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MOLECULAR PHYSIOLOGY OF XYLAN DEGRADATION

BY THERMOANAEROBES

By

Yong -Eok Lee

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

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ABSTRACT

MOLECULAR PHYSIOLOGY OF XYLAN DEGRADATION

BY THERMOANAEROBES

By

Yong -Eok Lee

Physiological, biochemical and molecular mechanisms of xylan degradation were investigated in *Thermoanaerobacter* strain B6A-RI, which was described as a new thermoanerobe species. This organism produced endoxylanase, β-xylosidase, arabinofuranosidase, acetyl esterase and xylose isomerase, and the first three activities were produced coordinately. When grown on xylan, the xylanolytic enzyme activities were cell associated rather than secreted. The organism produced multiple endoxylanases which were glycosylated and thermostable. Endoxylanase (*xynA*), β-xylosidase (*xynB*) and xylose isomerase (*xylA*) genes were cloned into *Escherichia coli* and sequenced, and the expressed enzymes were purified and characterized. *XynB* and *xynA* but not *xylA* were oriented in the same direction on the 28 kb DNA fragment. The thermostable β-xylosidase hydrolyzed xylopentose, xylotriose, xylobiose and PNPX, but had no activity on xylan. The deduced amino acid sequence of xylose isomerase showed very high homology to those from thermoanaerobes but not mesophilic aerobes. *xynA* gene was similar to genes from family F β-glycanases grouped by hydrophobic cluster analysis. Endoxylanase A cleaved xylan and xylooligosaccharides, but not xylobiose. Deletion of part of the N-terminal region of *xynA* resulted in a

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mutant enzyme that retained activity but lost thermostability. Deletion of part of the C-terminus did not alter thermostability or activity. Individual substitution, by site directed mutagenesis, of Asp-504 and Asp-569 by asparagine and Glu-567 by glutamine completely destroyed endoxylanase activity, and implicated their role in the general catalysis mechanism predicted for hydrolytic enzymes such as lysozyme. Negatively charged cell surface structures were visualized on cells grown on xylan by scanning and transmission electron microscopy. The presence of these putative xylanosome structures coincided with the production of cell bound endoxylanases and the ability of the cells to bind tightly to xylan. The xylanosome was specific for cellular adhesion to and degradation of xylan but not cellulose.

To my late father, Jung-Woo Lee, my mother, Young-Sook Kim,
my wife, Pyung-Ok Lim, and my son, John Lee
with love

To

To my late father, Jung-Woo Lee, my mother, Young-Sook Kim,
my wife, Pyung-Ok Lim, and my son, John Lee

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ACKNOWLEDGMENTS

I would like to acknowledge my gratitude to the many people who have made the completion of this dissertation possible.

First and foremost I want to thank Dr. J. G. Zeikus for his guidance and intellectual support throughout this project. He gave me a opportunity to study and allows me to develop and mature as a scientist.

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I am very grateful to my parents for their love and support.

Words cannot express my thanks to my wife, Pyung-Ok, for all of her understanding, patience, friendship and love. Without her emotional encouragement the completion of this degree would not have been possible.

And most importantly, thanks and praise to the LORD, who truly " causes all thing to work for good for those who love Him".

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G+C	guanine-plus-cytosine
kDa	kilo-dalton
MOPS	3-(N-morpholino)propanesulfonic acid
MES	2-(N-morpholino)ethanesulfonic acid
PAS	periodic acid, Schiff's reagent
TLC	thin layer chromatography
CMC	carboxymethyl cellulose
ORF	open reading frame
kb	kilo-base
Mr	molecular weight
S.D.	Shine-Dalgarno
PNPX	p-nitrophenyl-b-D-xylopyranoside
FPLC	fast performance liquid chromatography
EDAC	1-(3-dimethylaminopropenyl)-3-ethylcarbodiimide hydrochloride

RBB-xylan

Tm

HEPES

SDS

PAGE

G+C

kDa

MOPS

MES

PAS

TLC

CMC

ORF

kb

Mr

S. D.

PNPX

FPLC

EDAC

ABBREVIATIONS

RBB-xylan	4-O-methyl-O-glucurono-D-xylan-Remazole Brilliant Blue R
Tm	midpoint thermal denaturation temperature
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
G+C	guanine-plus-cytosine
kDa	kilo-dalton
MOPS	3-(N-morpholino)propanesulfonic acid
MES	2-(N-morpholino)ethanesulfonic acid
PAS	periodic acid, Schiff's reagent
TLC	thin layer chromatography
CMC	carboxymethyl cellulose
ORF	open reading frame
kb	kilo-base
Mr	molecular weight
S. D.	Shine-Dalgarno
PNPX	p-nitrophenyl-b-D-xylopyranoside
FPLC	fast performance liquid chromatography
EDAC	1-(3-dimethylaminopropenyl)-3-ethylcarbodiimide hydrochloride

Chapter I

Introduction and Objectives

INTRODUCTION

A. Structure and composition of lignocellulosic materials

In recent years there has been considerable interest in the utilization of plant material as a source of fuels and chemicals. Plant material is composed mainly of cellulose, hemicellulose and lignin. For example, wheat contains approximately 25-40% cellulose, 25-50% hemicellulose and 10-30% lignin (16). Cellulose is a linear polymer of glucose molecules linked in the β-1,4 configurations. Cellulose is organized in laminar crystallites which are bundled into microfibrils in the plant cell wall. Each microfibril contains regions of amorphous cellulose and is coated and intertwined with hemicellulose. Hemicellulose or xylan is a linear heteropolymer of xylose, possessing side chains of 4-O-methyl-D-glucuronic acid and L-arabinose, which is also linked in the β-1,4-configuration. The hemicellulose is in turn crosslinked to lignin which is a polymer of p-hydroxyphenylpropane units connected by C-C and C-O-C linkages.

B. Hemicellulose

[1] Abundance of hemicellulose

Hemicellulose is the term given to low molecular weight polysaccharides that occur together with cellulose in plant tissues. Hemicellulose comprises up to one third of the content of dry cellulosic biomass (97). After cellulose, it is the next most abundant renewable polysaccharide in nature. Xylan, a major component of plant hemicellulose, can be converted to monosaccharides by enzymatic hydrolysis. However, xylan-degrading enzyme systems have been studied less than the cellulose-

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INTRODUCTION

A. Structure and composition of lignocellulosic materials

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degrading enzyme systems. One of the reasons for this might be the heterogeneous composition of hemicellulose. The hydrolysis of this polymer does not lead to a single product, differing from the degradation of cellulose, which results in the formation of glucose. Presently, large amounts of hemicellulosic materials are wasted or dumped, contributing to pollution from the effluent of mills in the pulp and paper industry.

[2] Structure and composition of hemicellulose

Unlike cellulose, hemicelluloses show variability in both structure and composition and are heteropolymers of various hexoses, pentoses and uronic acids. Most hemicelluloses contain 2 to 6 different sugar residues and are often classified according to the major carbohydrate backbone, for example, as glucans, xylans, mannans, galactans and galacturonans (96). Mannans are the predominant hemicellulose in gymnosperms, whereas xylans predominate in angiosperms (97).

C. Occurrence, structure and composition of xylans

Xylans are the most abundant noncellulosic polysaccharides in angiosperms, where they account for 20 to 30% of the dry weight of woody tissue. In gymnosperms, where galactoglucomannans and glucomannans form the major hemicelluloses present, xylans are less abundant, accounting for about 8% of tissue dry weight (2). A substantial proportion of all known xylans are acidic due to the attachment of 4-O-methyl-D-glucuronic acid groups. Typical xylans from gymnosperms, for example, contain 14 to 18% 4-O-methyl-D-glucuronic acid, with low variable levels of L-arabinofuranose side chains (101).

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β-1,4-Xylans are mainly found in secondary walls, the major component of mature cell walls in woody tissue (97). Although they also represent the major hemicellulose in the primary walls of monocots, xylan generally constitutes a minor component of the primary wall in dicots (2, 64). Xylans from monocots are of two types; highly branched arabinoxylans, containing no glucuronic acid, as found in cereal endosperms, and less-branched arabinoxylans with uronic acid and galactose side chain types occurring in more highly lignified tissue (104).

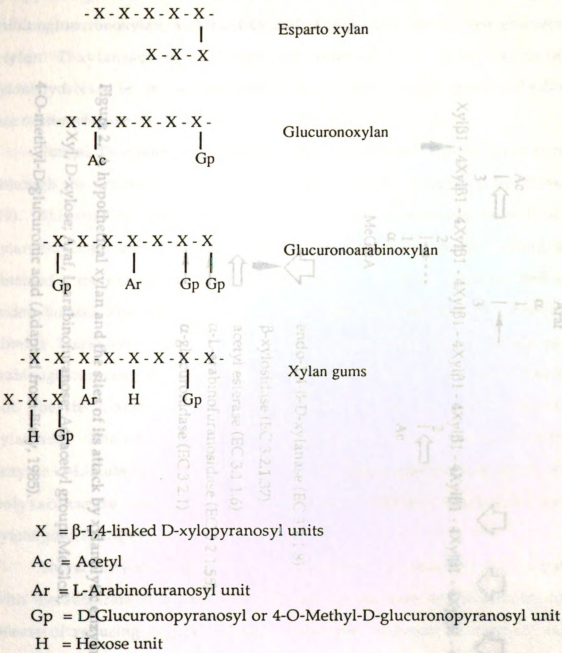
The backbone of xylan consists of a polymer of β-1,4-linked xylopyranose units. Side chains are linked to the main chain via β-1,6-xylopyranose, α-1,3-arabinofuranose, or α-1,2-(4-O-Me)-glucosyluronate bonds (96). Figure 1 shows possible configurations for various xylans.

D. Enzymes involved in the degradation of xylan

Complete breakdown of a branched acetyl xylan requires the action of several hydrolytic enzymes (Fig. 2). The best known are endo-1,4-β-xylanase (xylanase), which attack the polysaccharide backbone, and β-xylosidase, which hydrolyze xylooligosaccharides to D-xylose. The resulting D-xylose is isomerized to xylulose by xylose isomerase and further catabolized after phosphorylation by xylulokinase. The xylanolytic enzymes have recently been reviewed elsewhere (9, 19, 105).

[1] D-xylanases

D-xylanases have been reported to occur in bacteria, fungi and protozoa (19). In general, D-xylanases are predominantly extracellular in bacteria and fungi though some microorganisms, for example, rumen bacteria (15, 40) and protozoa (3) also have intracellular D-xylanas



X = β-1,4-linked D-xylopyranosyl units

Ac = Acetyl

Ar = L-Arabinofuranosyl unit

Gp = D-Glucuronopyranosyl or 4-O-Methyl-D-glucuronopyranosyl unit

H = Hexose unit

Figure 1. Possible configurations for xylans (Adapted from Thompson, 1983).

Xyl, D-xylose; Araf, L-arabinofuranose; Ac, acetyl group; MeGlcA, 4-O-methyl-D-glucuronic acid (Adapted from Biely, 1985).

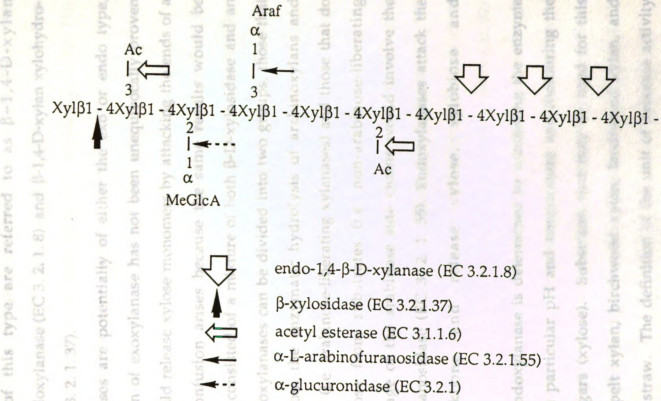


Figure 2. A hypothetical xylan and the sites of its attack by xylanolytic enzymes: Xyl, D-xylose; Araf, L-arabinofuranose; Ac, acetyl group; MeGlcA, 4-O-methyl-D-glucuronic acid (Adapted from Biely, 1985).

D-xylanases are enzymes capable of hydrolyzing the β-1,4-D-xylopyranosyl linkages of the β-1,4-D-xylans, namely arabinoxylan, arabinoglucuronoxylan, arabino-4-O-methyl-D-glucuronoxylan and glucuronoxylan. D-xylanases of this type are referred to as β-1,4-D-xylan xylanohydrolase or endoxylanase (EC 3. 2. 1. 8) and β-1,4-D-xylan xylohydrolase or exoxylanase (EC 3. 2. 1. 37).

Fungal D-xylanases are potentially of either the exo or endo type, although the production of exoxylanase has not been unequivocally proven (19). This enzyme would release xylose monomers by attacking the ends of a xylan molecule and confusion arises because the same results would be obtained if the enzyme consisted of a mixture of both β-D-xylosidase and an endoxylanase. The endoxylanases can be divided into two groups, those that liberate L-arabinose from the enzymatic hydrolysis of arabinoxylans and arabinoglucuronoxylans (i.e. arabinose-liberating xylanases) and those that do not liberate L-arabinose from substrates (i.e. non-arabinose-liberating xylanases). The cleavage of the arabinose side chains would involve the enzyme α-L-arabinofuranosidase (EC 3. 2. 1. 55). Endoxylanases attack the polysaccharide backbone and release xylose, xylobiose and xylooligosaccharides.

The activity of endoxylanase is determined by incubating the enzyme with the substrate at a particular pH and temperature and measuring the release of reducing sugars (xylose). Substrates that may be used for this purpose include oat spelt xylan, birchwood xylan, larchwood xylan, and arabinoxylan from rice straw. The definition of the unit of xylanase activity usually given is the amount of enzyme required to produce 1 μmol of reducing sugar in a specified time under the conditions of the assay.

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Endo-1,4-β-D-xylanases usually have pH optima in the region pH 3.5 to 6.5. The enzymes produced by fungi have pH optima in the range 3.5 to 5.5. pH optima of bacterial xylanases tend to be around 6.0 (19, 101, 105). Temperature optimum and temperature stability values may vary significantly with source (Table 1). Thermostable xylanases have been reported from a number of microorganisms including *Bacillus* sp. C-125 (39), *Clostridium acetobutylicum* (54), *Clostridium stercorarium* (5), *Caldocellum saccharolyticum* (57), *Clostridium thermocellum* (33), *Streptomyces lividans* (66) and *Trichoderma harzianum* (93, 94).

The molecular weight of xylanases varies greatly depending on the microbial source (105). Generally the molecular weights of the xylanases like those of other hemicellulases, are relatively low, ranging from 15 kDa to 85 kDa (Table 1).

[2] β-D-Xylosidase (EC 3. 2. 1. 37)

β−D-Xylosidase hydrolyzes xylose units from the nonreducing terminus of D-xylan chains with the products retaining configuration (20, 92). β-xylosidase activity decreases with increasing xylooligosaccharide chain length (40, 92). β-xylosidase also have substantial transferase activity (20, 92). The enzyme is usually assayed colorimetrically since it hydrolyzes p-nitro-β-D-xylopyranoside to D-xylose and p-nitrophenol. Activity can also be assayed by detecting cellobiose hydrolysis leading to the formation of glucose. Some of the microorganisms reported to produce β-D-xylosidase are listed in Table 2.

[3] Other enzymes liberating the side-chain sugars and their synergistic effect on xylan degradation

Table 1. General properties of xylanases from bacteria and fungi

Organism	Strain	Mol wt[a]	Optimum pH	Optimum temp (°C)	pI	End product	Reference
Aspergillus niger	14	24,000	4.0	50	4.2	X1, X2, X3	31, 32
Bacillus circulans	WL-12	15,000	5.5-7.0	ND	9.1	X2, X3, X4	21
		85,000	5.5-7.0	ND	4.5	X1, X2, X3	
B. pumilus	IPO	24,000	6.5	45-50	ND	X2, X3	77
B. stearothermophilus	21	39,500	7.0	60	4.83	X2, X3	68
B. subtilis	PAP115	32,000	5.0	50	ND	X1, X2, X3	7
C. acetobutylicum	ATCC 824	29,000	5.5-6.0	60	8.5	X2, X3, X4	54
		65,000	5.0	50	4.4	ND	
C. stercorarium		44,000	6.5	75	4.5	X2, X3	5
		62,000	6.0	75	4.4	X2, X3	
		72,000	6.0-7.0	75	4.4	X2, X3	
C. thermolacticum		39,000	6.0-6.5	80	4.9	X2, X3	17

[a]Data from SDS-PAGE

X1 = xylose, X2 = xylobiose, X3 = xylotriose

Table 1. General properties of xylanases from bacteria and fungi (cont.).

