsp. *enterica* serovar Typhi, STY0171 (NP_454763), YicJ (NP_458178), YihP (NP_458032), and STY0050 (NP_454653); Telon, *Thermosynechococcus elongatus* BP-1, Tlr1521 (NP_682311); Tery, *Trichodesmium erythraeum* IMS101, Tery3039 (ZP_00073765); Tfusca, *Thermobifida fusca*, Tfus0314 (ZP_00056973); Xaxo, *Xanthomonas axonopodis* pv. *citri* strain 306, XylB1 (AAM36146), XylB2 (AAM39065), XAC0165 (AAM35057), XAC4183 (AAM39018), XylP (NP_644556), YnaJ (NP_644495); Ypes, *Yersinia pestis*, YPO0995 (NP_404610).

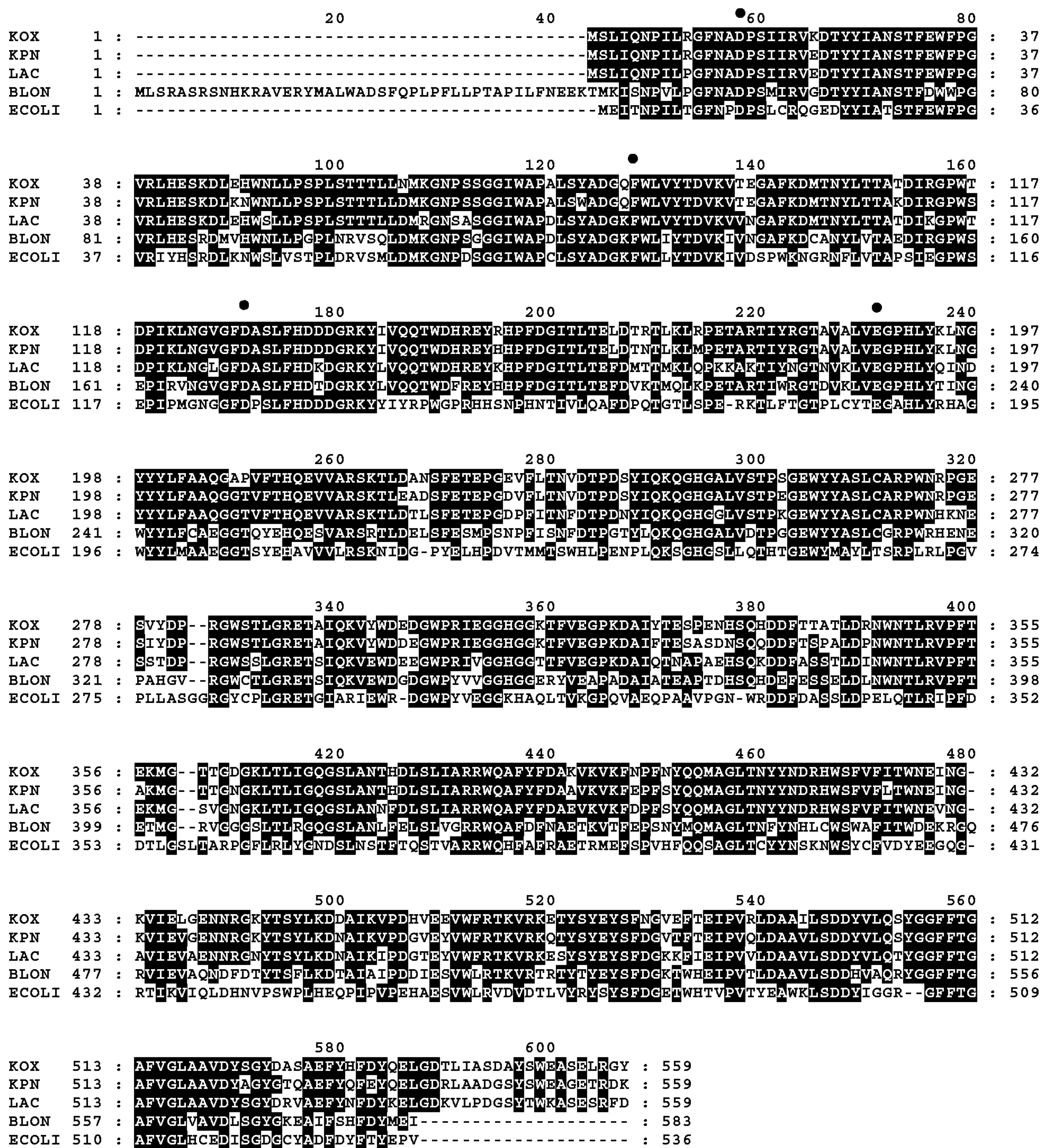


FIG. 3. Comparison of translated sequences for *K. oxytoca* XynB (KOX) and homologs from *K. pneumoniae* (KPN), *L. lactis* (LAC), *B. longum* (BLON; Blon1245), and *E. coli* K12 YagH. Residues which are identical in three of the five sequences are shaded in black. Solid circles above residues D15, F85, D128, and E188 in KOX correspond to residues D18, F114, D158, and E221 from *C. japonicus* ArbA that are involved in catalytic activity and substrate binding.

regulatory sites for xylose (XylR) and CRP (CcpA) were absent in the intergenic region of *xylR-xylABM xynTBX* in *L. lactis* (39).

Sequence comparisons and phylogenetic analyses of XynB homologs. A BLASTP search of protein sequences identified more than 70 homologs of XynB, 48 of which were selected for phylogenetic analyses (Fig. 2A). Experimental verification of hydrolase activity is limited in this group and includes only *xynB* from *Bacillus pumilus* (26, 47), *xylB* from *Bacillus* sp. strain KK-1 (10), *xsa* from *Selenomonas ruminantium* (44), *xylB* from *Butyrivibrio fibrisolvens* (43), *arbA* from *Cellvibrio japonicus* (36), and Abn from *Bacillus thermodenitrificans* TS-3 (41). Xylosidase activity was reported in GenBank entries for *xynF* from *Caldicellulosiruptor saccharolyticum* and *xynA* from *Azo-*

