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XYLAN DEGRADATION BY THE THERMOPHILE CLOSTRIDIUM STERCORARIUM: CLONING AND EXPRESSION OF XYLANASE, ß-D-XYLOSIDASE, AND α-L-ARABINOFURANOSIDASE GENES IN ESCHERICHIA COLI

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Summary: Seven genes related to arabinoxylan degradation were isolated from a genomic library of the thermophilic bacterium *Clostridium stercorarium*. The cloned genes include a xylanase gene (*xynA*), two ß-D-xylosidase genes (*bxlA* and *bxlB*), two α-L-arabinofuranosidase genes (*arfA* and *arfB*), and two genes (*celW* and *celX*) encoding enzymes termed celloxylanases, which hydrolyze both xylans and ß-D-cellobiosides. The genes *xynA*, *celX*, and *bxlB* were found to encode the major xylanolytic enzyme activities induced by growth of *C. stercorarium* on xylan.

Xylan is a major component of the hemicellulose fraction of plant biomass. It constitutes after cellulose the most abundant renewable resource for the production of fermentable sugars by enzymatic saccharification. The xylan of grasses and softwood is mostly arabinoxylan, a branched heteroglycan with a backbone of (1→4)-linked ß-D-xylopyranosyl residues substituted at position 3 by α-L-arabinofuranosyl residues. Enzymatic hydrolysis of arabinoxylan involves endo-ß-1,4-xylanases (1,4-ß-D-xylanohydrolase; EC 3.2.1.8), ß-D-xylosidase (1,4-ß-D-xylohydrolase; EC 3.2.1.37), and α-L-arabinofuranosidases (EC 3.2.1.55) (1,2).

Some potential applications for xylanolytic enzymes in the pulp and paper industry would require the use of thermostable enzymes. Thermophilic microorganisms have therefore attracted considerable attention as sources of thermoactive enzymes for xylan hydrolysis. *Clostridium stercorarium* is a thermophilic anaerobic bacterium capable of degrading a wide variety of carbohydrates including xylans (3). Several xylanolytic enzymes have been isolated from supernatants of *C. stercorarium* cultures

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grown on xylan. These enzymes include three xylanases, a ß-D-xylosidase, and an α-L-arabinofuranosidase (4,5). Furthermore, two *C. stercorarium* enzymes hydrolyzing ß-D-cellobiosides as well as xylans have also been purified (5,6). In this communication we report the molecular cloning and preliminary characterization of several *C. stercorarium* genes related to arabinoxylan degradation.

Materials and methods

Bacterial strains and plasmids. *C. stercorarium* NCIB 11745 was obtained from the National Collection of Industrial Bacteria, Aberdeen, UK. *E. coli* strains JM83 and JM109 were grown in LB broth (7) and used as recipients for molecular cloning. Plasmids pUC18 and pUC19 were used as cloning vectors (8). Transformants were selected with ampicillin (100 μg).

Recombinant DNA techniques. The construction of the genomic *C. stercorarium* cosmid library has been described previously (9). Preparation of plasmid DNA, subcloning, and physical mapping were performed using standard procedures (10). Biotinylation of DNA and Southern hybridization was carried out as described by Soutschek-Bauer et al. (11).

Screening methods. Recombinant clones carrying xylanase genes were identified by assaying cell-free extracts prepared by freeze-thaw lysis (12) for xylanase activity. ß-D-Xylosidase and α-L-arabinofuranosidase producing clones were detected by their fluorescence under UV illumination in the presence of the fluorogenic substrates 4-methylumbelliferyl-ß-D-xyloside and 4-methlumbelliferyl-α-L-arabinofuranoside.

Enzyme assays. Xylanase and ß-glucanase were assayed by incubation for 60 min at 60°C in a 1% (w/v) solution of oat spelts xylan or barley ß-glucan in 0.1 M succinate buffer, pH 6.0. Reducing sugars released from the substrate were determined with the 3,5-dinitrosalicylic acid reagent (13). One enzyme unit corresponds to the release of 1 μmole of xylose equivalent. ß-D-Glucosidase, ß-D-cellobiosidase, ß-D-xylosidase, and α-L-arabinofuranosidase activities were determined by measuring the release of p-nitrophenol from the corresponding p-nitrophenyl-glycoside. Assay mixtures (1 ml) containing 2 mM substrate in 0.1 M succinate buffer, pH 6.0, were incubated for 30 min at 60°C. Reactions were stopped by addition of 2 volumes of 1 M Na_2CO_3. The optical density of the liberated p-nitrophenol was measured at 395 nm. One unit of activity is defined as the amount of enzyme liberating 1 μmole p-nitrophenol per min.