Organism	Strain	Mol wt[a]	Optimum pH	Optimum temp (°C)	pI	End product	Reference
Streptomyces sp.	3137	25,000	5.0-6.0	60-65	10.3	X1, X2	60
		25,000	5.0-6.0	60-65	10.1	X1, X2	
		50,000	5,5-6.5	60-65	7.1	X1, X2	
S. lividans		43,000	6.0	60	5.2	X1, X2	66
Schizophyllum commune		22,000	5.0	50	4.0	X1, X2	74
Trichoderma harzianum		20,000	5.0	50	9.4	X1, X2	106
T. longibrachiatum	Rifai	21,500	5.0	55	9.45	X2, X3	82
		33,000	5.0	60	9.25	X1, X2	
T. viride		22,000	5.0	53	9.3	X1, X2	99
Thermoascus aurantiacus	C436	32,000	5.1	80	7.1	X2	95

[a]Data from SDS-PAGE

X1 = xylose, X2 = xylobiose, X3 = xylotriose

Table 2. General properties of the purified β–xylosidases from different microorganisms

Organism	pH optimum	Temp optimum (°C)	pI	Mol wt [a]	Mol wt of subunit [b]	Reference
Aspergillus niger	6.7-7.0	42	ND	78,000		43
A. fumigatus	4.5		75	5.4	360,000	48
Bacillus pumilus	7.0	ND	4.4	130,000	70,000	76
B. stearothermophilus	6.0	70	4.1	150,000	75,000	68
C. acetobutylicum	6.0-6.5	45	5.85	224,000	85,000, 63,000	53
Chaetomium trilaterale	4.5	ND	4.86	240,000	118,000	100
Emericella nidulans	4.5-5.0	55	3.25	240,000	116,000	62
Penicillum wortmanni	3.3-4.0	ND	5.0	100,000		20
Trichoderma viride	4.5	55	4.45	102,000		62

[a] Data from gel-filtration

[b] Data from SDS-PAGE

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Relatively little is known about the enzymes which liberate the side-chain sugars, which include α-L-arabinofuranosidase and α-glucuronidase, or the enzymes involved in the degradation of acetyl xylan, such as acetyl esterase.

α-L-Arabinofuranosidases have been reviewed by Kaji (44), who subdivided them into two groups, an *Aspergillus niger* type of α-L-arabinofuranosidase, which can hydrolyze the terminal non-reducing α-L-arabinofuranoside residues from arabinoxylans, and a *Streptomyces purpurascens* type of α-L-arabinofuranosidase, which cannot hydrolyze terminal non-reducing α–L-arabinofuranoide residues from arabinoxylan. There are claims that α-L-arabinofuranosidase activity is an inherent property of some xylanase (19). α-L-Arabinofuranosidases have been purified from several microorganisms (34, 38, 52) and cooperative interactions between α-L-arabinofuranosidase and xylanase have been demonstrated (34).

α–Glucuronidase was detected from *Agaricus bisporus* and *Pleurotus ostreatus*. These enzymes act synergistically with xylanases and liberates 4-O-methylglucuronic acid from 4-O-methylglucuronic acid-substituted xylooligomers (80).

Esterases that deacetylate acetyl xylan have been reported to occur in fungi (12, 79) and bacteria, including *Fibrobacter succinogenes* (63), and *Caldocellum saccharolyticum* (56). The fungal esterases act synergistically with xylanases in the degradation of acetyl xylan. In the absence of esterase, xylanases break only a limited number of glycosidic bonds. Both the rate and the extent of breakdown of xylan increases when esterase is present. The two activities also act synergistially to liberate acetyl residues (10).

[4] Xylose isomerase

Xylose isomerase (EC 5. 3. 1. 5) is an intracellular enzyme that catalyzes the isomerization reaction between D-xylose and D-xylulose during xylose metabolism in various microorganisms. Because this enzyme can also convert D-glucose to D-fructose, the enzyme is often referred to as glucose isomerase (91). Production of glucose isomerase has been reported from a large number of bacteria and actinomycetes that can grow on xylose as an energy source. Among these, *Streptomyces* species have been studied most extensively and used as a source of industrial enzymes (90, 91). Most microorganisms require xylose to induce glucose isomerase production and enzyme synthesis is catabolite repressed by glucose. Most purified xylose isomerases have molecular weights ranging from 80,000 to 190,000 and are composed of 2 or 4 identical subunits (1, 14). The pH optima for different xylose isomerases lie in the range of 6.5 to 9.5 (14). Temperature optima for enzyme activity and stability vary greatly depending on the source of xylose isomerase. Xylose isomerase is a metalloenzyme, and it requires a divalent cation such as Mn^{++}, Co^{++}, or Mg^{++} for both catalytic activity and enzyme stability.

E. Occurrence of multiple xylanases in microorganisms

Multiple xylanases have been reported in several microorganisms (105). Five different xylanases have been purified and characterized from *Aspergillus niger* species (24, 25, 42, 43, 86). The occurrence of multiple xylanases have also been detected in *Clostridium stercorarium* (5). *Streptomyces* species (51, 60, 67), *Trichoderma harzianum* (93, 94, 106), *Aeromonas* sp. strain 212 (69) and *Penicillium janthinellum* (92.

The extent of xylanase multiplicity in microorganisms remains to be answered, particularly since a zymogram technique has detected five major and ten minor xylanases in the culture filtrate of *Aspergillus niger* 14 (11). The nature and relevance of minor xylanases may not be determined, since often the major xylanolytic activities are the enzymes purified. Minor xylanases may have functions that are not required in large quantities such as hydrolysis of bonds which are not prevalent within the polymer. These minor xylanases may be produced in larger amounts if the culture conditions were altered, or they may be lost during the purification procedure either due to adsorption on to the substrate, or degradation.

Xylan is a heteropolymer and its structure differs from plant to plant. Therefore the production of multiple xylanases, with each enzyme with having specialized functions, may be one strategy whereby a microorganism achieves efficient xylan hydrolysis. However the regulation, the substrate cross-specificity (87, 98), and the posttranslational modifications of these enzymes must be considered, because each of these factors can result in the occurrence of multiple enzymes with xylanolytic activity.

In fungi, some of the multiple xylanases may also be allozymes, products of different alleles of the same gene. Multiple xylanases may be proteolytic degradation products or differential glycosylation products (5, 6, 24, 25, 50, 110). In some cases, each of the multiple xylanases may be a distinct gene product as found with *T. harzianum* which has three distinct xylanases (106), *Bacillus* sp. C-125 with at least two types of xylanases (39), and *C. acetobutylicum* with two different endoxylanases which have been characterized (54).

Multiplicity in β-xylosidase also has been suggested by the detection of multiple β-xylosidases in *A. niger* 14 (81), in *Trichoderma harzianum* E58

with two β-xylosidases (106), and the presence of four β-xylosidases from *Penicillium wortmanni* IFO 7237 (61).

The complexity of xylan must be considered in questions concerning xylanase multiplicity. Multiple xylanases and β–xylosidases may be more effective in the hydrolysis of xylan due to the heterologous nature of this polymer, as the different forms may also have varying abilities to interact with other xylanases in xylan hydrolysis. The significance of multiple xylanases may lie in the hydrolysis of other xylose-containing polysaccharides, or of secondary substrates. As xylan is important in fiber cohesion, the primary functions of multiple xylanases may in fact relate to disruption of the fibers and exposure of the other lignocellulosic components to other hydrolases. Such functions might relate to debranching and transferase activity in some xylanases rather than in hydrolysis of xylosidic linkages alone. In addition the possession of a number of enzymes which catalyze the same reaction, but which function under different conditions, such as temperature or pH, may be of great advantage to the organism when competing for substrates in a changing environment.

F. Regulation of xylanases

In non-cellulolytic bacteria and yeasts, xylanolytic enzymes appear to be inducible: xylanase and β-xylosidase are produced in high amounts during growth on xylan and synthesis of the enzyme is catabolite repressed by well metabolized carbon sources such as glucose or xylose (9). Xylan cannot enter the cells, so that the signal for accelerated synthesis of xylanolytic enzymes must involve low molecular weight fragments, such as xylobiose and xylotriose. The oligosaccharides are formed by hydrolysis of xylan in the medium by small amounts of enzymes produced constitutively (9).

G. Biotechnology of xylanases

[1] Bioconversion of hemicellulose to chemicals

Bioconversion of lignocellulose to fermentative products requires that the highest yield of various polymeric sugars is obtained. Current thought holds that the complete xylanolytic (and cellulolytic) systems are required to achieve maximum hydrolysis of this complex substrate, to small oligosaccharides and monomers. For the utilization of hemicellulose, such a system would require xylanases, β-xylosidases and other hemicellulases. This could be achieved in a number of ways, either by using the xylanase system of a very effective xylanolytic microorganism, or alternatively using a combination of specialized enzymes from different microorganisms, or from microbes grown under different conditions for enzyme induction. With the current research into alteration of catalytic activity through the use of site-directed mutagenesis, protein engineering may provide new enzymes with unique properties which would facilitate in the bioconversion of lignocellulose.

[2] Biobleaching

In paper manufacturing, xylanase treatment has been suggested because it can reduce energy cost of refining pulp (73, 78) and eliminate the formation of toxic compounds. Multiple xylanases will probably be required to maximize xylan removal from these pulps (105). The feasibility of using xylanase for cellulose purification during rayon manufacture, would be highly dependent on the intrinsic selectivity of the enzymes, the absence of cellulolytic activities, and the availability of enzyme preparations in inexpensive bulk quantities (105). One strategy for obtaining cellulase-free xylanase is the isolation of xylanolytic microorganisms which are free of

cellulases. This could be achieved by one of two means, the isolation of cellulase deficient mutant strains, or alternatively another strategy would be the cloning of xylanase genes into noncellulolytic organisms such as *Escherichia coli* which has been achieved from numerous organisms (7, 50, 65, 75, 83, 88, 111). This could result in the overexpression of the enzyme and excretion from the cell, which would be advantageous due to the ease of working with cell-free systems.

[3] Other applications of xylanases

Along with cellulases and pectinases, the use of xylanase has also been suggested in applications such as the clarification of juices (9, 18), the preparation of dextrans for use as food thickeners (96), and the production of fluids and juices from plant materials (9, 107). Also there are some applications for xylanases in the baking of high fiber breads to enhance rising and water retention for the manufacture of low calorie bread (58), and for the pretreatment of animal feed to enhance fiber utilization and reduce manure production.

[4] Thermostable xylanases for industrial processes.

The efficient use of enzymes in many industrial processes requires that the overall amount of substrate converted per unit quantity of enzyme is maximized. Reaction temperature is an important physical factor that can be optimized. For every 10°C increase in temperature, reaction rates approximately double. Therefore, using thermostable enzymes, the amount of enzyme required can be reduced, or the conversion time shortened. There are a number of advantages of running processes at elevated temperatures, microbial contamination can be reduced at higher temperatures, and

solubility of substrates can be increased. Thermophilic organisms are good sources of thermostable enzymes and recombinant DNA technology provides the opportunity for overproduction of these enzymes by cloning of the genes into mesophilic hosts, with the production of enzymes which usually retain thermostability and can be purified almost to homogeneity in a one step heat treatment. Protein engineering techniques such as site-directed mutagenesis can also produce catalytically more efficient enzymes or enzymes specific in activity, for industrial applications.

H. Molecular biology of xylan degradation

[1] Cloning of the genes involved in xylan degradation

Recombinant DNA technology offers opportunities for the isolation and molecular characterization of individual genes encoding for xylanolytic enzymes. Cloning of hemicellulases could facilitate xylan bioconversion by introducing a number of different enzymes into one organism to provide numerous activities required to degrade this heterologous substrate, creating a hemicellulolytic enzyme system. Such a system could be introduced into an industrially important xylose-fermenting organism so that the direct fermentation of xylan would be possible.

All the cloning work published so far has been restricted to bacterial genes. Several xylanases from *Bacillus* sp. have been cloned and expressed in *E. coli* (108, 109). Xylanases from *Aeromonas* sp. (50), *Bacteroides* spp. (88, 102, 103), *Butyrivibrio fibrisolvens* (55, 59), *Fibrobacter succinogenes* (41), *Ruminococcus flavefaciens* (22), *Cellulomonas* sp. (8) and *Clostridium* sp. (23, 111) were also cloned and characterized. Relatively few xylanases have been cloned from thermophilic organisms (28, 57).

In comparison to the studies on xylanases there have been only a few reports on the cloning of β-xylosidase genes. Two β-xylosidase genes were isolated from *Bacillus pumilus* (76) and cloned and expressed in *E. coli*. β-Xylosidases from *Butyrivibrio fibrisolvens* (85) and from *Caldocellum saccharolyticum* (57) were also cloned and expressed in *E. coli*.

[2] Domains in xylanases

Cellulases and xylanases hydrolyze β-1,4-glycosidic bonds between pyranose units, but they show subtle differences in specificity. Structural features common to such enzymes may be related to their general catalytic activities as glycosidases. Those peculiar to each enzyme may be related to their specificities, i.e., exoglycanase or endoglycanase, cellulase or xylanase. Such features should be reflected in the amino acid sequences of the enzymes. Cellulases contain two distinct functional domains, a catalytic domain (CD) and a cellulose-binding domain (CBD) (4, 29, 71), but the existence of similar domains in xylanases remains to be elucidated.

Cellulases and xylanases can be grouped into nine families of related enzymes on the basis of amino acid sequence identities in their putative catalytic domains (4, 37, 49) and hydrophobic cluster analysis (30). Most xylanases belong to either family F or family G. Family F xylanases are usually large molecular weight proteins and some of them have cellulase activity. Family F β-1,4-glycanases include *xyn*A from *Bacillus* sp. strain C-125 (36), *xyn*A from *Butyrivibrio fibrisolvens* (59), *Cel*B, *xyn*A and ORF4 from *Caldocellum saccharolyticum* (57, 84), *cex* from *Cellulomonas fimi* (70), *xyn*Z from *Clostridium thermocellum* (33), *xyn* from *Cryptococcus albidus* (13), *xyn*A and *xyn*B from *Pseudomonas fluorescens* subsp. *cellulosa* (35, 46) and *xyn* from *Thermoascus aurantiacus* (89). Family G xylanases are small

molecular weight enzymes and include *xyn* from *Bacillus circulans* (109), *xynA* from *B. pumilus* (27), *xyn* from *B. subtilis* (72) and *xynB* from *Clostridium acetobutylicum* (112).

It seems that various β-1,4-glycanases arose from a few progenitor sequences by mutation and domain shuffling. In this regard, it should be noted that some enzymes show a mixed specificity in that enzymes that hydrolyze β-1,4 bonds in cellulosic substrates may also hydrolyze xylan, chitin, and related substrates. This could in part provide an explanation as to the significant homology in the putative catalytic domains of cellulases and xylanases.

I. Objectives

This research was undertaken because little is known about the physiological, biochemical and molecular genetic features of xylanolytic enzymes from thermophilic bacteria that are known to produce intrinsically thermostable and thermoactive enzymes (e.g., amylase, cellulase, protease, or alcohol dehydrogenase).

In order to elucidate the molecular features of xylanases produced by thermoanaerobes, their physiological function and compartmentalization, their biochemical mode of action, and molecular characteristics of the genes involved in xylan degradation, the following major research objectives were undertaken:

(i) Isolation and general characterization of xylanolytic microorganisms for selection of a model thermoanaerobe.

(ii) Physiological characterization and regulation of xylanase activity in a model organism.

(iii) Purification and biochemical characterization of thermophilic endoxylanase, β-xylosidase and xylose isomerase activities.

(iv) Cloning, organization, sequencing, and expression of endoxylanase, β-xylosidase and xylose isomerase genes.

(v) Identification of the catalytic and thermostability domains of endoxylanase.

(vi) ultrastructural studies to characterize any cell surface features involved in xylan adhesion and hydrolysis.