TABLE 2. β-Xylosidase activities in permeabilized cell preparations of *K. oxytoca* M5A1 and recombinant *E. coli*[a]

Strains and properties	Growth media	β-Xylosidase activity (nmol min^{-1} mg^{-1})
K. oxytoca M5A1	LB + 5% glucose	0.5
	LB + 2.5% glucose + 2.5% xylose	0.5
	LB + 5% xylose	28
E. coli KO11(pLOI3705)	LB + 5% glucose	7.5
	LB + 2.5% glucose + 2.5% xylose	43
	LB + 5% xylose	140 (5.0)
E. coli TOP10F′ (pLOI3708)	LB, no sugar, without IPTG	520
	LB, no sugar, with IPTG	3,100 (105)
E. coli KO11(pLOI3708)	LB + 5% xylose	1,020
E. coli KO11(pLOI3709)	LB + 5% xylose	1,100
E. coli KO11 (pNEB193)	LB + 5% xylose	0.4
E. coli TOP10F′	LB, no sugar	<0.4

[a] Values represent an average of two or more experiments which differed by less than 25%. Arabinosidase activities are shown in parentheses.

spirillum irakense without supporting data. Most XynB homologs are from gram-positive organisms consistent with an early gram-positive ancestor.

Operons that include xylobiose transporters adjacent to hydrolases appear common in nature and may provide an evolutionary advantage (Fig. 2A). Among the 39 XynB homologs for which flanking sequences were available, 12 xylosidase genes were adjacent to a putative permease and 5 were adjacent to a putative ATP-dependent ABC transporter. Of the 12 with adjacent permeases, 5 encoded homologs of *K. oxytoca* XynT. All appear to contain operons resembling *K. oxytoca xynTB*: *B. subtilis* (*ynaJ-xynB*), *E. coli* (*yagG-yagH*), *C. acetobutylicum* (CAC3451-CAC3452, *xynD*), *L. lactis* (*xynT-xynB*), and *K. pneumoniae* (*xynT-xynB*). Nineteen of the XynB homologs were found adjacent to other glycosyl hydrolase genes, a particularly common feature in organisms that degrade xylan and cellulose. Four were adjacent to genes directly involved in xylose catabolism.