Electrophoresis and zymogram technique. Polyacrylamide gel electrophoresis was performed in 10% polyacrylamide gels in the presence of 0.1% SDS). Xylanase activity was detected by in situ staining with Congo red of polyacrylamide gels containing 0.1% xylan (14). Glycosidases were identified by fluorescence upon incubating the gels in a 2 mM solution of the corresponding 4-methylumbelliferyl-glycoside.

Materials. Xylan (oat spelts), p-nitrophenyl-glycosides, and 4-methylumbelliferyl-glycosides were from Sigma Chemical Co. Barley ß-glucan was purchased from Biocon (Cork, Ireland).

Results

In order to detect xylanolytic genes, a genomic library of *C. stercorarium* DNA previously constructed in the cosmid vector pHC79 (9) was screened for the expression of xylanase, ß-D-xylosidase, and α-L-arabinofuranosidase. Out of 1140 clones tested, 101 positive clones were identified. These cosmid clones were divided into distinct groups on the basis of substrate range, zymogram patterns, and Southern hybridization. One representative clone from each group was further analyzed by subcloning in pUC vectors. Zymograms from subclones were compared with those from the original cosmid clones to ensure that the structural genes were kept intact.

Seven subclones were obtained and their phenotype was characterized by assaying cell-free extracts for various xylanolytic and cellulolytic enzyme activities (Table 1). The restriction maps of these clones are shown in Fig. 1. Southern hybridization of the insert DNA to genomic *C. stercorarium* DNA confirmed that the cloned DNA fragments originated from *C. stercorarium* and that no apparent DNA rearrangements had occurred during cloning (data not shown). The molecular weights of the gene products as determined from zymograms upon SDS-polyacrylamide gel electrophoresis are presented in Table 2.

Deletion analysis and DNA sequencing shows that the various xylanases expressed by pXYN10 are encoded by a single gene,

Table 1. Substrate specificity of enzymatic activities expressed by recombinant clones

Clone	Specific activity (mU/mg protein)				
	Xylan	ß-Glucan	PNPC[a]	PNPX[b]	PNPA[c]
pXYN10	3140	12	0	0	0
pCEL10	1280	150	430	0	0
pCEL20	1120	125	390	0	0
pBXL10	0	0	0	30	0
pBXL20	0	0	2	740	120
pARF10	0	0	0	95	140
pARF20	0	0	0	0.4	210

[a] p-Nitrophenyl-ß-D-cellobioside; [b] p-nitrophenyl-ß-D-xyloside; [c] p-nitrophenyl-α-L-arabinofuranoside.