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Chapter II

Deoxyribonucleic acid homology and other comparisons among xylanase producing thermoanaerobic bacteria, with a proposal for new taxonomic designations

ABSTRACT

Two thermophilic, anaerobic xylan degrading bacteria, B6A-RI and LX-11 were isolated from Frying Pan Springs in Yellowstone National Park. The organisms grew chemoorganotrophically utilizing xylan and starch, but not cellulose, and a number of di- and monosaccharides including glucose and xylose. Both organisms had the same optimum temperature and pH for growth of 60°C and pH 6.0 respectively. Fermentation products included acetate, ethanol, lactate, CO_2 and H_2. Both organisms grew as rods, although B6A-RI formed longer cells than LX-11, and both deposited sulfur on the cells. The major difference between the two organisms was in spore formation, LX-11 was found to sporulate whereas B6A-RI was not. Comparison of LX-11 and B6A-RI was made with other thermophilic anaerobic xylan degrading bacteria in respect to DNA:DNA hybridization and total protein analysis in order to determine the relatedness of these organisms. From these findings three different groups emerged and new taxonomic assignments were proposed. *Clostridium thermocellum* LQRI was least related to the other seven strains and is placed in group I retaining its original taxonomic assignment without change. *Clostridium thermosulfurogenes* strain 4B and the new isolates B6A-RI and LX-11 are closely related and fall into group II for which a genus name *Thermoanaerobacterium* gen. nov. is given. Isolate LX-11 is given a taxonomic assignment as *Thermoanaerobacterium xylanolyticum* sp. nov. Isolate B6A-RI is named and classified as *Thermoanaerobacterium saccharolyticum* sp. nov., and *Thermoanaerobacterium thermosulfurigenes* strain 4B (original *C. thermosulfurogenes*) is the type species for the genus. Group III is termed *Thermoanaerobacter* and includes *Thermoanaerobacter ethanolicus* strain JW200, *Clostridium thermohydrosulfuricum* strain 39E

and strain E100-69, and *Thermoanaerobium brockii* strain HTD4. *T. brockii* strain HTD4 is renamed as *Thermoanaerobacter brockii* comb. nov. and *C. thermohydrosulfuricum* E100-69 is renamed as *Thermoanaerobacter thermohydrosulfuricus* comb. nov. *C. thermohydrosulfuricum* strain 39E is nearly identical to *T. ethanolicus* strain JW200 and are considered to belong to the same species. Therefore, strain 39E is renamed as *Thermoanaerobacter ethanolicus* strain 39E, with *T. ethanolicus* strain JW200 as the type strain.

INTRODUCTION

In recent years, understanding of the biology of thermophilic microorganisms has been greatly advanced. Thermophilic bacteria, which are found in both the Archaebacteria and the Eubacteria kingdoms, have been found in association with all types of thermal habitats including hot springs (3). Intensive studies on the biology of anaerobic thermophiles have been initiated in the last fifteen years in order to learn more about their function in nature, and because of their potential applications in biotechnology (3, 35). Most studies on eubacterial thermoanaerobes are focused on saccharolytic bacteria that form ethanol and lactate because of their importance in carbohydrate turnover in thermal environments and because of the utility of their thermostable enzymes in industrial processes (12). Saccharolytic thermoanaerobic eubacteria include species in the genus *Thermoanaerobacter, Thermoanaerobium* and *Clostridium*. Although several strains in these groups have been isolated and characterized, the taxonomy of these groups is uncertain and remains in a state of confusion.

Little is known on the microbial transformation of hemicellulose in thermoanaerobic habitats. Wiegel et al. (31, 34) studied a variety of thermophilic, anaerobic, saccharolytic bacteria, including *Thermoanaerobacter ethanolicus, Thermobacteroides acetoethylicus, Thermoanaerobium brockii,* and *Clostridium thermocellum,* which all ferment xylan albeit at a very slow rate. Studies were also conducted on *Thermoanaerobacter* strain B6A, an organism isolated from an algal mat present in Big Spring, Thermopolis, Wyoming (29), which was shown to extensively degrade xylan (28). Xylan, a major polysaccharide in hemicellulose, can be hydrolyzed by microbial enzymes to xylose (2).

Thermostable xylanolytic enzymes have potential applications in the pulp and paper industry (21, 22), and so thermophilic microorganisms have attracted considerable attention as sources of thermostable xylanases. In the present study we describe the general morphological, cellular and metabolic characteristics of two new xylanolytic species that were isolated from Frying Pan Springs in Yellowstone National Park, Wyoming by selective enrichment with xylan as the energy source for growth. This site was chosen because of its low pH (below pH 6.0) as we were interested in isolating xylan degrading anaerobes which grow at low pH and may have industrial importance. We also compare these strains with other described thermophilic saccharolytic anaerobes which have xylanolytic activity to investigate the taxonomic relationship between these organisms. These strains include *Thermoanaerobacter ethanolicus* (32), *Thermoanaerobium brockii* HTD4 (37), *Clostridium thermocellum* LQRI (13), *Clostridium thermohydrosulfuricum* E100-69, 39E (7, 36), *Clostridium thermosulfurogenes* 4B (25) and *Thermoanaerobacter* strain B6A (29).

Although morphological and biochemical characteristics are useful, DNA-DNA hybridization techniques have been especially successful in resolving taxonomic relatedness between closely related organisms (species level and below). In this study we performed DNA-DNA hybridization and total protein analysis to investigate the taxonomic relationships of xylanolytic thermoanaerobic species. The results of our protein profile and DNA-DNA relatedness studies indicate that there are three independent groups in saccharolytic thermoanaerobes and these results are supported by biochemical and physiological data, as well as partial 16S-rRNA cataloging studies of Bateson et al. (1). On the basis of our results, we propose new taxonomic assignments for these organisms. In addition, we propose that

Thermoanaerobacter and *Thermoanaerobacterium*, like *Clostridium*, be recognized as genera which can be comprised of sporeforming species.

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MATERIALS AND METHODS

Bacterial strains and culture conditions

The strains used in the present study are listed in Table 1. Stringent anaerobic culture techniques described previously (36) were employed for medium preparation and cultivation of organisms. Cultures were grown in crimp-sealed anaerobic tubes (Bellco Glass, Vineland, NJ) that contained 10 ml of medium with 0.5% substrate and N_2 gas headspace. Mass cultures to obtain cells for DNA isolation were carried out in a 1 L round bottom flask that contained 500 ml medium under a N_2 gas phase. *Clostridium thermocellum* LQRI was grown on GS medium (20) with 0.5% cellobiose. All other strains were grown on yeast extract (TYE) medium (36) with either glucose or xylose (0.5%) as a substrate. CM5 medium (29) without yeast extract but with vitamin solution and glucose was used to induce spore formation of strain LX-11. All cultures were incubated at 60°C without shaking. Xylanase activity was detected by the addition of the chromogenic substrate 4-O-methyl-O-glucurono-D-xylan-Remazol Brilliant Blue R (RBB) (Sigma Chemical Co., St. Louis, Mo.) to agar medium.

Isolation and enrichment

Sediments from the Frying Pan thermal acid spring in Yellowstone National Park was used for enrichment of xylanolytic thermoanaerobes. After several transfers in TYE medium with 0.5% oat spelt xylan, the enrichment culture was plated onto homologous media containing agar at 2.5% and with RBB-xylan (0.2%) as substrate, in an anaerobic glove box (Coy Lab Products, Ann Arbor, Mich.). The plates were incubated anaerobically at 60°C using a modified paint tank (8). After 3 days, the colonies which gave a

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TABLE 1. Strains used in this study.

Name as Received	Strain	Other Strain Designations[a]	Source	Reference(s)
Clostridium thermocellum	LQRI	ATCC 35609 DSM 2360	farm soil	13
Isolate	B6A-RI	ATCC 49915 DSM 7060	thermal spring	This study
Isolate	LX-11	ATCC 49914	thermal spring	This study
Thermoanaerobacter	B6A	None	thermal spring	29
Clostridium thermosulfurogenes	4B	ATCC 33743 DSM 2229	thermal spring	25
Thermoanaerobium brockii	HTD4	ATCC 33075 DSM 1457	thermal spring	37
Thermoanaerobacter ethanolicus	JW200	ATCC 31550T DSM 2246T	thermal spring	32
Clostridium thermohydrosulfuricum	39E	ATCC 33223	thermal spring	36
Clostridium thermohydrosulfuricum	E100-697	ATCC 35045T DSM 567T7	farm soil	7

[a] ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Federal Republic of Germany; T = type strain.

clear halo were picked from plates and placed into liquid media tubes in the anaerobic glove box.

Morphological and biochemical studies

Bacterial morphology and sulfur formation were investigated using a phase-contrast microscope model BHS (Olympus). Agar-coated glass slides (23) were used for photomicroscopy. To prepare specimens for electron microscopy, cells were harvested by centrifugation at 5,000 X g for 10 min and fixed overnight in 2.5% glutaraldehyde in 0.1 M Na_2HPO_4-KH_2PO_4 (pH 7.2) buffer solution at 4°C. The cells were embedded in agar and, after a buffer wash, postfixed for 1 h at room temperature in 1% OsO_4 in the same buffer described above. Cells were then dehydrated through an ethanol series, treated with propylene oxide, and embedded in either Poly/Bed 812 (Polysciences, Inc., Warrington, Pa.) or VCD/HXSA (Ladd Research Industries, Inc., Burlington, Vt.) epoxy resin. Thin sections were cut with a diamond knife mounted on an Ultratome III (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead citrate, and examined with an electron microscope (CM-10; Philips Electronic Instruments, Co., Mahwah, N.J.). Growth was determined by measuring the increase in turbidity at 660 nm with a spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). Fermentation products were measured directly in liquid or gas samples removed from the culture tubes by gas chromatography as described previously (6). Routine biochemical tests were performed by standard methods of Smibert & Krieg (27).

Preparation of high molecular weight DNA

The cells were harvested at the early exponential phase of growth and

washed with saline-EDTA (0.15 M NaCl, 1 mM EDTA, pH 8.0). DNA was isolated and purified by the method of Marmur (18). The purity of the DNA was tested by spectrophotometry from the ratio of A_{260} to A_{280}.

Determination of DNA base compositions

The midpoint thermal denaturation temperatures (T_m) were determined as described by Mandel & Marmur (17) with a Gilford Response^{TM} spectrophotometer equipped with a thermoprogram (Gilford Instrument Laboratories, Inc., Oberlin, Oh.) The temperature was raised 0.5°C/min, starting at 70°C, and denaturation was monitored at 260 nm. The guanine-plus-cytosine (G+C) contents were calculated by using the thermoprogram and the equation described by Mandel & Marmur (17). DNA of *Escherichia coli* B (Sigma Chemical Co.) (57.0 mol%G+C) was used as the reference.

DNA-DNA hybridizations

DNAs were labeled by nick translation with a commercial kit (Bethesda Research Laboratories, Gaithersburg, Md.), using [1',2',5-^{3}H]dCTP (NEN). The average specific activity obtained with this procedure was 5 x 10^5 cpm/μg of DNA. The labeled DNAs were denatured before hybridization by being heated at 100°C for 5 min and then placed on ice. The S1 nuclease procedure described by Johnson (9) was used for the DNA homology experiments. Each reassociation reaction mixture contained 10 μl of heat denatured labeled DNA (0.03 to 0.05 μg), 50 μl of denatured unlabeled DNA (20 μg), 25 μl of 5.28 M NaCl in 1 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) (pH 7.0), and 25 μl of deionized formamide. The mixtures were reassociated at 53°C (25°C below the thermal melting point in this buffer

syste

preci

filter

scint

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reant

Poly

expo

4,000

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blue

acid-c

Enzyn

mixtu

system) for 24 h. The S1-resistant duplexes and 50 μg of yeast t-RNA were co-precipitated by adding 10% trichloroacetic acid and collected on glass fiber filters (Whatman GF/C). Radioactivity was measured with a Packard gamma scintillation counter. Sheared salmon sperm DNA was hybridized against radiolabeled DNA in each experiment to determine the background level of reannealing of the test DNA.

Polyacrylamide gel electrophoresis of soluble proteins

For total cellular protein analysis, 10 ml of cultures of the late exponential growth phase of cultures were harvested by centrifugation at 4,000 X g for 15 min. The cells were washed with saline-EDTA (pH 7.0) and were suspended in 0.3 to 0.5 ml of deionized water and sonicated on ice for 5 min with an ultrasonicator (Kontes, Vineland, N.J.). The cell debris were removed by centrifugation (15,000 X g) for 5 min and the protein concentration of the supernatant determined by the method of Lowry et al. (16); 50 μg of protein from each strain was used as a sample for gel electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) by the discontinuous buffer system of Laemmli (11) was performed with a model Protean II vertical slab gel unit (Bio-Rad). The separating gel contained acrylamide at a final concentration of 7.5% and the stacking gel contained 4% acrylamide. The gel was stained with Coomassie blue (0.125% Commassie blue R-250, 50% methanol, 10% acetic acid) and destained in methanol-acetic acid-distilled water (4:1:5 vol/vol).

Enzyme assays

Endoxylanase activity was determined by incubating a 1 ml reaction mixture containing 1% oat spelt xylan (Sigma Chemical Co.) and enzyme

solution in 50 mM acetate buffer (pH 5.5) for 1 hr at 65°C. The reducing sugar formed was determined by the dinitrosalicylic acid method (19) by using D-xylose as standard. One unit of xylanase was defined as the activity releasing 1 μmol of xylose in 1 min.

RESULTS

Enrichment and isolation

Frying Pan Springs in Yellowstone National Park are located in the Sylvan Springs thermal area, and are small, shallow hydrothermal areas. Samples were taken during the summer and at this time the pH of these springs ranged from 4.5 to 6.0 and the temperature was around 60°C. Collection was made by passing a jar close along the surface of the bottom of the spring and collecting the sediment. Enrichments for thermophilic anaerobic xylan utilizing bacteria were made by subculturing in TYE medium containing xylan and transferring onto agar plates, where single colonies were picked to obtain isolates in pure culture. The two isolates studied in more detail were LX-11 and B6A-RI.

Morphology and ultrastructure

From the light microscope, morphological differences between LX-11 and B6A-RI were very apparent. The former organism forms short rods, either singly or in pairs (Fig. 1A), whereas B6A-RI is composed of much longer rods which occur in chains of varying length (Fig. 1B). Both strains utilize thiosulfate as an electron acceptor and convert thiosulfate to elemental sulfur which is deposited on the cell and in the medium (Fig. 1A and 1B). The organisms also differ in their growing patterns. In stationary phase, cells of B6A-RI become more elongated and tend to aggregate and eventually settle to the bottom of culture vessels; occurrences which are not observed with LX-11. The most striking morphological difference is that LX-11 forms spores (Fig. 2) whereas despite numerous attempts under various conditions, B6A-RI has not been found to sporulate. Approximately 20% of LX-11 cells contain

Figure 1. Phase-contrast photomicrographs of sulphur-depositing cultures of *Thermoanaerobacter* strain LX-11 (A) and strain B6A-RI (B) grown on LPBB, 0.5% (w/v) glucose, 0.1% (w/v) yeast extract and 20 mM $Na_2S_2O_3$.

Note that phase-bright sulfur accumulates in the medium and on the cells. Bar = 3 μm.

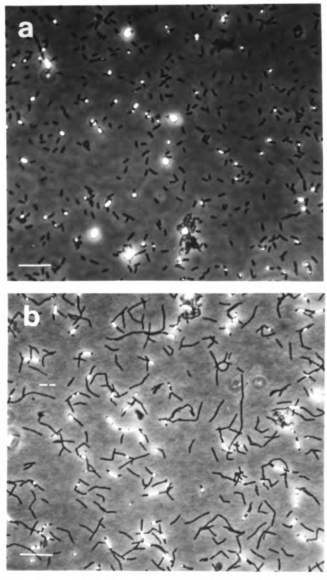
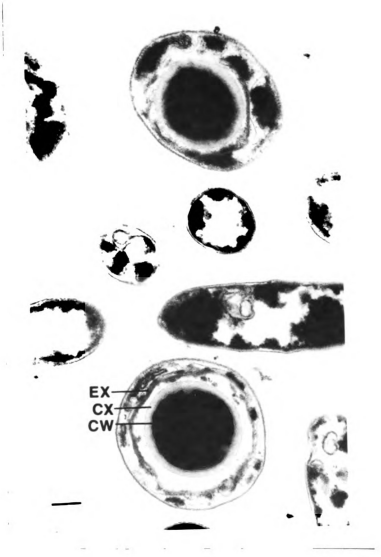

a
b

Figure 2. Electron micrograph of an endospore of *Thermoanaerobacter* strain LX-11 in thin section.

The exosporium (EX) surrounds the spore coat, which encloses the cortex (CX) and the cell wall (CW). Bar = 0.2 μm.


EX
CX
CW

spores, which are located in the terminal part of the cell. The mature spores always remained within the mother cell, rather than free in solution.