XynB homologs can be readily organized into four groups based on the phylogenetic tree structure (Fig. 2A) and their genomic neighboring genes. Group I is the most cohesive group and includes *K. oxytoca* XynB and 11 other members. This group contains all organisms with putative *xynTB* operons and all organisms with *xynB* adjacent to xylose catabolism genes. Two members of this group are adjacent to putative ABC transporters. Within Group I, *K. oxytoca* XynB shared the highest homologies (Fig. 3) with XynB from *K. pneumoniae* (identifier RKP04766, 89% identity; Integrated Genomics, Inc.) (28), XynB from *L. lactis* (81% identity), and Blon1245 (68% identity) from *Bifidobacterium longum*. These four genes form a closely related subgroup with lower homology to other xylosidases (<50% identity). The *E. coli* homolog YagH also showed significant homology (46% identity). The similarities of XynB and XynT to respective homologs in *L. lactis* and *K. pneumoniae* are consistent with a gram-positive ancestor of *L. lactis.* It is interesting that the cryptic homologs in *E. coli* are located in a cluster of genes surrounded by insertion sequences

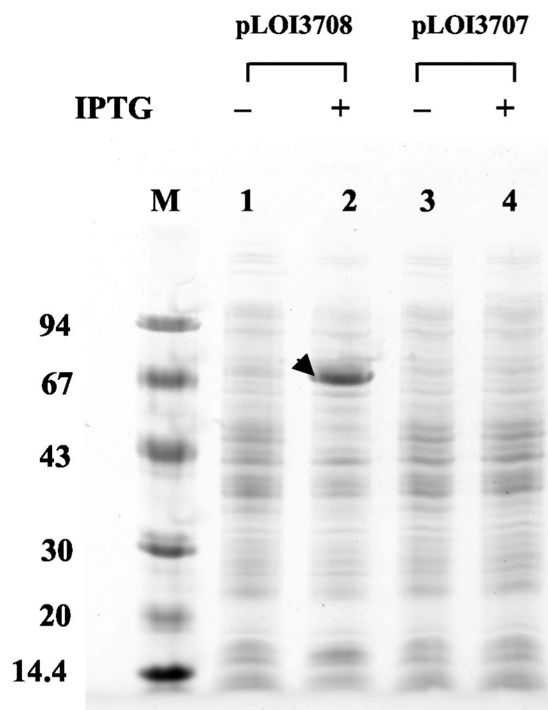
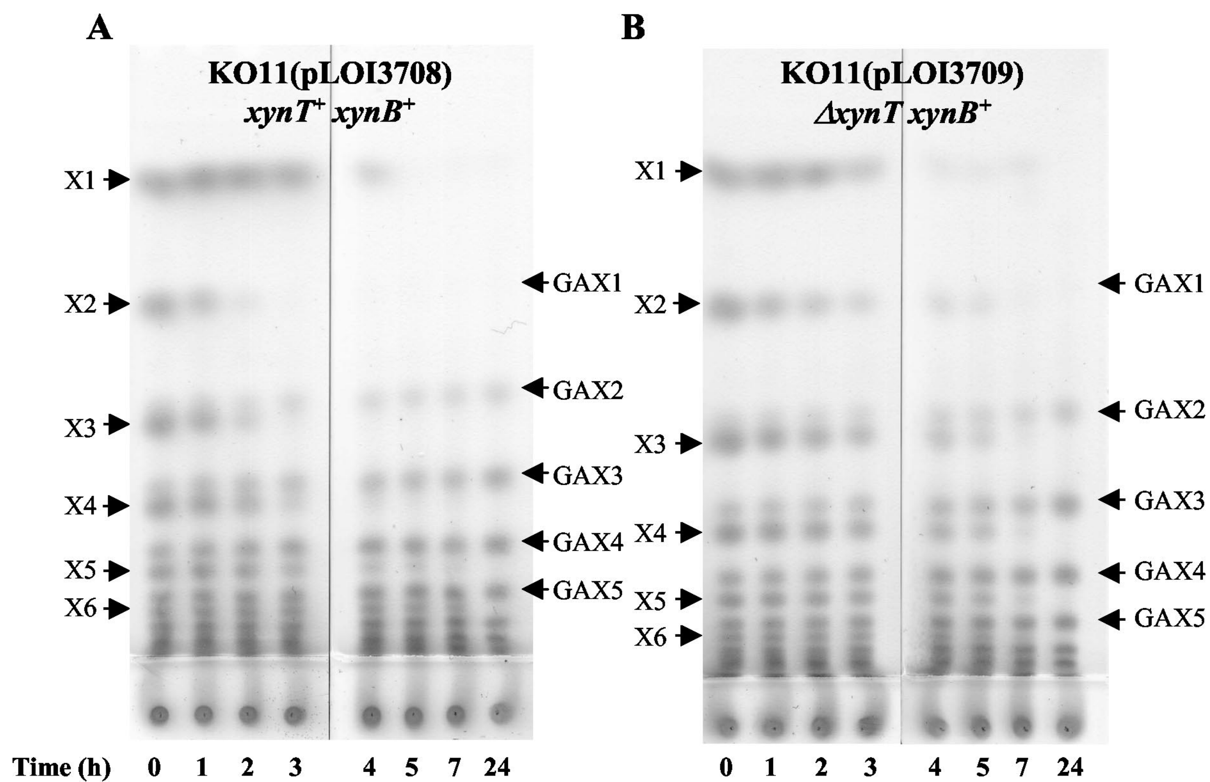