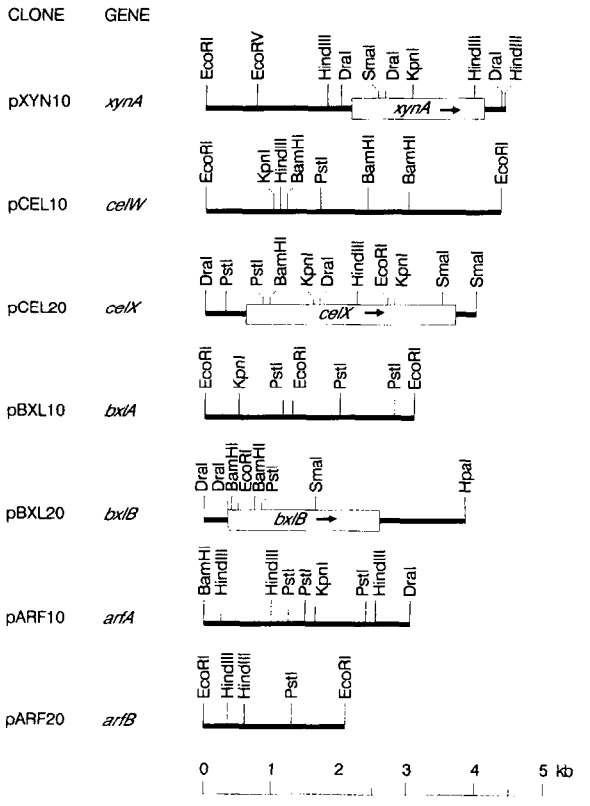

Fig. 1. Restriction maps of cloned *C. stercorarium* DNA inserts expressing xylanolytic enzymes. Open boxes indicate the positions of genes determined by DNA sequence analysis. The arrows denote the direction of transcription.

designated *xynA* (unpublished results). The encoded isoenzymes were characterized as endo-1,4-ß-xylanases devoid of other enzymatic activities. On the other hand, plasmids pCEL10 and pCEL20 express unrelated enzymes with broad substrate specificities, which hydrolyze xylans, ß-glucans, and ß-cellobiosides. The gene present on pCEL20 was found to be identical with the previously isolated ß-cellobiosidase gene *celX* (9). Activity staining revealed that xylanase and ß-D-cellobiosidase activities reside in the same polypeptides of 80 kDa and 120 kDa, respectively.

Plasmids pXL10 (*bxlA*) and pXL20 (*bxlB*) code for ß-D-xylosidases, both of which hydrolyzed xylobiose. It should be noted that the *bxlB* gene product also showed significant α-L-arabinosidase activity (Table 1). The glycosidase ecoded by the *arfA* gene of pARF10 was active against aromatic α-L-arabinosides and ß-D-xylosides but devoid of xylobiase activity. On the other

Table 2. Enzyme designation and molecular weight of gene products

Gene	Encoded enzyme	Molecular weight of gene product(s)
xynA	Endo-ß-1,4-xylanase	70.000, 62.000, 42.000
celW	Celloxylanase	42.000
celX	Celloxylanase	120.000, 80.000
bxlA	ß-D-Xylosidase	ND[a]
bxlB	ß-D-Xylosidase	85.000
arfA	α-L-Arabino/ß-D-xylosidase	49.000
arfB	α-L-Arabinofuranosidase	30.000

[a] ND, Not determined. Enzymatic activity was not detectable in zymograms after SDS-polyacrylamide gel electrophoresis.

hand, the *arfB* gene of pARF20 expressed a specific α-L-arabinosidase with little or no activity towards other arylglycosides.

Discussion

Several xylanase isoenzymes ranging in size from 72 kDa to 42 kDa have been purified from *C. stercorarium* culture supernatants (4,5). However, their immunological cross-reactivity suggests that these isoenzymes arose by partial proteolysis of a common precursor (4). Expression of the *xynA* gene in *E. coli* also yielded multiple protein species with molecular weights between 70.000 - 42.000. DNA sequencing revealed that the cloned DNA fragment contains a single open reading frame translating into a protein of 70.7 kDa (unpublished results). This strongly suggests that the *xynA* gene codes for the precursor of the major xylanase activities of *C. stercorarium*.

Both the *celW* and *celX* gene encode enzymes which hydrolyze xylans as well as ß-cellobiosides and ß-glucans. This novel type of enzyme has been designated "celloxylanase" (15) implicating a function both in xylan and cellulose degradation. Enzyme with similar substrate specificities are also produced by other cellulolytic bacteria (16-19). Two celloxylanases with molecular weights of about 80.000 (celloxylanase I) and 120.000 (celloxylanase II) have been purified from *C. stercorarium* (5,6). Their molecular weights and enzymatic properties correspond closely to those of the *celX* gene products expressed in

E. coli. The presence of a single long open reading frame encoding a protein of 115.3 kDa was confirmed by DNA sequence analysis (unpublished results). Celloxylanase II might therefore represent the primary *celX* translation product, which is converted to celloxylanase I by proteolytic cleavage.

The observed properties of the *bxlB* gene product agree with those of the ß-D-xylosidase induced by growth of *C. stercorarium* on xylan (5). On the other hand, the major α-L-arabinosidase activity of *C. stercorarium* has not been sufficiently characterized to establish its role in arabinoxylan hydrolysis and its relationship to the cloned *arf* genes.

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