Metabolism

In order to assist in the taxonomic assignment of B6A-RI and LX-11, their characteristics were compared with those of other thermoanaerobes. The organisms selected are listed in Table 1. LX-11, B6A-RI, and B6A utilized the same range of substrates, growing well on xylan and starch but unable to degrade cellulose (Table 2). Growth occurred on a number of disaccharides including maltose, lactose, sucrose and cellobiose, and of the monosaccharides tested, glucose, xylose, galactose, mannose, fructose and the sugar alcohol mannitol supported growth (Table 2). The fermentation products formed by LX-11 and B6A-RI included acetate and ethanol in approximately equal ratios and hydrogen and carbon dioxide (results not shown). Substrate utilization by the other thermoanaerobic strains were similar to B6A-RI and LX-11 with the exception of *C. thermosulfurogenes* 4B which could not utilize lactose, and *T. ethanolicus* JW200 which could not ferment mannitol. The metabolic properties of strain B6A-RI and *Thermoanaerobacter* strain B6A were identical. *C. thermocellum* LQRI was the only cellulolytic species among this group of bacteria.

Growth of LX-11 and B6A-RI occurred on xylan, and xylanase activities were determined and compared with other xylan utilizing thermoanaerobic bacteria (Table 3). B6A-RI and LX-11 produced high levels of xylanase during growth on xylose, in comparison with the other organisms, with the exception of *C. thermocellum* LQRI which has active xylanase along with an active cellulase system.

TABLE 2. Comparison of substrate utilization by thermoanaerobes.

	Thermoanaerobic strains							
Substrate	B6A-RI[a]	LX-11[b]	B6A[c]	JW200[d]	39E[e]	E100[f]	HTD4[g]	4B[h]
Glucose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Xylan	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	ND	ND	+	+
Galactose	+	+	+	+	ND	+	ND	+
Sucrose	+	+	+	+	+	+	+	+
Cellulose	-	-	-	-	-	-	-	-
Mannitol	+	+	+	-	ND	ND	ND	+
Cellobiose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	ND	ND	ND	ND
Mannose	+	ND	+	+	ND	+	ND	ND

[a]Isolate B6A-RI (this study).
[b]Isolate LX-11 (this study).
[c]*Thermoanaerobacter* strain B6A (14, 24, 29).
[d]*Thermoanaerobacter ethanolicus* strain JW 200 (32).
[e]*Clostridium thermohydrosulfuricum* strain 39E (36).
[f]*C. thermohydrosulfuricum* strain E100-69 (neotype) (7).
[g]*Thermoanaerobium brockii* strain HTD4 (37).
[h]*Clostridium thermosulfurogenes* 4B (25).
ND = Not determined.

TABLE 3. Comparison of xylanase activity from various thermoanaerobes.

Strains	Growth (A_{660})	Final pH	Activity (U/ml)	Protein conc. (mg/ml)	Specific Activity (U/mg)
B6A-RI	1.40	5.1	0.108	1.66	0.065
LX-11	1.30	4.2	0.067	2.00	0.033
39E	1.15	5.8	0.029	1.98	0.015
4B	1.05	5.5	0.023	2.25	0.010
JW 200	1.25	5.9	0.017	2.99	0.006
HTD4	0.85	5.5	0.006	2.40	0.003
E100	1.02	5.1	0.005	1.85	0.003
LQRI	0.92	6.5	0.206	1.50	0.137

B6A-RI = New isolate.
LX-11 = New isolate.
39E = *Clostridium thermohydrosulfuricum* strain 39E.
4B = *Clostridium thermosulfurogenes* strain 4B.
JW 200 = *Thermoanaerobacter ethanolicus* strain JW 200.
HTD4 = *Thermoanaerobium brockii* strain HTD4.
E100 = C. *thermohydrosulfuricum* strain E100-69 (neotype).
LQRI = C. *thermocellum* strain LQRI.

The strains were cultivated in TYE media containing 0.5% xylose except C. thermocellum (GS medium containing 0.5% cellobiose). Xylanase activity was assayed by incubating the enzyme at 65°C for 30 min. in sodium acetate buffer (50 mM, pH 5,5) contining 1% (w/v) xylan (oat spelt, Sigma). The reducing sugar formed was determined by dinitrosalicyclic acid (DNS) method. One unit of xylanase activity is defined as the amount of enzyme that released 1 µmol of xylose equivalent per minute.

Molecular taxonomic analyses

In order to characterize strain LX-11 and B6A-RI further, and to compare these two new isolates with other xylanolytic thermoanaerobic eubacteria, further studies were made by analyzing G+C content, DNA-DNA hybridization and total protein content.

DNA base composition

The G+C content of both of the new isolates B6A-RI and LX-11 was 36 mol% (Table 4). This was the same for *C. thermosulfurogenes* 4B and *C. thermohydrosulfuricum* E100-69. For the other organisms the G+C content was very similar ranging from 34 to 35 mol%, with the exception of *C. thermocellum* LQRI with a G+C content of 43 mol% (Table 4).

DNA-DNA hybridization

In order to determine the relatedness of these xylanolytic thermoanaerobes DNA-DNA hybridization studies were performed (Table 4). The results clearly show that there is little relatedness between *C. thermocellum* LQRI and the other thermoanaerobes, as the relative amount of DNA-DNA homology was 13% or lower, thus *C. thermocellum* LQRI was placed in a separate group. The high level of binding within the remaining organisms gave rise to two other groups. Significant similarity was formed between LX-11 and *C. thermosulfurogenes* 4B, with 89% homology which together with the new isolate B6A-RI formed group II. *C. thermohydrosulfuricum* 39E and *T. ethanolicus* JW200 were very similar, with 96% homology, and formed group III together with *T. brockii* HTD4 and *C. thermohydrosulfuricum* E100-69.

TABLE 4. Comparison of DNA homology between thermoanaerobic strains that display xylanase activity.

Strain	Mol% G+C content[b]	% Relative Binding with Labeled DNA from[a]							
		LQRI	B6A-RI	LX-11	4B	HTD4	JW200	39E	E100
Group I									
C. thermocellum LQRI	43	100[c]	13	8	5	5	5	6	6
Group II									
Strain B6A-RI	36	30	100	43	53	17	19	19	23
Strain LX-11	36	35	61	100	92	17	18	22	20
C. thermosulfurogenes 4B	36 (33)[d]	40	87	86	100	17	17	20	23
Group III									
T. brockii HTD4	34 (30-31)[e]	45	29	24	19	100	84	86	57
T. ethanolicus JW200	35 (32)[f]	40	28	44	18	84	100	97	60
C. thermohydrosulfuricum 39E	35	44	29	24	21	81	94	100	58
C. thermohydrosulfuricum E100-69	36 (36)g	34	34	25	20	70	67	79	100

[a]Comparisons were made in triplicate.
[b]Values in parentheses were obtained from the literature.
[c]The levels of binding between homologous strains was arbitarily defined as 100%.
[d]Reference 25.
[e]Reference 37.
[f]Reference 32.
gReference 33.

Electrophoretic analysis of whole-cell proteins

Cellular protein patterns obtained by gel electrophoresis are useful for differentiation of species and have a strong relationship to the degree of DNA-DNA homology. To get comparable protein patterns for all strains, the organisms were cultivated in the same medium (TYE medium) except for *C. thermocellum* (GS medium) and were harvested at the end of the exponential growth phase. The soluble protein patterns of the eight strains are shown in Fig. 3. A considerable difference in protein banding patterns was evident between *C. thermocellum* LQRI and the other organisms. Proteins from the new isolates B6A-RI and LX-11 and *C. thermosulfurogenes* 4B were all similar, as were proteins from *T. brockii* HTD4, *T. ethanolicus* JW200 and *C. thermohydrosulfuricum* 39E and E100-69.

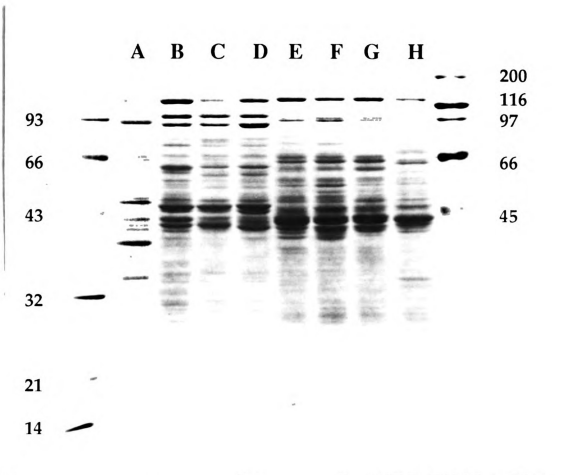


Figure 3. Electrophoretic comparison of cellular proteins from thermoanaerobic strains that display xylanase activity.

Lane A, *Clostridium thermocellum* LQRI; lane B, *Thermoanaerobacter* strain B6A-RI; lane C, new isolate strain LX-11; lane D, *Clostridium thermosulfurogenes* 4B; lane E, *Thermoanaerobium brockii* HTD4; lane F, *Thermoanaerobacter ethanolicus* JW200; lane G, *Clostridium thermohydrosulfuricum* 39E; lane H, *Clostridium thermohydrosulfuricum* E100-69. Numbers indicate the size (kDa) of the molecular weight markers.

DISCUSSION

This study describes two new thermophilic anaerobic bacteria, B6A-RI and LX-11 isolated from Frying Pan Springs in Yellowstone National Park. A comparison was made of these organisms with other xylanolytic thermoanaerobes in order to address their taxonomic affiliation. Traditionally, morphological and biochemical characteristics have been considered important in the classification of bacteria. At present, spore formation is considered a valuable taxonomic criterion to distinguish between organisms, but often sporulation can only be observed under very specialized laboratory conditions and is often misleading for taxonomic assessment of anaerobic bacteria as some organisms originally thought to be non-sporeformers have been found to sporulate (15).

Although classification of bacteria based on similarities in phenotypic characteristics has been successful in the past, this approach is not precise enough for differentiating between superficially similar organisms or for determining phylogenetic relationships among the bacteria within a group. More and more organisms are being classified based on differences at the molecular level with respect to G+C content, 16S ribosomal RNA sequences and DNA-DNA hybridization, all characteristics which cannot be influenced by conditions of growth in the laboratory.

In this present paper we have compared the new isolates B6A-RI and LX-11 with other thermophilic, xylanolytic anaerobes. Strain B6A-RI appeared identical in metabolic and morphological properties to *Thermoanaerobacter* strain B6A (29) but differed from strain LX-11 which formed spores. The physiological and biochemical properties of these saccharolytic thermoanaerobic strains indicated that the current taxonomic

assignments and nomenclatures of these strains based on phenotypes were not adequate and should be provided with new taxonomic assignments. Thus, it is intended here to re-examine the taxonomic position of xylanolytic *Clostridium* species in comparison with *Thermoanaerobacter* and *Thermoanaerobium* species based on differences at the molecular level.

The G+C values of all the organisms were very similar and did not allow for group separation. Based on DNA-DNA homology data, protein banding patterns and metabolic studies, we place the strains under study in 3 different groups and propose new taxonomic assignments in which sporeformers and non-sporeformers are placed in the same genus and species (Table 5). *Clostridium thermocellum* strain LQRI stands out as an organism that is least related to the other 7 strains. This culture forms group I and it is suggested that it retains its original taxonomic assignment without any change.

Clostridium thermosulfurogenes strain 4B and the new xylanolytic isolates LX-11 and B6A-RI are closely related and fall in group II for which a genus name *Thermoanaerobacterium* gen. nov. is given. Strain LX-11 is given a taxonomic assignment as *Thermoanaerobacterium xylanolyticum* sp. nov. due to its xylanolytic properties. Strain B6A-RI is named and classified as *Thermoanaerobacterium saccharolyticum* sp. nov. Both strain B6A-RI and *Thermoanaerobacter* strain B6A behave like true saccharolytic strains producing amylases while growing on starch as well as on other substrates (14, 24). *Thermoanaerobacterium thermosulfurigenes* strain 4B is the type species for the genus *Thermoanaerobacterium*.

Group III includes *Thermoanaerobacter ethanolicus* strain JW200, *Clostridium thermohydrosulfuricum* strain 39E and strain E100-69, and *Thermoanaerobium brockii* strain HTD4 due to their close relatedness. This

TABLE 5. Proposed nomenclature for xylanolytic thermoanaerobic bacterial species of uncertain phylogenetic affiliation.

Group	Proposed Name	Former Name	Strain	Habitat
I.	*Clostridium thermocellum*	*Clostridium thermocellum*	LQRI	farm soil
II.	*Thermoanaerobacterium thermosulfurigenes*	*Clostridium thermosulfurogenes*	4B	thermal spring
	Thermoanaerobacterium xylanolyticum	This study	LX-11	thermal spring
	Thermoanaerobacterium saccharolyticum	This study *Thermoanaerobacter*	B6A-RI B6A	thermal spring
III.	*Thermoanaerobacter brockii*	*Thermoanaerobium brockii*	HTD4	thermal spring
	Thermoanaerobacter thermohydrosulfuricus	*Clostridium thermohydrosulfuricum*	E100-69	farm soil
	Thermoanaerobacter ethanolicus	*Thermoanaerobacter ethanolicus,*	JW200	thermal spring
		Clostridium thermohydrosulfuricum	39E	thermal spring

group is termed *Thermoanaerobacter*. *Thermoanaerobium brockii* strain HTD4 is renamed *Thermoanaerobacter brockii* comb. nov., *Clostridium thermohydrosulfuricum* strain E100-69 is renamed as *Thermoanaerobacter thermohydrosulfuricus* comb. nov. *C. thermohydrosulfuricum* strain 39E described by Zeikus et al., (36) is nearly identical to *T. ethanolicus* strain JW200 and, thus both strains are considered to belong to the same species. Perhaps, *T. ethanolicus* strain JW200 is an asporogenous mutant of strain 39E. Therefore, strain 39E is renamed as *Thermoanaerobacter ethanolicus* strain 39E, and *Thermoanaerobacter ethanolicus* strain JW200 is the type strain. A taxonomic key for preliminary identification of saccharolytic, ethanologenic thermoanaerobes is outlined in Table 6.

This proposed taxonomic arrangement of these organisms is supported by previous studies by other workers. J. Wiegel first suggested that *T. brockii, T. ethanolicus,* and *C. thermohydrosulfuricum* 39E could be close taxonomic relatives because they display a similar substrate range and temperature range for growth and a biphasic growth curve (30). Kondratieva et al. (10) recognized the similarity of *Thermoanaerobium lactoethylicum* to both *T. brockii* and *T. ethanolicus* and suggested that the genus name *Thermoanaerobium,* and not *Thermoanaerobacter,* be used for these species. Bateson et al. (1) have reported that the 16S rRNA sequences of *T. brockii, T. ethanolicus* and *C. thermohydrosulfuricum* were nearly identical but significantly different from *C. thermosulfurogenes*. This is supported by 16S rRNA cataloging studies of Cato and Stackebrandt (4) in which it was found that sporeforming *Clostridia* do not form one phylogenetic homologous family but six sublines which embrace both sporeforming and non-sporeforming species as well.

The genera *Thermoanaerobacter* (32) and *Thermoanaerobium* (37)

TABLE 6. Taxonomic key for preliminary identification of saccharolytic, ethanologenic thermoanaerobes.

A. Does not reduce thiosulfate, ferments cellulose.

1. *Clostridium thermocellum*

B. Does reduce thiosulfate, does not ferment cellulose.

1. Reduces thiosulfate to elemental sulfur

Thermoanaerobacterium thermosulfurigenes

Thermoanaerobacterium saccharolyticum

Thermoanaerobacterium xylanolyticum

2. Reduces thiosulfate to H_2S

Thermoanaerobacter brockii

Thermoanaerobacter ethanolicus

Thermoanaerobacter thermohydrosulfuricus

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were used to describe the first thermophilic anaerobic rod-shaped, Gram-positive, non-sporeforming bacteria that produce ethanol and lactate as principal saccharide fermentation products. *Thermoanaerobacter* is listed under irregular, non-sporeforming Gram-positive rods in Bergey's Manual of Systematic Bacteriology (30). At present, the genus *Thermoanaerobacter* is comprised of two described species, *T. ethanolicus* (32), and *T. finnii* (26). Strain JW200 has been designated the neotype strain of *Thermoanaerobacter ethanolicus*.

The genus *Thermoanaerobium* is also comprised of only two described species, *T. brockii* (37) and *T. lactoethylicus* (10), with *T. brockii* strain HTD4 as the neotype strain. Previously organisms belonging to the genus *Thermoanaerobacter* and *Thermoanaerobium* were considered to be strict anaerobes, motile or non-motile rods, Gram-positive to variable that were non-sporeforming, except for *T. finnii* (26) and formed chains of cells comprised of uneven lengths including mini cells. Recently, *T. brockii* was shown to form spores (5). All species were saccharolytic and degraded starch but not cellulose and formed ethanol and/or lactate as major end products with lower levels of H_2/CO_2, and acetate. Consequently, it was not possible to adequately distinguish between *Thermoanaerobacter* versus *Thermoanaerobium* strains based on these morphological, cellular and nutritional properties alone. Using the criteria detailed in this paper of distinguishing between organisms based on differences at the molecular level, we propose the following classification for *Thermoanaerobacter* and *Thermoanaerobium*.