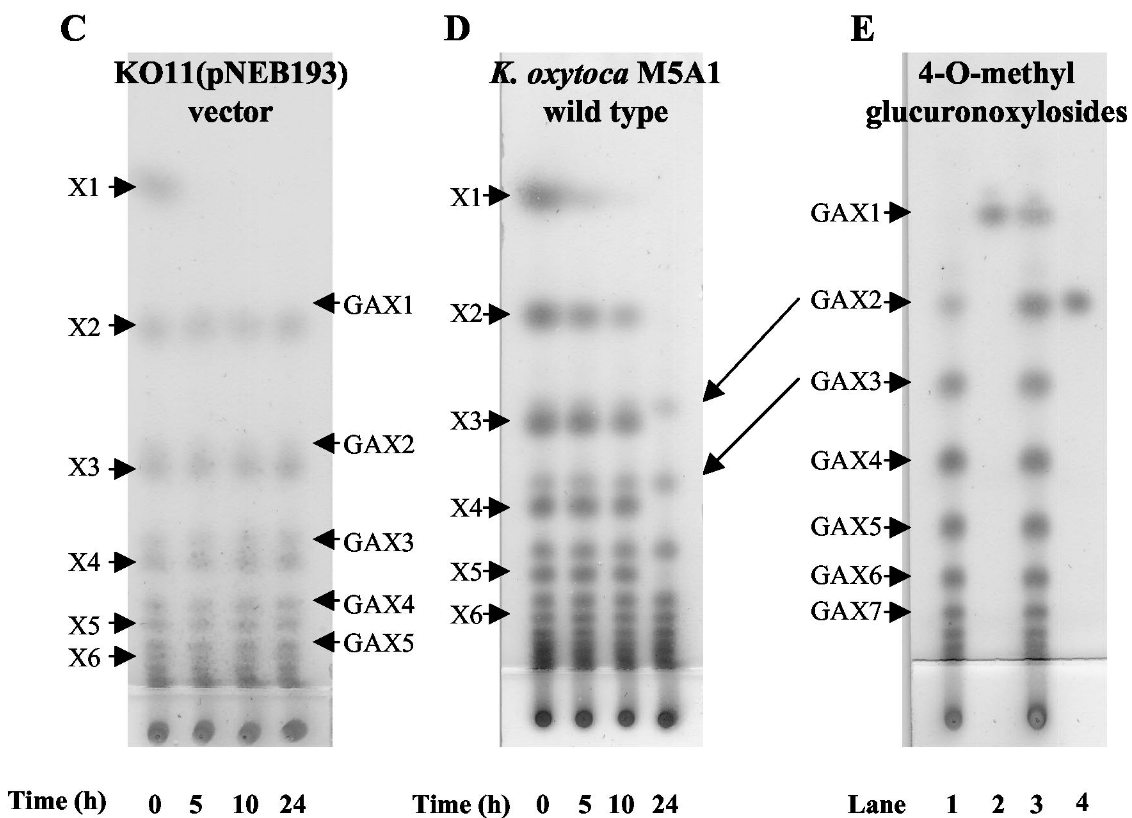
FIG. 4. SDS-PAGE comparison of proteins in recombinant strains of *E. coli* TOP10F′ containing *xynTB* coding regions in the forward (pLOI3708) and reverse (pLOI3707) orientations with respect to the *lac* promoter. Cells were grown and harvested as described for xylosidase assays. Lanes: M, protein standards with molecular mass in kilodaltons indicated on the left; 1, TOP10F′(pLOI3708) without IPTG; 2, TOP10F′(pLOI3708) with 1 mM IPTG; 3, TOP10F′(pLOI3707) without IPTG; 4, TOP10F′(pLOI3707) with 1 mM IPTG. The arrow denotes a 65-kDa band in lane 2 corresponding to XynB.

which may indicate a transposon-mediated acquisition. Limited sequence is available in the region encoding XynB homologs for four members of this group. *B. subtilis* and *C. acetobutylicum* also contained adjacent homologs of XynT and XynB that are more distantly related.

XynB homologs in groups II and III are more diverse than those in Group I. Group II primarily contains XynB homologs that are adjacent to transport genes unrelated to *K. oxytoca* XynT. In groups III and IV, most homologs are located adjacent to hydrolases for carbohydrate polymers. Group IV also includes XynB homologs that exhibit arabinase/arabinosidase activities (ArbA from *C. japonicus* [36], Abn from *B. thermodenitrificans* TS-3 [41], and XynF from *C. saccharolyticus*).

All XynB homologs contain the COG3507 domain characteristic of glycosyl hydrolase family 43 (5). The ArbA protein from *C. japonicus* is the only member in family 43 with a known three-dimensional structure (36). Although overall sequence identity to *K. oxytoca* XynB was less than 23%, residues (D38, F114, D158, and E221) that are involved in catalytic activity and ligand binding in *C. japonicus* ArbA were found to align with residues D15, F85, D128, and E188 in *K. oxytoca* XynB (Fig. 3). Accepting substitutions of F to Y or W, further comparisons revealed that the identities and spacings of these four residues were conserved in all but 2 of the 48 homologs examined.


A
KO11(pLOI3708)
xynT+ xynB+
X1
X2
X3
X4
X5
X6
GAX1
GAX2
GAX3
GAX4
GAX5
Time (h) 0 1 2 3 4 5 7 24
B
KO11(pLOI3709)
ΔxynT xynB+
X1
X2
X3
X4
X5
X6
GAX1
GAX2
GAX3
GAX4
GAX5
0 1 2 3 4 5 7 24


C
KO11(pNEB193)
vector
X1
X2
X3
X4
X5
X6
GAX1
GAX2
GAX3
GAX4
GAX5
Time (h) 0 5 10 24
D
K. oxytoca M5A1
wild type
X1
X2
X3
X4
X5
X6
Time (h) 0 5 10 24
E
4-O-methyl
glucuronoxylosides
GAX1
GAX2
GAX3
GAX4
GAX5
GAX6
GAX7
Lane 1 2 3 4

Sequence comparisons and phylogenetic analyses of XynT homologs. A BLASTP search using *K. oxytoca* XynT identified 100 homologous transporters (Fig. 2B), all of which contain the MelB domain (COG2211) characteristic of Na^+/melibiose symporters. For most members, additional sequence data was available for adjacent genes. Multiple XynT homologs were found in some organisms, including three in *C. acetobutylicum*, *B. subtilis*, and *Leuconostoc mesenteroides*, four in *Microbulbifer degradans*, and two in both *Xanthomonas axonopodis* and *X. campestris.* None of the XynT homologs have previously been shown to function in xylobiose transport. XylP from *L. pentosus* has been shown to transport an α-xyloside, isoprimeverose[α-D-xylopyranosyl-(1,6)-D-glucopyranose] (8).