Description of *Thermoanaerobacterium* gen. nov.

Ther.mo.an.ero.bac.teri.um. Gr. n. thermos hot; Gr. pref. annot; Gr.n.aerair; Gr.n. bacterion a small rod; M.L. neut. n. *Thermoanaerobacterium*, rod which grows in absence of air at high temperatures.

Cellular characteristics. Straight rods 0.5 x > 15 μm. Forms long filaments, cells motile, peritrichous flagella. Gram negative cell wall. Catalase negative. Reduces thiosulfate to elemental sulfur. Deposits elemental sulfur on cells when grown with thiosulfate. Spores may be detected. Surface colonies are about 0.5-5 mm in size. The optimum temperature for growth is 60°C. The G+C content of the DNA is 25-40 mol%. The principal products of carbohydrate fermentation are ethanol, acetate, lactate, H_2, and CO_2. The type species is *Thermoanaerobacterium thermosulfurigenes* comb. nov.

Description of *Thermoanaerobacterium thermosulfurigenes* (25)

ther.mo.sul.fur.i'ge:nes. Gr. adj. thermos hot. L.n. sulfur brimestone, Gr. suffix-genes born form; N.L. neut. adj. thermosulfurigenes releasing sulfur in heat.

Cellular characteristics. Straight rods 0.5 x > 2 μm. Stains Gram-negative; exponential phase cells motile by peritrichous flagellation. Forms long filaments and deposits yellow elemental sulphur on cells and in the medium when grown with thiosulfate. Neither sulfite nor sulfide is detected. Swollen, white refractile, spherical endospores formed. No outer wall membranous layer present in thin sections. Sporulation occurs in xylose- or pectin-containing media. Agar embedded colonies are fluffy, 0.5-1.5 mm in diameter, and not pigmented. DNA base composition of 32.6 (+0.04)

mol%G

C

maxim

minim

Compl

tetracyc

ml^{-1}) or

N

of carb

polygal

galacto

aesculi

methar

lactate

sulfite

hydrol

volcan

Natior

33743,

Descri

Spores

mol%G+C. Cytochromes undetectable; catalase negative.

Growth characteristics. Optimum temperature for growth is 60°C, maximum 75°C, minimum 35°C, pH range for growth; optimum 5.5-6.5 minimum 4.0, maximum <7.6. Obligate thermophile and anaerobe. Complete growth inhibition by penicillin, streptomycin, cycloserine, tetracycline, chloramphenicol (each at 100 μg ml^{-1}), sodium azide (500 μg ml^{-1}) or O_2 (0.203 X 10^5 Pa). Growth is inhibited by 2% NaCl and by sulfite.

Metabolic characteristics. Chemoorganotroph. Utilizes a wide variety of carbohydrates as energy sources including pectin, arabinose, cellobiose, polygalacturonose, rhamnose, amygdalin, salicin, inositol, mannitol, xylose, galactose, glucose, mannose, maltose, starch, melibiose, sucrose, trehalose or aesculin. No growth on H_2/CO_2, lactose, cellulose, tartrate, lactate, pyruvate, methanol or glycerol. Fermentation products of glucose are ethanol, H_2, CO_2, lactate and acetate. Methanol and isopropanol formed on pectin. Sulfate, sulfite and nitrate not reduced. Pectin methylesterase and polygalacturonase hydrolase are produced.

Habitat. Algal-bacterial mat ecosystems associated with thermal, volcanic springs. This strain was isolated from Octopus Spring, Yellowstone National Park, U.S.A.

Type strain. *Thermoanaerobacterium thermosulfurigenes* 4B (ATCC 33743, DSM 2229).

Description of *Thermoanaerobacterium xylanolyticum* sp. nov.

xy.lan.o.ly'ti.cum. Gr. n. xylanosum, xylan; Gr. adj. lyticus, dissolving; N. L. adj. xylanolyticum, xylan dissolving.

Cellular characteristics. Rods approximately 0.8-1.0 μm by 2.0-7.0 μm. Spores are spherical and terminal. Cells are motile. The organism is Gram-

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negative. Surface colonies are circular with smooth edges, surface texture rough, cloudy to white and about 2-5 mm in size. The mol% G+C of the DNA is 36.1. Catalase negative.

Growth characteristics. Anaerobic. Can initiate growth in a N_2-gassed media without addition of a chemical (cysteine or Na_2S) reducing agent. pH optimum for growth is about 6.0. The optimum temperature for growth is 60°C with a growth temperature range between 45-70°C. Growth is inhibited by penicillin G (200 μg/ml), neomycin sulfate, ampicillin, streptomycin sulfate, rifampicin, polymyxin B, erythromycin, tetracycline, acridine orange (all at 100 μg/ml). Yeast extract stimulates growth. Growth is inhibited even by 1% NaCl.

Metabolic characteristics. Utilizes mannose, sucrose, cellobiose, arabinose, rhamnose, galactose, fructose, maltose, pyruvate, lactose and glucose. Cellulose, melibiose, melezitose, xylitol, ribose, raffinose and lactate are not fermented. Fermentation end products include ethanol, acetic acid, H_2 and CO_2. Lactate not detected in fermentation broth.

Habitat. Geothermal areas of Yellowstone National Park, Wyoming.

Type strain. *Thermoanaerobacterium xylanolyticum* strain LX-11 (ATCC 49914).

Description of *Thermoanaerobacterium saccharolyticum* sp. nov.

sac.cha.ro.ly'ti.cum. Gr. n. sacchar, sugar; Gr. adj. lyticus, dissolving; N.L. neut. adj. saccharolyticum, sugar-dissolving.

Cellular characteristics. Rods approximately 0.8-1.0 by 3.0-15 μm with some cells occasionally being as long as 30 μm. Elongated cell morphology during nutrient limitation or stationary phase. The cell walls contain three electron-dense layers of 5 nm thickness, alternating with electron light layers

of similar thickness. Gram-negative but a distinct outer membrane characteristic of many Gram-negative bacteria is not observed. Cell division proceeds by formation of well-defined division septa, often producing daughter cells of unequal length. Cells motile, peritrichous flagella. Cells survived heating at 85°C for 15 min, but not at 90°C for 5 min. Cells contain a plasmid of 1.5 Md. Surface colonies on agar plates are soft, tan, circular, convex with hollow centers ("donut" shape). Colony sizes range from 0.5-4.0 mm after 4 days growth at 55°C. The mol% G+C of the DNA is 29-31 (T_m). Catalase negative.

Growth characteristics. Anaerobic, will initiate growth in a N_2-gassed media without the addition of chemical reducing agents. Addition of reducing agents (cysteine or Na_2S) did not generally stimulate growth. pH optima for growth is 6.0, and minimum and maximum pH for growth initiation is 3.5 and below 9.0, respectively. The temperature data for growth are T_{max} 68-70°C, T_{opt} 60°C, and T_{min} 45°C. Growth is inhibited by penicillin G (200 μg/ml), chloramphenicol (100 μg/ml), neomycin (100 μg/ml), or O_2 (0.2 atm). The culture is resistant to 2% NaCl. Yeast extract stimulates growth.

Metabolic characteristics. Ferments a wide variety of carbohydrates including glucose, fructose, mannose, galactose, maltose, cellobiose, sucrose, lactose, trehalose, xylose, starch, rhamnose, raffinose and xylan. Cellulose, ribose, melibiose, melezitose, xylitol, or sorbitol were not fermented. No growth occurs in absence of a fermentable carbohydrate. Fermentation products from either glucose or xylan include ethanol, acetic acid, lactic acid, H_2 and CO_2. L-Rhamnose is fermented to equimolar amounts of 1,2-propandiol, plus a mixture of ethanol, acetic acid, lactic acid, H_2 and CO_2.

Habitat. Geothermal sites in Wyoming (Yellowstone National Park and thermopolis areas) and Nevada (Steamboat area).

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Type strain. *Thermoanaerobacterium saccharolyticum* strain B6A-RI (ATCC 49915).

Description of *Thermoanaerobacter brockii* (37) comb. nov.

brock-i.i M.L. gen. no. brockii of Brock; named after Thomas Dale Brock, who pioneered studies on physiological ecology of extreme thermophiles.

Cellular characteristics. Rods measuring 1.0 μm by 2-20 μm. Cells frequently uneven in length (mini-cells) and occurring in chains, pairs and filaments. Gram positive and round, terminal endospores detected. DNA base composition of 30.0 - 31.4 (± 1) mol% G+C. Cytochrome pigments and catalase absent. Mono-layered cell wall architecture without an outer wall membrane.

Colony Characteristics. Colonies are uniformly round, mucoid, non-pigmented, flat, and grow to a diameter of 0.2 - 0.3 cm in 48 h.

Growth Characteristics. Obligately anaerobic. Optimum temperature for growth 65-70°C, maximum < 85°C and minimum > 35°C. pH optimum for growth ~7.5, no growth above 9.5 or below 5.5.

Metabolic characteristics. Chemoorganotroph. Utilizes a variety of saccharides as energy sources including starch, maltose, glucose, lactose, sucrose and cellobiose. Growth inhibited by air, penicillin, cycloserine, streptomycin, tetracycline and chloramphenicol. Fermentation end products are ethanol, lactic acid, acetic acid, hydrogen and carbon dioxide. Reduces thiosulfate to hydrogen sulfide.

Habitat. Anaerobic thermal features associated with volcanic activity including springs, decomposing photosynthetic biomass and sediments.

Type strain. *Thermoanaerobacter brockii* HTD4 (ATCC 33075, DSM

1457). This strain was isolated from a Washburn thermal springs edge sediment located in Yellowstone National Park, U.S.A.

Description of *Thermoanaerobacter thermohydrosulfuricus* comb. nov.

ther.mo.hy.dro.sul.fur'i.cus. M.L. masc. n. thermos hot; M.L. masc. adj. hydrosulfuricum pertaining to hydrogen sulfide; M.L. masc. adj. thermohydrosulfuricum indicating that the organism grows at high temperatures and reduces sulfite to H_2S.

Cellular characteristics. Rods approximately 0.3-0.6 μm by 2.0-13.0 μm Rods are single or in short chains, in some strains long filamentous group of cells. Cells are motile and peritrichous. Spores are spherical, terminal, sporangia swelling the cell. Sporulating cultures showing thinner, more elongated cells than non-sporulating cultures. Cells are Gram-variable. Cell wall is composed of two layers. The outermost layer consist of hexagonally shaped particles. The center-to-center distance between adjacent particles is 13.5 nm; they are composed of glycoprotein containing glucose, galactose, mannose and rhamnose, which have a molecular weight of approximately 140,000. The outermost layer completely covers the cell and is resistant to digestion by proteolytic enzymes. Cell walls contain meso-DAP. The mol% of G+C is 35-37 (T_m). Catalase negative.

Growth characteristics. Obligately anaerobic. The optimum temperature is 67-69°C; the maximum temperature at which growth occurs is 76-78°C. Growth at 37°C is poor with no growth at 28°C. Growth occurs at pH 5.5-9.2; optimum growth from 6.9-7.5. Growth is inhibited by H_2 in the gas phase and by lactate.

Metabolic characteristics. H_2-CO_2 are produced in the media containing liver infusion. H_2S is produced from tryptophan, peptone, and yeast extract.

Sulfite and thiosulfate are reduced to H_2S; sulfate is not reduced. Acetyl methyl carbinol is not produced. Fructose, galactose, glucose, mannose, xylose, cellobiose, maltose, sucrose, trehalose, pectin, esculin and salicin are fermented. Fermentation of dextrin, potato starch, mannitol, dulcitol and sorbitol, and coagulation of litmus milk are variable. Inositol, erythritol, glycerol, lactate, tartrate and cellulose are not fermented; nitrite but not nitrate is reduced, coagulated albumin is not hydrolyzed and indole is not produced. Products of metabolism in PYG broth are acetate and lactic acids, ethanol, CO_2 and H_2; formic, butyric, isovaleric, and isocaproic acids, and propanol and isopropanol may be detected. Methanol is a major metabolic end product if grown in pectin. Reduces thiosulfate to hydrogen sulfide.

Habitats. Isolated from extraction juices of beet sugar factories; from mud and soil; from hot springs in Utah and Wyoming and from a sewage plant in Georgia.

Neotype strain. *Thermoanaerobacter thermohydrosulfuricus* E100-69 (DSM 567 = NCIB 10956).

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Chapter III

Regulation and characterization of xylanolytic enzymes of *Thermoanaerobacter* strain B6A-RI

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ABSTRACT

During growth on xylan and xylose *Thermoanaerobacter* strain B6A-RI produced endoxylanase, β-xylosidase, arabinofuranosidase and acetyl esterase and the first three activities appeared to be produced coordinately. Under non-limiting xylan growth conditions these enzyme activities were predominanatly cell associated, however, during growth on limiting concentrations of xylan the majority of endoxylanase activity was extracellular rather than cell associated. Endoxylanase, β-xylosidase and arabinofuranosidase activities were induced by xylan, xylose and arabinose respectively. Acetyl esterase activity was constitutive and endoxylanase activity was catabolite repressed by glucose. Endoxylanase activity was purified using gel filtration with Sephacryl S-300 and affinity chromatography with xylan coupled to Sepharose CL-4B preequilibrated to 45°C with 50 mM sodium acetate buffer (pH 4.0) and eluted with 0.1% soluble xylan. A single area of activity was identified on the zymogram which was composed of multiple bands, and one major protein complex with a molecular weight of approximately 160 kDa, and a minor protein complex with a molecular weight of approximately 130 kDa on SDS-PAGE. The endoxylanase activity stained with Schiff's reagent indicative of glycoproteins. The purified endoxylanase displayed a specific activity of 41 units/mg protein on xylan, and a pH and temperature optima of 6.0 and 70°C respectively.

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INTRODUCTION

β-1,4-xylans are heterogeneous polysaccharides found in the cell walls of all land plants and in almost all plant components (35). After cellulose, xylan is the most abundant renewable polysaccharide in nature, accounting for 20 to 30% of the dry weight of woody tissue.

Complete breakdown of a branched acetyl xylan requires the action of several hydrolytic enzymes (6, 25, 39). The most important microbial enzymes involved in xylan degradation are endo-1,4-β-D-xylanase (EC 3.2.1.8) and β–D-xylosidase (EC 3.2.1.37). Endo-1,4-β-D-xylanase acts on chains of xylans, arabanoxylans and 4-MeO-glucuronoxylans. β-xylosidase breaks down short oligosaccharides from the nonreducing end to xylose, and has substantial transferase activity (25). A number of other glycosidases may play a more minor role in polysaccharide degradation, including α-L-fucosidase cleaving terminal L-fucoside from xyloglucans and rhamnogalacturonans and α-L-rhamnosidase (EC 3.2.1.40) releasing L-rhamnose from the nonreducing end of partially degraded rhamnogalacturonans. α-D-Glucuronidase releases D-glucuronic acid from xylan and α-L-arabinofuranosidase (EC 3.2.1.55) removes arabinosyl side chains. Often xylans are present in the partially acetylated form, which come under attack from acetyl esterases (EC 3.1.1.6).

These xylan degrading enzymes are produced by a wide variety of microorganisms including aerobic and anaerobic mesophiles and thermophiles (6, 32). The majority of the studies have been carried out in aerobic, mesophilic fungi and bacteria (6, 25, 32, 39).

Enzymatic hydrolysis products from xylan, namely xylose can be subsequently converted into liquid fuel, single-cell protein, solvents and

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other chemical products using selected fermentative microorganisms (6, 8). Such bioconversion processes are particularly attractive for the elimination of residues and wastes produced by agriculture and forestry. Furthermore, it may be possible to use xylanases in biopulping processes for the preparation of cellulose pulp, bio-bleaching and purification of fibers for rayon manufacture (6). Another potential application of xylanases may be in the food and feed manufacture such as improving baking with high fiber materials, clarification of fruit juices and wine, improved animal feed properties by fiber modification and production of food thickeners.

The feasibility of using xylanase for cellulose bleaching or purification from hemicellulose will be highly dependent on the intrinsic selectivity of the enzymes, the absence of cellulolytic activity and the availability of enzyme in inexpensive bulk quantities. Stability is also a highly desirable characteristic for industrial enzymes, and potential sources for thermostable xylanases include several thermophilic microorganisms (5, 10, 23, 31).