Based on primary structure and adjacent genes, XynT homologs can be organized into at least five groups (Fig. 2B). Group I contains seven members, including XynT from *K. oxytoca*. As with XynB, *K. oxytoca* XynT was most similar to *K. pneumoniae* and *L. lactis* homologs (94 and 83% identity, respectively), providing further support for the cotransfer of *xynT* and *xynB*. Two other transporters, XynD from *Prevotella bryantii* strain B_14 (accession number CAD21012) and a partial ORF from *Bacteroides ovatus* (S55894), also shared significant homology with *K. oxytoca* XynT (39 and 41% identity, respectively). The other two members of this group (*B. subtilis* YnaJ and *C. acetobutylicum* CAC3451) were more distantly related to XynT (both 34% identity) but contained adjacent genes encoding homologs of *K. oxytoca* XynB. Remaining members of the XynT tree exhibited lower homologies to *K. oxytoca* XynT (less than 30% identity). All but Group V (cyanobacteria) included transporters that are adjacent to hydrolases. Lactose, raffinose, and galactoside symports were found in Group III.

Regulation of *K. oxytoca xynB* expression. The presence of putative XylR and CRP sites in the promoter region of the *K. oxytoca xynTB* operon (Fig. 1C) indicates possible regulation by xylose (XylR) and by glucose (CRP). Since *E. coli* and *K. oxytoca* are closely related with extensive similarity in regulatory systems and protein sequences, the effect of these sugars was investigated in both organisms (Table 2). Expression of *xynB* was controlled more tightly in M5A1 than in KO11(pLOI3705). However, activity levels were consistently higher in KO11. Expression of *xynB* was induced 56-fold in *K. oxytoca* by xylose in the absence of glucose and was repressed to basal levels in the presence of both sugars. In KO11(pLOI3705), xylose induced *xynB* expression by 19-fold in the absence of glucose. In the presence of both sugars, expression was partially repressed (sixfold induction) in KO11(pLOI3705). These results demonstrate that the native *xynTB* operon may be subject to CRP, inducer exclusion, and induction by xylose in both *K. oxytoca* and KO11. Differences in xylosidase activities and the extent of regulation can be attributed largely to the higher gene dosage of *xynTB* in KO11 harboring pLOI3705, a high-copy plasmid.

Additional plasmids were constructed to remove the native regulatory elements and place the *xynTB* operon directly under the control of the *lac* promoter in pNEB193. Despite the presence of $lacI^q$ in the host, *E. coli* TOP10F′(pLOI3708) expressed much higher levels of xylosidase in the presence and absence of IPTG than recombinant KO11(pLOI3705) containing the native regulatory elements. Little or no xylosidase activity was observed in TOP10F′ or in KO11(pLOI3706), which lacks *K. oxytoca* DNA. The high level of *xynB* induction in TOP10F′(pLOI3708) was apparent in protein gels (Fig. 4). A prominent 65-kDa protein band corresponding to XynB was clearly visible in IPTG-induced cells carrying the *xynTB* genes. This band was absent in an analogous construct in which the direction of *xynTB* transcription was reversed (pLOI3707). Although no new band corresponding to XynT (54 kDa) was detected, this membrane protein may be expressed at lower levels or may be poorly solubilized.

Several xylosidases have been reported to also serve as efficient arabinosidases (43, 44). However, this does not appear to be the case for XynB. Recombinant XynB was 30-fold less active with *p*-nitrophenyl-α-L-arabinofuranoside as a substrate than the xylopyranoside derivative (Table 2) and was inactive with *p*-nitrophenyl-α-L-arabinopyranoside (data not shown).

Xylodextrin utilization by ethanologenic derivatives of *E. coli* strain KO11. The biological activities of XynT and XynB were confirmed in small-scale fermentations using a homologous series of soluble xylodextrins (partial acid hydrolysate of birchwood methylglucuronoxylan) as natural substrates (Fig. 5A and B). KO11(pLOI3708) harboring the full-length *xynT* and *xynB* genes metabolized xylodextrins more rapidly than KO11(pLOI3709) containing an internal deletion in *xynT*. Differences between these strains were particularly evident between 2 to 7 h of incubation. Since both strains expressed equivalent levels of xylosidase activity (Table 2), the increased rate of metabolism by KO11(pLOI3708) can be attributed to XynT-mediated transport of xylobiose or xylodextrins. After 24 h of incubation, xylodextrins X2 through at least X6 had been fully metabolized by both strains. Similar results were also obtained for a comparison between the library clones pLOI3704 with a truncated *xynT* gene and pLOI3705 containing the full *xynTB* operon (data not shown). Only xylose was metabolized by control strains of *E. coli* containing the vector