Thermophilic anaerobes produce active saccharidases that enable rapid hydrolysis of polymers such as cellulose and starch (41). Detailed characterization of xylanolytic enzyme complexes present in thermoanaerobes has not yet been reported. *Clostridium thermocellum* produces a cellulosome which organizes cellulases for rapid degradation of cellulose (2). Little is known about the hemicellulases from thermophilic anaerobic bacteria that grow rapidly on insoluble xylan. Wiegel et al. (36, 37) studied a variety of thermophilic, anaerobic, saccharolytic bacteria, including *Thermoanaerobacter ethanolicus*, *Thermobacteroides acetoethylicus*, *Thermoanaerobium brockii*, and *Clostridium thermocellum*, which all ferment xylan albeit at a very slow rate. Studies were also conducted on *Thermoanaerobacter* strain B6A an organism isolated from an

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algal mat present in Big Spring, Thermopolis, Wyoming (34) which was shown to extensively degrade xylan (35). Later *Thermoanaerobacter* strain B6A was shown to possess a number of saccharidases including amylase, glucose isomerase, and high levels of endoxylanase (17).

Recently we isolated two xylanolytic species from Frying Pan Springs in Yellowstone National Park, Wyoming, a thermal spring with a pH below 6.0, (19). One of the isolates,*Thermoanaerobacter* strain B6A-RI, is a model xylanolytic thermoanaerobe because it has very active xylanases but it is not cellulolytic. The purpose of the present study was three fold: first to identify the kinds and complexity of xylanase activities present; second to determine the regulation of xylanolytic enzymes with respect to the growth substrate, and thirdly, to partially purify and characterize the endoxylanase complex.

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MATERIALS AND METHODS

Chemicals and gases

All chemicals were reagent grade or better and were obtained from Sigma Chemcal Co., St. Louis, Mo., or Mallinckrodt, Inc., Paris, Ky. All gases used were at least 99.9% pure and were passed over copper-filled Vycor furnaces (Sargent Welch Scientific Co., Skokie, Ill.) to remove oxygen.

Organism and culture conditions

Thermoanaerobacter strain B6A-RI was grown on TYE medium at 60°C as described elsewhere (19).

Measurement of growth and end products

Cell growth was determined by measuring optical density at 660 nm. Xylose was measured as reducing sugar with dinitrosalicyclic acid (DNS) reagent (21). Ethanol and acetate were determined by flame ionization gas chromatography. Cell free culture media (1 ml) was acidified with 100 ml of 10 N H_3PO_4, and 2 ml were injected into a Hewlett Packard HP 5890A gas chromatograph equipped with a Chromosorb 101 (80/100 mesh) column (Supelco). The column was operated at 200°C, and the detector and injector temperatures were 250°C and 190°C, respectively.

Enzyme production and batch culture studies

Thermoanaerobacter strain B6A-RI was grown in 750 ml fermentation vessels (New Brunswick Sci., New Brunswick, NJ) containing 500 ml of medium and 1.0%(w/v) oatspelt xylan, which was added before autoclaving. The vessel was rendered anaerobic by the addition of 0.01%(w/v) Na_2S. The

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vessel was agitated at 200 rpm, sparged with nitrogen and carbon dioxide (95:5), and the temperature maintained at 60°C, without pH control. Samples (3 ml) were taken at intervals for determination of growth, reducing sugar, fermentation products and enzyme activities. For enzyme activities, 1.5 ml of culture was centrifuged at 16,000 x g for 5 min in an Eppendorf microcentrifuge, and the supernatant taken as the extracellular fraction. The cells were washed twice in 50 mM MOPS buffer (pH 6.0) and resuspended in 1.5 ml of the same buffer. These cells were sonicated for 2 min and used to measure cellular, i.e., both soluble and cell bound activity.

Enzyme activities

All enzyme assays were incubated at 65°C. Endoxylanase activity was determined using oat spelt xylan. The assay consisted of 1%(w/v) xylan with 50 mM sodium acetate (pH 5.5) and enzyme to give a final volume of 0.2 ml. The reaction mixture was incubated for 2 h and xylose quantitated by addition of 0.8 ml DNS reagent (21) and absorbance measured at 640 nm.

The β-xylosidase assay consisted of 1 mM p-nitrophenyl β-D-xylopyranoside, 50 mM sodium acetate (pH 5.5) and enzyme to give a final volume of 0.4 ml. The reaction mixture was incubated for 10 min and terminated by the addition of 0.8 ml of 0.5 M sodium carbonate and read at 410 nm.

Arabinofuranosidase activity was determined in a reaction mixture containing 2 mM p-nitrophenyl α-L-arabinofuranoside, 50 mM sodium-acetate buffer (pH 5.5) and enzyme to give a final volume of 0.5 ml. The reaction was incubated for 30 min and terminated by the addition of 0.33 ml of 1 M sodium carbonate and read at 410 nm.

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The acetyl esterase assay consisted of 1 mM α-napthylacetate, 50 mM MES (pH 6.0) and enzyme to give a final volume of 0.5 ml. The reaction was incubated for 25 min and then 0.5 ml of 0.01% (w/v) Fast Corinth V salt in 1 M sodium acetate buffer (pH 4.3) containing 10% (v/v) Tween 20 (prepared shortly before use) was added, and the solution incubated at room temperature for 10 min before being read at 535 nm. All enzyme assays were linear over the period of the assays. β-xylosidase and arabinofuranosidase activities were expressed as μmoles of p-nitrophenol released per min (international units) per ml of enzyme source, and acetyl esterase activity was determined as μmoles of α-napthol per min (U) per ml of enzyme source. Endoxylanase activities were expressed as μmoles of reducing sugar (xylose equivalents) released per min (U) per ml of enzyme source.

Synthesis of xylan-coupled Sepharose CL-4B and purification procedure

Synthesis of xylan-coupled Sepharose CL-4B was based on the procedure described by Sundberg and Porath (29). 5 g oat spelt xylan (Sigma Chemical Co.) was mixed with 35 g suction dried Sepahrose CL-4B (Pharmacia LKB Biotechnology, Piscataway, NJ) in 90 ml 0.5 M sodium carbonate (pH 13) in a 500 ml flask. 10 ml epichlorohydrin (Sigma Chemical Co.) was added to the mixture and incubate at 60°C for 2 h with vigorous shaking in a water bath. The mixture was washed with excess water on a glass filter-funnel to remove uncoupled reagents and suspended in distilled water.

Purification of endoxylanases

Thermoanaerobacter strain B6A-RI was grown on TYE medium with 0.5% xylose to mid exponential phase, centrifuged and the culture supernatant used as a source of endoxylanases. The culture supernatant was

concentrated by ultrafiltration using YM 100 membrane with a 100 kDa molecular weight cut-off (Amicon Co., Danverse, MA), and loaded on Sephacryl S-300 column (2.5 x 50 cm, Pharmacia) prequibrated with 50 mM NaCl and 50 mM sodium acetate buffer (pH 5.5) and eluted with the same buffer. The active fractions were pooled and concentrated by ultrafiltration using a YM 30 membrane with a 30 kDa molecular weight cut-off (Amicon).

Using the procedure described by Rozie et al. (26), cross linked xylan was prepared as an affinity adsorbent. However, the flow rate with this material was very slow due to its gelatinous nature. To improve the flow rate, oat spelt xylan was coupled to Sepharose CL-4B by the modification of method of Sundberg and Porath (29). For affinity chromatography a Pharmacia column (1.5 x 33 cm) fitted with a thermostatic jacket connected to a water bath for temperature control was filled with xylan-coupled Sepharose CL-4B. The column was equilibrated with 50 mM sodium acetate buffer (pH 4.0) at room temperature and protein sample was loaded once the column had equilibrated to 45°C. After washing with 50 mM sodium acetate buffer (pH 5.5) at room temperature the xylanases were eluted with soluble xylan (0.1%) in 50 mM sodium acetate buffer (pH 5.5) at room temperature. The active fractions were pooled and incubated at 60°C for 2 - 4 h to digest xylan coeluted with enzyme and the hydrolysis products were removed by dialysis against 50 mM sodium acetate buffer (pH 5.5)

Electrophoresis and zymograms

SDS-polyacrylamide gel electrophoresis (PAGE) was performed in 7.5% gels by the method of Laemmli (13). The gels were stained for endoxylanase activity in a modification of the cellulase assay described by Béguin (4). A suspension (0.1% final concentration) of soluble xylan was incorporated into

the separating gel before the addition of ammonium persulfate and polymerization. Protein samples were prepared by heating for 5 min at 100°C in the presence of sample buffer (3% SDS, 10% glycerol, 5% β-mercaptoethanol in 31 mM Tris hydrochloride (pH 6.8), and then applied to the gel.

Upon completion of electrophoresis, the gel was washed twice for 30 min in 50 mM sodium acetate buffer (pH 5.5) containing 25% isopropanol and once in 50 mM sodium acetate buffer for 15 min. The gel was then incubated in 50 mM sodium acetate buffer for 5 min at 60°C. The gel was stained in a 0.2% (w/v) Congo red solution for 10 min at room temperature with gentle shaking and destained with 1 M NaCl until excess stain was removed from the active band. To facilitate photographic documentation, the gel was rinsed in 5% acetic acid which caused the background to turn dark blue, emphasizing the bands of activity. Proteins were stained with Coomassie brilliant blue R250, destained and photographed.

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RESULTS

Regulation of xylanolytic enzyme activity levels

Thermoanaerobacter strain B6A-RI was grown on various carbon sources and endoxylanase, β-xylosidase, acetyl esterase and arabinofuranosidase were compared with respect to activity (Table 1). High levels of β-xylosidase were produced during growth on all of the substrates with the exception of glucose, however, addition of xylose to a glucose grown culture resulted in enzyme synthesis (Table 1). Endoxylanase activity was most significant during growth of *Thermoanaerobacter* strain B6A-RI on xylose and xylan. Arabinose although the end product of arabinofuranosidase activity acted as an inducer for this enzyme, and acetyl esterase activity was constitutive.

Growth and xylanolytic enzyme synthesis

Thermoanaerobacter strain B6A-RI grew readily in batch culture on xylose with a doubling time of 1.13 h (Fig. 1). Although this organism produces acetate, ethanol, lactate, CO_2 and H_2, only acetate and ethanol were determined under these conditions. β–xylosidase, acetyl esterase and arabinofuranosidase activities were produced during growth on xylose and were detected in cells. Endoxylanase activity was also synthesized during growth on xylose, but most activity was detected in the culture supernatant, not in the cells.

Growth of *Thermoanaerobacter* strain B6A-RI on xylan was much slower than on xylose and a significantly longer lag time was observed (Fig. 2). An increase in reducing sugars occurred during xylan fermentation with the maximum amount formed during the early phase of growth (i.e. before

Table 1. Effect of growth substrate on xylanolytic enzyme levels of *Thermoanaerobacter* strain B6A-RI.

Substrate	O.D.	Enzyme activity[a]			
		β-xylosidase (mU/ml)	endoxylanase (mU/ml)	arabinosidase (mU/ml)	acetyl esterase (mU/ml)
Glucose	1.04	11	3	5	41
Xylan	N.D.	272	57	7	43
Maltose	1.00	58	3	5	37
Xylose	0.96	87	26	11	46
Arabinose	0.90	73	6	107	35

All substrates were present at 0.5%(w/v) except xylan which was present at 1%(w/v).
[a]Enzyme unit is expressed as μmoles of reducing sugar produced per min.
Enzyme activities comprise of the cellular fraction from cells in the late exponential phase.

Figure 1. Fermentation and xylanolytic enzyme synthesis time course of *Thermoanaerobacter* strain B6A-RI during growth on xylose.

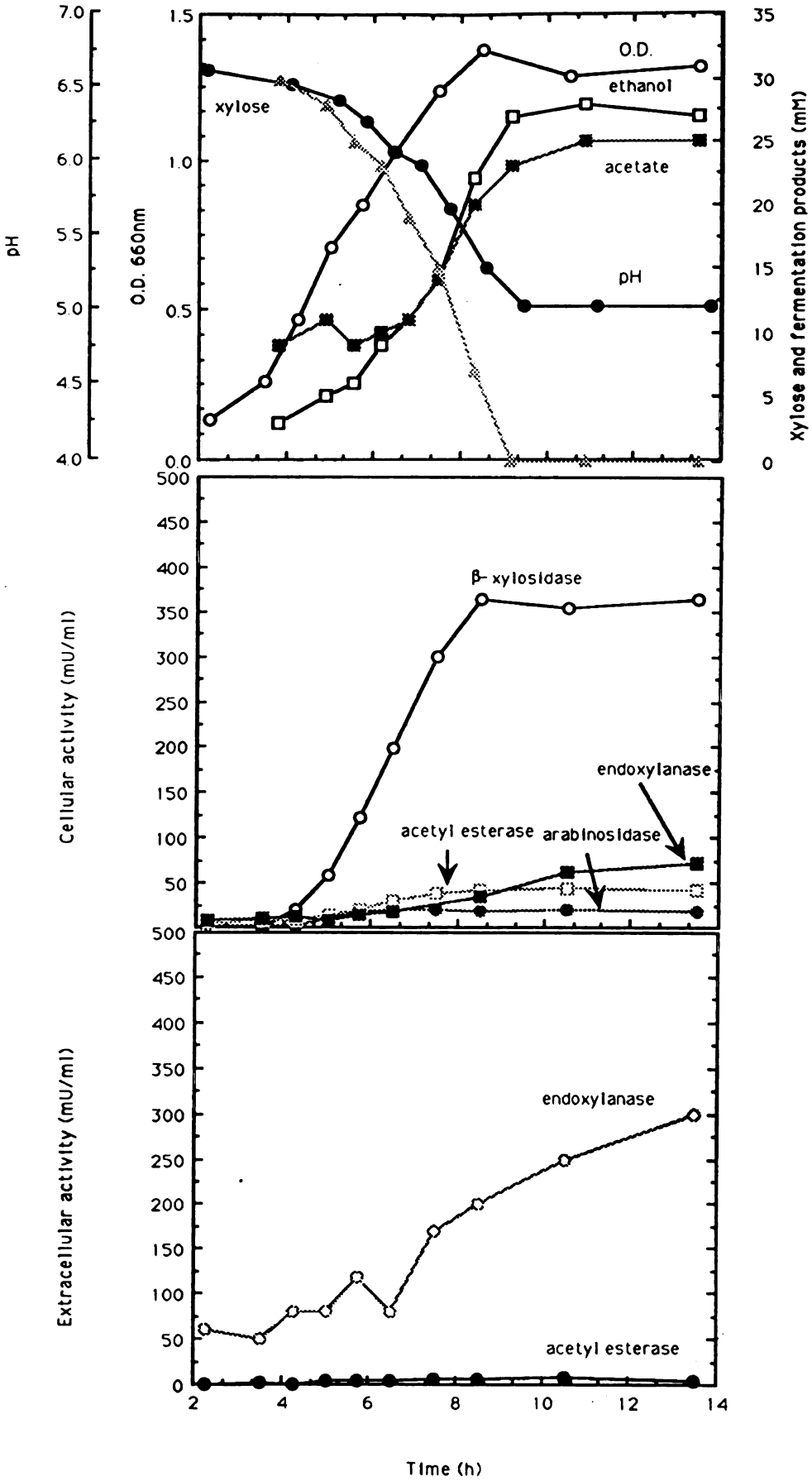
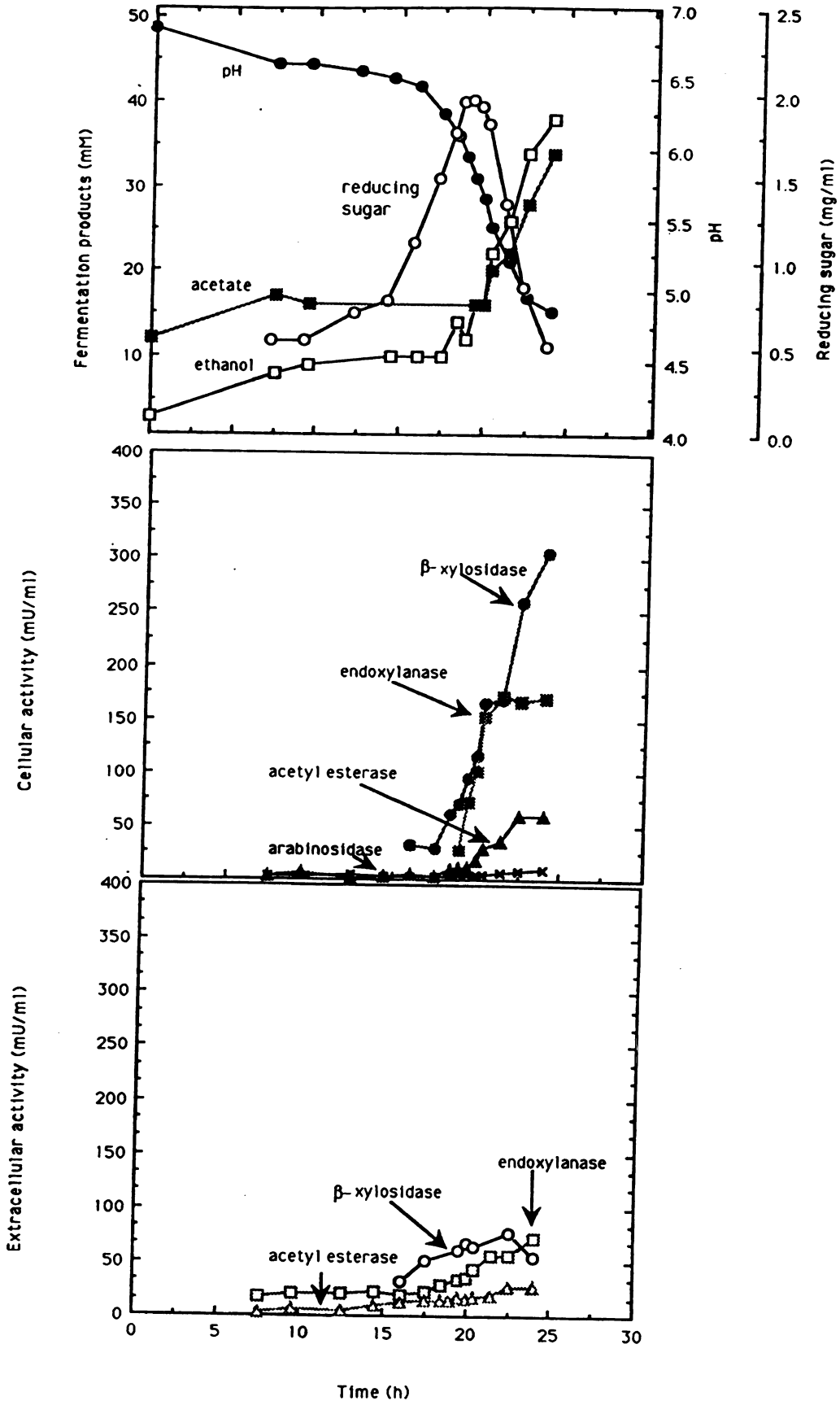