FIG. 5. Thin-layer chromatograms of xylodextrins during fermentation. β-Xylosidase activities for all strains are provided in Table 2. (A) KO11(pLOI3708) containing the full coding regions for *xynT* and *xynB*. (B) KO11(pLOI3709) containing the coding region for *xynB* and a nonfunctional *xynT* (internal deletion). Note that xylosidase activities in permeabilized cell preparations of KO11(pLOI3708) and KO11(pLOI3709) were equivalent. (C) KO11(pNEB193) containing the vector alone. (D) *K. oxytoca* M5A1. (E) Cochromatography of GAX1 and GAX2 standards with spent medium from a 24-h xylodextrin fermentation with KO11(pLOI3708). Lanes: 1, spent medium; 2, GAX1 alone; 3, spent medium plus GAX1 and GAX2; 4, GAX2 alone. The labels X1 to X6 refer to the number of xylosyl residues, xylose to xylohexose. Methylglucuronoxylosides containing 1 to 7 xylosyl residues are labeled GAX1 to GAX7, respectively. Arrows connecting panels D and E are included to illustrate positions corresponding to GAX2 and GAX3 in both chromatograms. Xylodextrins were identified by comparison to xylobiose, xylotriose, and cellobiosides as standards (6). Longer methylglucuronoxylosides (GAX3 to GAX7) were inferred by migration rates relative to xylodextrins with equivalent numbers of carbohydrate residues.