pH
O.D. 660nm
Xylose and fermentation products (mM)
O.D.
ethanol
xylose
acetate
pH
Cellular activity (mU/ml)
β- xylosidase
endoxylanase
acetyl esterase
arabinosidase
Extracellular activity (mU/ml)
endoxylanase
acetyl esterase
Time (h)

Figure 2. Fermentation and xylanolytic enzyme synthesis time course of *Thermoanaerobacter* strain B6A-RI during growth on xylan.


Fermentation products (mM)
pH
reducing sugar
acetate
ethanol
Reducing sugar (mg/ml)
Cellular activity (mU/ml)
β-xylosidase
endoxylanase
acetyl esterase
arabinosidase
Extracellular activity (mU/ml)
endoxylanase
β-xylosidase
acetyl esterase
Time (h)

significant amounts of fermentation products were formed), and then was consumed during the exponential phase. All xylanlolytic enzyme activities were synthesized during growth. Interestingly, although similar levels of endoxylanase are produced when *Thermoanaerobacter* strain B6A-RI is grown on xylan or xylose, the activity was mainly cell associated during growth on the insoluble substrate in comparison to the predominantly extracellular activity found during growth on xylose. The possibility that xylanase is adsorbed onto xylan, therefore reducing the amount of activity that can be detected in the culture supernatant, cannot be excluded, but high levels of activity observed tend to suggest that at least some of the enzyme is nonadsorbed.

Acetyl esterase activity was mainly cell associated, with lower levels occurring in the supernatant and intracellular fractions. Only low levels of cell bound arabinofuranosidase activity could be detected, and this may reflect the fact that oatspelt xylan is particularly low in arabinoside side chains, consisting mainly of xylose units, thus, not inducing this enzyme.

Cellular location of endoxylanase

The cellular location (i.e. cell bound versus excreted) of cellulases, xylanases and amylases can be affected by substrate availability. Consequently, experiments were performed such that the concentration of xylan was varied during growth of *Thermoanaerobacter* strain B6A-RI in batch culture, and levels of endoxylanase activity associated with the cell versus the culture supernatant were determined (Table 2). At low concentrations of xylan, endoxylanase was predominantly extracellular and with increasing concentrations of the substrate, activity was predominatly cell associated.

Table 2. Effect of xylan concentration on cell bound versus excreted endoxylanase activity in *Thermoanaerobacter* strain B6A-RI.

Xylan concentration %(w/v)	Extracellular activity (mU/ml)	Cellular activity (mU/ml)	Ratio of extracellular/ cellular activity
0.05	10	2	5
0.10	24	8	3
0.15	40	26	1.6
0.20	46	38	1.2
0.30	62	74	0.8

Thermoanaerobacter strain B6A-RI was grown in 10 ml pressure tubes on TYE medium with different concentrations of xylan. Cells were harvested at midexponential phase and endoxylanase activity determined.

Purification of endoxylanase activity by affinity chromatography

Extracellular endoxylanase existed as a high molecular weight complex (greater than 1000 kDa) which could not be separated by standard methods e.g. ammonium sulfate precipitation, anion exchange chromatography and cation exchange chromatography using a number of different buffer systems at different pH values. When analyzed using SDS-PAGE and zymograms, endoxylanase was composed of multiple bands, including proteins without endoxylanase activity.

In the purification scheme employed, gel filtration was used to elute a high molecular weight fraction which was pooled and concentrated. The results showed that most activity was associated with the high molecular weight fraction. This partially purified endoxylanase was further purified by affinity chromatography using xylan-coupled Sepharose CL-4B.

Initially binding of the high molecular weight endoxylanase to the affinity column material did not occur at 4°C or at room temperature. As this procedure relies on interaction of the enzyme active site with the substrate, the temperature of the column was increased to 45°C, in an attempt to facilitate this interaction. Initially the pH of the column was 5.5, however, there was limited binding of endoxylanase at this pH, and the pH was lowered to 4.0 with a significant increase in affinity of the enzyme for the column matrix. This approach was successful and a single peak of activity eluted, which appeared on SDS-PAGE as a major band of 160 kDa and a minor 130 kDa band (Fig. 3a).

The *in situ* zymogram technique has been used for analysis of thermostable cellulases and xylanases from *C. thermocellum* (22, 27), and proved to be a very convenient method for preliminary characterization of these endoxylanase activities from *Thermoanaerobacter* B6A-RI. To

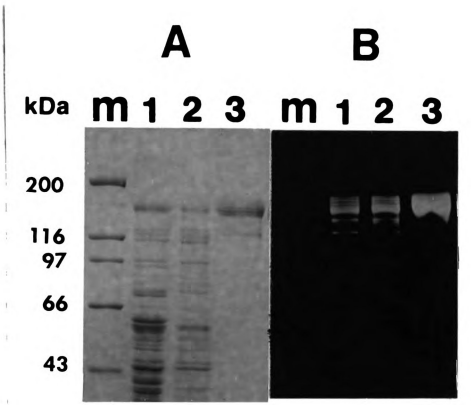


Figure 3. SDS-PAGE (A) and a zymogram (B) of endoxylanase purification from *Thermoanaerobacter* strain B6A-RI grown on xylose.

10 μg of protein were applied to each lane. Lane m, size marker; lane 1, extracellular fraction from the culture grown on xylose; lane 2, after gel filtration using a Sephacryl S-300 column; lane 3, after affinity chromatography using xylan-coupled Sepharose CL-4B.

determine that the protein bands seen in SDS-PAGE had endoxylanase activity, a zymogram was performed by renaturing the endoxylanase after SDS-PAGE and detection *in situ* (Fig. 3b). From the zymogram, multiple endoxylanase activities with high molecular weight were detected indicating that more than one enzyme with the ability to hydrolyze xylan was present.

PAS (Periodic Acid, Schiff's reagent) staining indicated that the endoxylanases from *Thermoanaerobacter* B6A-RI are glycoproteins (data not shown).

Characterization of the endoxylanase activity

Using the endoxylanase complex eluted from the affinity column, temperature and pH optima were determined. The pH optimum for the endoxylanase was 6.0 (Fig. 4) and the temperature optimum was 70°C (Fig. 5). The specific activity of the endoxylanase complex on xylan was 41 units/mg protein. The products of xylan hydrolysis by the affinity purified endoxylanase complex were mainly xylobiose and xylotetriose as determined by TLC (data not shown).

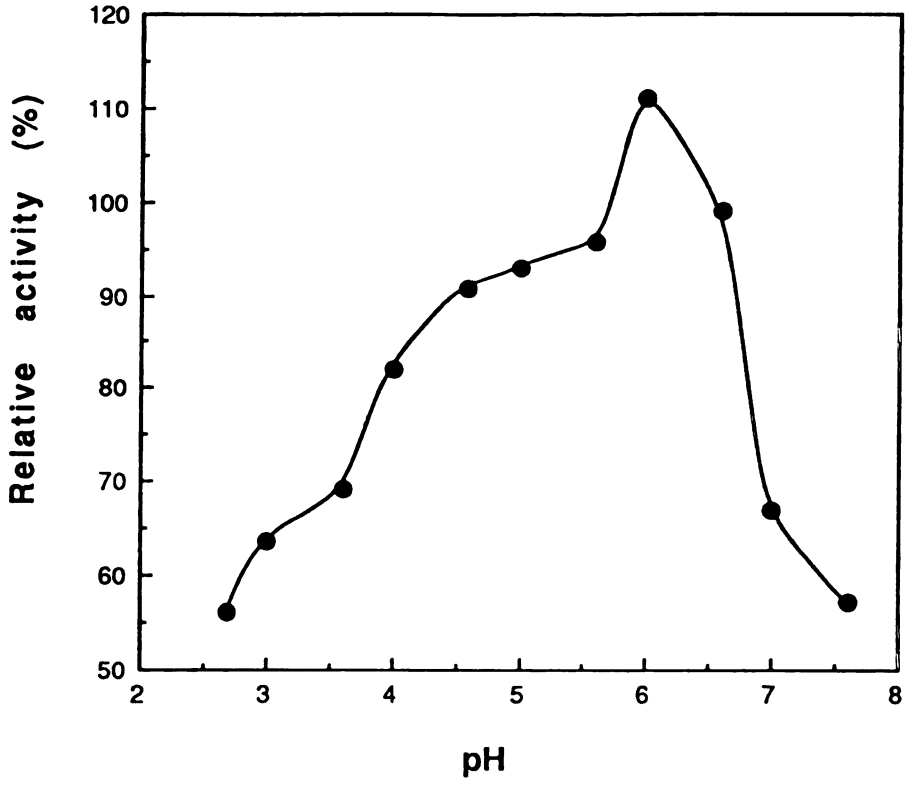


Figure 4. The pH profile of partially purified endoxylanase from *Thermoanaerobacter* B6A-RI.

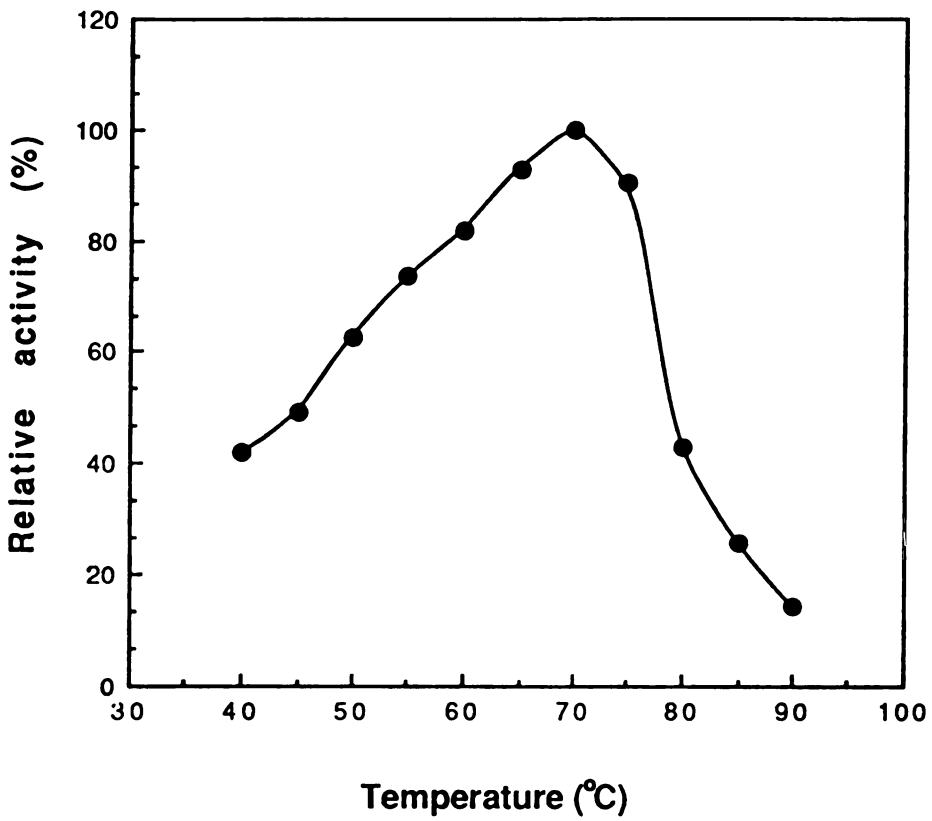


Figure 5. The temperature profile of partially purified endoxylanase from *Thermoanaerobacter* B6A-RI.

DISCUSSION

These data represent the first detailed characterization of the xylanolytic activities present in *Thermoanaerobacter* strain B6A-RI. This organism degrades xylan by the coordinate production of a complex of hydrolyzing enzymes which act together in a concerted manner to effectively degrade the substrate. This organism produces the highest level of xylanases reported for a non-cellulytic producing thermoanaerobe. During growth on insoluble xylan, cells of *Thermoanaerobacter* strain B6A-RI produce a cell bound endoxylanase complex comprised of multiple proteins which is excreted when the insoluble growth substrate is limiting. In addition, intracellular β-xylosidase, acetyl esterase and arbinofuranosidase are produced. Synthesis of the xylanolytic outfit is regulated by pentose containing substrates.

Acetyl esterase activity was constitutive in *Thermoanaerobacter* strain B6A-RI. Xylose and xylan acted as inducers for β-xylosidase and endoxylanase, arabinose induced arabinofuranosidase. Xylan and xylose are also required to induce xylanase and β-xylosidase production in *Butyrivibrio fibrisolvens* (28), and xylose induces xylanase, xylanopyranosidase and arabinofuranosidase activity in *Clostridium acetobutylicum* (18), and xylanase and β-xylosidase in *Bacillus pumilus* (24).

In most xylanolytic bacteria endoxylanase activity appears to be mainly extracellular during growth on xylan, as found in *B. fibrisolvens* (11, 28), *Thermoanaerobacter ethanolicus* (36), and *C. acetobutylicum* (18). β-xylosidase appears to be mainly cell associated in *B. fibrisolvens* (11, 28) and equal levels were found in the extracellular fraction and cell bound fraction in cultures of *C. acetobutylicum* (18). This differs from the findings in this

study where highest levels of all of the xylan degrading enzymes were found associated with the cell during growth on xylan, except under conditions of xylan limitation in which endoxylanase becomes predominantly extracellular. Similar findings have been reported for pullulanase from *Clostridium thermohydrosulfuricum* which was predominantly cell bound during growth of the organism on 0.5% starch (1). However, when the organism was grown in continuous culture with growth-limiting amounts of starch, pullulanase and α–amylase were overproduced and a partial disintegration of the cell surface occurred, which was associated with the formation of membrane "blebs" and extracellular vesicles (1).

In *Clostridium thermocellum* a cell surface glycoprotein complex, termed the cellulosome, containing enzymes necessary for the degradation of cellulose has been demonstrated. This complex exists in cell surface bound and cell-free forms, and has been shown to be responsible for cellular adherence to cellulose and for the degradation of cellulose to cellobiose by the intact organism (2, 16). The cellulosome constitutes the majority of the endoglucanase activity (~70%) and about one third of the total extracellular protein, and possesses the major proteins so far reported for the entire cellulolytic apparatus in this organism (15). Recently, xylanase activity has also been found to be localized in the cellulosome (22). Our studies in progress include examining *Thermoanaerobacter* B6A-RI for the presence of a xylanosome comprised of endoxylanases, xylan binding proteins and other xylanolytic enzymes.