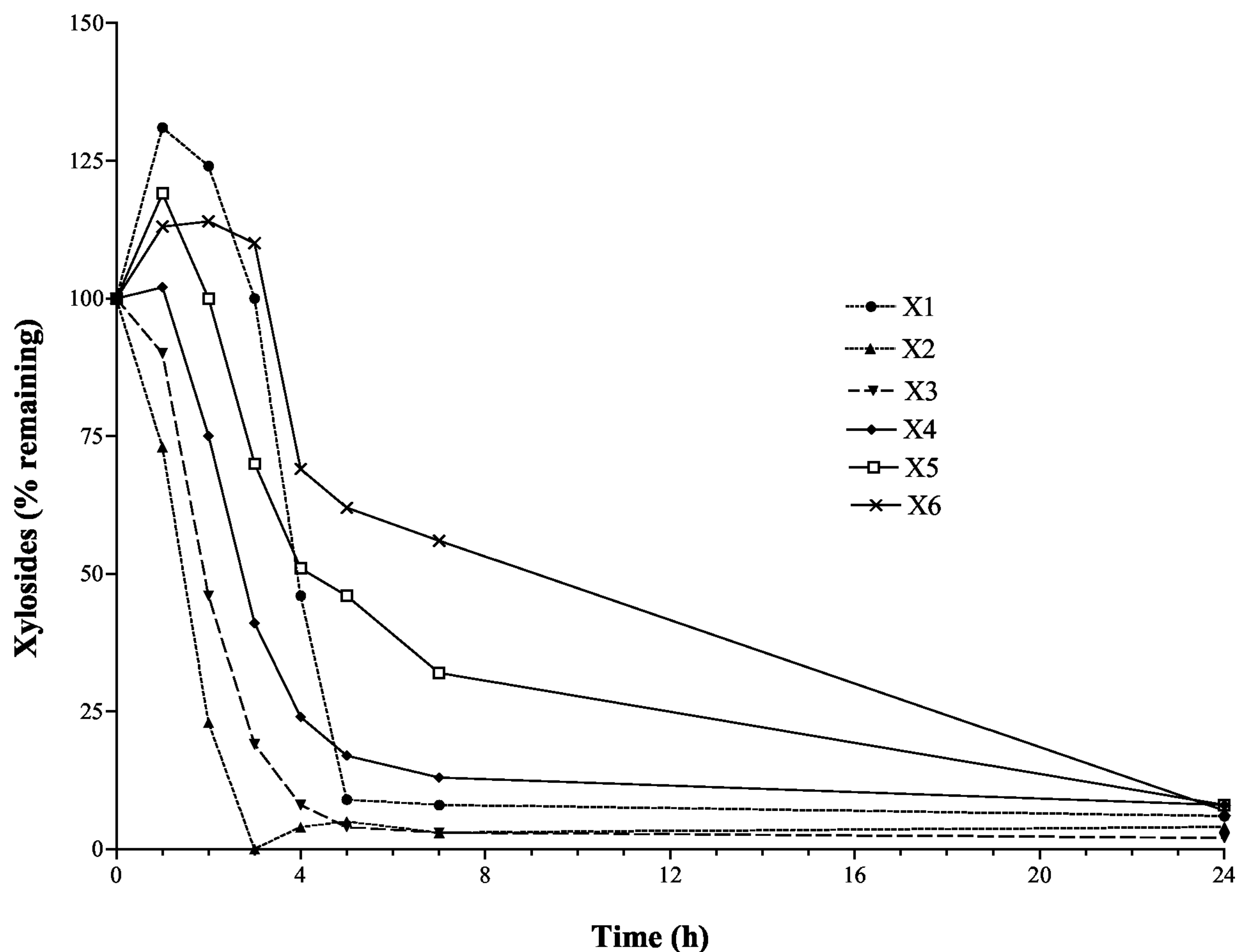


FIG. 6. Relative utilization of mixed xylodextrins by KO11(pLOI3708). Xylodextrins were separated by thin-layer chromatography and quantified by densitometry. The labels X1 to X6 refer to the number of xylosyl residues, xylose to xylohexose. Data are expressed as percentages of initial values at time zero.

alone (Fig. 5C). Native *K. oxytoca* M5A1 metabolized xylodextrins (Fig. 5D) more slowly than recombinant *E. coli* strains expressing the *xynB* or the *xynTB* operon, consistent with observed lower levels of xylosidase activity (Table 2).

Thin-layer chromatography and densitometry were used to monitor xylodextrin utilization by KO11(pLOI3708) (Fig. 6). The rate of utilization was inversely related to chain length, with xylobiose being metabolized most rapidly. Xylose accumulated initially together with smaller amounts of xylopentose and xylohexose. Despite the production of xylobiose and xylotriose as intermediates during the degradation of longer xylodextrins, both were near the lower limit of detection at incubation times longer than 3 h (Fig. 5A). After 24 h, xylodextrins up to six residues in length had been metabolized.

At least seven soluble components of birchwood methylglucuronoxylan hydrolysate were not metabolized by both *K. oxytoca* and the recombinant *E. coli* expressing XynB activities. Most were partially masked by more abundant xylodextrins but were clearly evident after fermentation (Fig. 5A, B, and D). Since the 4-O-methylglucuronic acid substitution of xylan is known to increase the resistance of substituted xylodextrins to chemical hydrolysis (21) and to block degradation by xylanases (11, 12, 48), GAX1 and GAX2 standards were prepared for comparison to the unknown components. Cochromatography of these standards with 24-h broth from xylodextrin fermentations with KO11(pLOI3708) identified the two most mobile spots as GAX1 and GAX2 (Fig. 5E). GAX1 migrated just above xylobiose. Although only a small amount of GAX1 was present before or after fermentation, GAX2 was a prominent component after fermentation, positioned immediately above the xylotriose region. GAX1 and GAX2 appear to represent part of a homologous series of compounds that migrate immediately above each xylodextrin containing an equal number of glycosyl residues (GAX1-GAX7). Resistance to hydrolysis by XynB xylosidase is consistent with a methylglucuronic acid substitution at the nonreducing terminus (18).

ACKNOWLEDGMENTS

This research was supported by grants from the U.S. Department of Agriculture (01-35504-10669 and 00-52104-9704), the U.S. Department of Energy (FG02-96ER20222), and the Consortium for Plant Biotechnology Research (GO12026-161). DNA sequences were kindly provided by Jack Shelton (Microbiology Sequencing Facility) and the ICBR DNA Sequencing Core at the University of Florida.

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