Many anaerobic cellulolytic bacteria possess high molecular weight, multisubunit cellulases which are often found associated with the cell surface (38) or sedimentable membranous fragments (9). *Bacteroides succinogenes* produces CMCase, β-glucosidase, xylanase and β-xylosidase during growth on

cellulose, and approximately 50% of each of the released enzymes was associated with sedimentable subcellular membrane vesicles which adhered to cellulose (7). In these cellulolytic anaerobes enzyme complexes analogous to the cellulosome may be present. In *Thermoanaerobacter* strain B6A-RI, the finding of predominantly cell bound endoxylanase activity, a strong affinity of the cells for the substrate and the presence of cell surface protrubances (Lee, Lowe, Pankratz & Zeikus, manuscript prepared for publication) suggests the presence of a xylanosome cell surface structure analogous to the cellulosome in *C. thermocellum.*

The genes encoding the major endoxylanase and β-xylosidase activities in *Thermoanaerobacter* strain B6A-RI have been cloned (Lee and Zeikus, manuscript prepared for publication), and the endoxylanase gene contains a leader sequence whereas the β-xylosidase gene does not, confirming the findings from this present study that endoxylanase activity is capable of excretion from the cell, whereas β-xylosidase intracellular in cell location.

Although there has been great interest in the purification of xylanases, relatively few attempts have been made using affinity chomatography (26, 30). This approach has been successful in this study with the finding that *Thermoanerobacter* B6A-RI produces multiple endoxylanases. In addition, endoxylanase activities were associated in a high molecular weight protein complex as evidenced from gel filtration, and it was difficult to separate this complex into individual components. The zymogram study showed broad activity bands due to the presence of multiple endoxylanases of similar molecular weight. PAS staining indicated that these proteins may be glycoproteins, therefore, this multiplicity may arise from different glycosylation of single parent enzyme. However, it can not be ruled out that this may be the result of proteolysis or RNA processing.

Thermoanaerobacter B6A-RI endoxylanase is active at high temperature and has a temperature optimum similar to other purified endoxylanases including those from some *Bacillus* sp. (12, 23), and *Clostridium stercorarium* (5). Interestingly the endoxylanase could only bind to the xylan-coupled Sepharose CL-4B at temperatures above 45°C and purification attempts at 4°C and at room temperature were not succesful. This suggests that the substrate binding site of the thermophilic endoxylanase from *Thermoanaerobacter* B6A-RI may open at high temperatures. It has been postulated that thermostable enzymes are compact molecules and need high temperatures for proper configuration for substrate binding and catalysis, and the findings in this study support this hypothesis.

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Chapter IV

Cloning, sequencing and biochemical characterization of endoxylanase from *Thermoanaerobacter* strain B6A-RI

ABSTRACT

A genomic library of *Thermoanaerobacter* B6A-RI was constructed in *Escherichia coli* HB101 using the cosmid vector pHC79. A recombinant plasmid, pXDM1, contained a DNA insert of 28 kb, and gave rise to clones which had both endoxylanase and β-xylosidase activities. Subcloning of the plasmid revealed that the two genes were clustered within a 20 kb DNA fragment. The gene encoding for β-xylosidase (*xynB*) was located upstream of the endoxylanase (*xynA*) gene. *xyn*B was found in a 2.5 kb fragment and *xynA* in a 5 kb fragment. A putative 33 amino acid signal peptide was present in *xynA*, which corresponded to the N-terminal amino acids. An open reading frame (ORF) of 3471 bp, corresponding to 1157 amino acid residues, was found. The molecular weight of the endoxylanase calculated from the deduced amino acid sequence was 130 kDa. *xynA* from *Thermoanaerobacter* B6A-RI, had strong similarity to genes from family F β–glycanases grouped by hydrophobic cluster analysis. The distribution of endoxylanase activity was approximately equal between the periplasm and cytoplasm in the clones containing *xynA*. The apparent pH and temperature optimum for the activity of the cloned endoxylanase was 70°C and 5.5 respectively. The cloned endoxylanase A was stable at 75°C with no detectable loss of activity over 60 min, and displayed a specific activity of 23 units/mg protein on oatspelt xylan. The cloned xylanase was an endo-acting enzyme able to cleave xylan and xylooligosaccharides, but not xylobiose.

INTRODUCTION

Hemicelluloses are the second most abundant polysaccharides in nature, and they are closely associated with cellulose and lignin in plant cell walls. The major component of hemicellulose is xylan, a heteropolymer with a backbone of β-1,4-linked D-xylose residues and short side chains of arabinose and methylglucuronic acid (2).

Biodegradation of xylan involves the action of two major xylanolytic enzymes, namely endoxylanase (1,4-β-D-xylan-xylanohydrolase; E.C. 3.2.1.8) and β-xylosidase (1,4-β-D-xylan-xylohydrolase; E.C. 3.2.1.37). The endoxylanase randomly cleaves the internal xylosidic linkages of the xylan backbone, yielding xylooligosaccharides of various lengths which are further hydrolyzed to xylose molecules by β-xylosidase. The hydrolysis of β-1,4-xylan plays an important role in the conversion of renewable resources such as straw and wood into easily fermentable products.

Some possible applications for xylanases in the pulp and paper industry would require cellulose-free xylanase preparations to prevent cellulose degradation (36). Thermophilic xylanases could have significant advantages over mesophilic xylanases because of their thermostability and catalytic efficiencies at high temperatures (9). Enzyme catalyzed xylan hydrolysis depends on the thermostability of xylanase in order to obtain higher reaction rates and greater solubility of reactants at high temperatures. Fungi such as *Thermoascus aurantiacus* (48) and *Humicolosa grisea* (27), and bacteria such as *Bacillus* sp. (37), *Clostridium stercorarium* (1), *Clostridium thermolacticum* (8) and *Clostridium thermocellum* (28) have been reported to produce thermostable xylanases.

Xylanases from various microorganisms have been cloned and expressed in *E. coli* (11, 20, 24, 25, 33, 41, 46, 47), but there have been very few reports on thermostable xylanases from thermophilic anaerobic bacteria (15, 24).

A number of thermoanaerobic bacteria have been known to have xylanase activity including *Thermoanaerobacter ethanolicus, Thermobacteroides acetoethylicus, Thermoanaerobium brockii,* (42, 43) and *C. thermocellum* (28) which all ferment xylan albeit at a very slow rate, and *Thermoanaerobacter* strain B6A (39) which was shown to extensively degrade xylan (38). However, comparatively little is known about the molecular and biochemical properties of the enzymes from these strains.

Thermoanaerobacter strain B6A-RI is an non-cellulolytic thermophilic anaerobic bacterium capable of degrading a wide range of carbohydrates including xylan (23). We have previously shown that the synthesis of endoxylanase and β-xylosidase in *Thermoanaerobacter* strain B6A-RI is induced by xylan and xylose. In this bacterium the degradation of xylan to xylooligosaccharides is catalyzed by extracellular or cell-bound endoxylanases followed by hydrolysis to D-xylose by β-xylosidase.

In order to investigate the molecular mechanism of xylan degradation by *Thermoanaerobacter* strain B6A-RI and to overproduce thermophilic xylanases, we have constructed a genomic library of this organism using the cosmid vector pHC79, from which we isolated a cosmid clone which had both the endoxylanase and β-xylosidase activities. In this report we describe the cloning and expression of *Thermoanaerobacter* xylanase genes in *E. coli,* and the biochemical and molecular properties of a recombinant endoxylanase which was purified to homogeneity.

MATERIALS AND METHODS

Bacterial strains and plasmids

The xylanolytic thermoanaerobic bacterium used in this study is *Thermoanaerobacter* strain referred to as strain B6A-RI (23). *Escherichia coli* strains HB101 (F$^-$*mcr*B *mrr hsd*S20[$r_B^-m_B^-$] *rec*A13 *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20[Sm^r] *xyl*5 λ^- *leu mtl*1) and DH5α (F- ϕ80d*lacZ* Δ[*lacZYA-argF*]U169 *deo*R *rec*A1 *end*A1 *hsd*R17[r_K^-, m_K^+] *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1) were used as the recipient strains for recombinant plasmids. Cosmid vector pHC79 was used for the construction of a genomic library, and plasmid pUC18 or pUC19 was used for subcloning.

Chemicals, media and culture conditions

All chemicals were of reagent grade and were of the highest purity available. *Thermoanaerobacter* strain B6A-RI was grown in TYE medium (50) containing 0.5% xylose at 60°C. LB medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) was used to grow the *E. coli* strains. For agar plates 1.5% (w/v) Bacto-agar (Difco Laboratories, Detroit, MI) was included. The medium was supplemented with ampicillin (50 μg/ml) for *E. coli* strains carrying a vector or recombinant plasmids.

Plate assay for the detection of thermostable endoxylanase

LB agar plates containing 0.2% (w/v) 4-O-methyl-D-glucurono-D-xylan-Remazole Brilliant Blue R (RBB-xylan), and methylumbelliferylxyloside (10 mM) (Sigma Chemical Co., St Louis, MO) were used for screening of the endoxylanase and β-xylosidase activity respectively. *E. coli* was plated onto

these media and incubated overnight at 37°C. The plates were transferred to 60°C for 2 to 4 hr to detect the enzyme activities.

Preparation of DNA

Thermoanaerobacter strain B6A-RI was grown anaerobically to early stationary phase at 60°C, and chromosomal DNA was isolated by a modification of the Marmur method (26) as described previously. Plasmid DNA was purified from the cleared lysate by the procedure described by Clewell (7), followed by ultracentrifugation in a cesium chloride-ethidium bromide density gradient. For screening of recombinant plasmids in transformants, the rapid alkaline extraction procedure of Birnboim and Doly (5) was used.

Construction of genomic library of *Thermoanaerobacter* B6A-RI and cloning procedure in *E. coli*

The basic procedure for cloning was conducted according to the method of Sambrook et al. (32). Chromosomal DNA of *Thermoanaerobacter* strain B6A-RI was partially digested with Sau3A and 30 to 40 kb fragments were isolated from an agarose gel by electroelution, using an electroelution apparatus, Elutrap (Schleicher & Schuell, Inc. Keene, NH). The cosmid vector pHC79 was linearized with BamHI and dephosphorylated with Calf intestine phosphatase. The partially digested chromosomal DNA fragments were ligated with linearized dephosphorylated pHC79 at a molar ratio of 1:2. The ligated DNA was packaged with a lambda *in vitro* packaging kit as described by the manufacturer (Amersham Co., Arlington Heights, IL). *E. coli* HB101 was infected with the recombinant phage preparation and plated onto LB agar plates containing ampicillin. The resulting transformants were screened on

LB agar plates containing 0.2% (w/v) RBB-xylan and ampicillin (50 μg/ml). Xylanase activtiy was detected as a clear halo around colonies on RBB-xylan plates after incubation at 60°C.

Restriction enzyme mapping and subcloning of the endoxylanase gene (*xynA*)

After digestion with the appropriate restriction endonucleases the desired fragments were eluted from the agarose gel by electroelution using the Elutrap, and subcloned into the plasmid pUC18. Each subclone was transformed into *E. coli* DH5α, spread on an LB agar plate containing ampicillin, and incubated overnight at 37°C. The endoxylanase and β-xylosidase activity was determined using RBB-xylan plates and LB agar plates containing methylumbelliferylxyloside, respectively and recombinant clones were identified by the restriction pattern of the plasmid DNA isolated by rapid alkaline extraction.

Southern hybridization

Chromosomal DNA from *Thermoanaerobacter* strain B6A-RI and *E. coli* HB101 were digested completely with restriction enzymes, electrophoresed through agarose gels and stained with ethidium bromide. For hybridization with radiolabed probes, the DNA was transferred to a Zeta-Probe blotting membrane (Bio-Rad Laboratories, Richmond, CA) by the method of Southern (34). Gel-purified DNA fragments were labeled with a random prime DNA-labeling kit (Boehringer Mannheim, Indianapolis, IN) and α-^{32}P dATP (3,000 Ci/mmol, New England Nuclear, Wilmington, DE) according to the manufacturers instructions. Hybridization of the DNA was by the method of Sambrook et al. (32).

DNA sequencing

Double-stranded DNA was denatured by the method of Zhang et al. (51) and was sequenced in both directions using the dideoxy-chain termination method and Sequenase version 2.0 kit (United States Biochemical Co. Cleveland, OH) according to the manufacturer's instructions. Additional sequencing primers were synthesized by the Michigan State University Macromolecular Facility.

DNA sequences were analyzed by using the GENEPRO software package (Hoefer Scientific Instruments, San Francisco, CA) and the University of Wisconsin Genetics Computer Group GCG package (version 7) (10).

Localization of endoxylanase in *E. coli* transformants

Cells were subjected to osmotic shock for release of periplasmic proteins as described previously (44). Following osmotic shock, the cells were suspended in 50 mM sodium acetate buffer, pH 5.5, and sonicated for five cycles of 20 seconds with 1 min intervals, keeping the material on ice. Cell debris was removed by centrifugation at 12,000 X g for 10 min, and the supernatant was retained as the cytoplasmic fraction.

Purification of cloned endoxylanase

Unless otherwise stated, all operations were performed at room temperature.

(i) **Preparation of cell extract:** One liter of an overnight culture of *E. coli* DH5α (pZEP12) grown in LB medium containing ampicillin (50 μg/ml) was centrifuged at 5,000 X g for 20 min. Cells were resuspended in 40 ml of 50 mM sodium actate buffer, pH 5.5, and disrupted by passage through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 20,000

lb/in^2. The cell extract was centrifuged at 12,000 X g for 20 min to remove cell debris, and the supernatant was used as the crude enzyme preparation.

(ii) Heat treatment: The cell extract of *E. coli* DH5α (pZEP12) was heated at 75°C for 10 min in a shaking water bath, and chilled in ice. The denatured proteins were removed by centrifugation at 12,000 X g for 20 min, and the soluble fraction used for endoxylanase purification. The heat-treated soluble fraction was concentrated by centrifugation on an Amicon YM 30 membrane with a 30 kDa molecular weight cut-off (Amicon Co., Danvers, MA).

(iii) Anion exchange column chromatography: The concentrated sample was loaded on a column (2.5 cm x 18 cm) of Q-Sepharose (Pharmacia, Piscataway, NJ) previously equilibrated with 50 mM sodium acetate buffer (pH 5.5). The column was washed with the same buffer and eluted with a 500 ml linear NaCl (0 to 1.0 M) gradient in the same buffer. The active fractions were collected and pooled.

(iv) Affinity column chromatography: The active fractions were pooled and concentrated by ultrafiltration using a YM 30 membrane (Amicon). For affinity chromatography a Pharmacia column (1.5 cm x 33 cm) fitted with a thermostatic jacket connected to a water bath, for temperature control was filled with xylan-coupled Sepharose CL-4B (prepared as described in Chapter III). The column was equilibrated with 50 mM sodium acetate buffer (pH 4.0) at room temperature and the protein sample was loaded once the column had equilibrated to 45°C. After washing with 50 mM sodium acetate buffer (pH 5.5) at room temperature the xylanases were eluted with soluble xylan (0.1%) in 50 mM sodium acetate buffer (pH 5.5) at room temperature. The active fractions were pooled and incubated at 60°C for 2-4 h to digest xylan coeluted with enzyme, and the hydrolysis products were removed by dialysis against 50 mM sodium acetate buffer (pH 5.5).

Enzyme assays and protein determination

Endoxylanase activity was measured by the release of reducing sugars from oat spelt xylan (Sigma Chemical Co., St. Louis, Mo.). β-xylosidase activity was determined by the formation of p-nitrophenol from p-nitrophenyl-β-D-xyloside (Sigma). The assays were performed as described previously (Lee. Lowe & Zeikus, manusrcipt prepared for publication).

One unit of activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min for endoxylanase, and 1 μmol of nitrophenol per min for β-xylosidase.

Protein concentration was determined by the dye-binding assay of Bradford (6) with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (Sigma) as the standard.

Electrophoresis and molecular weight determination

SDS-PAGE was performed as described previously (22) with 7.5% acrylamide using a Mini-PROTEAN II system (Bio-Rad Laboratories, Richmond, CA). Proteins bands were visualized by Coomassie brilliant Blue G-250 staining. The molecular weight of the denatured protein was estimated by SDS-PAGE with high-range molecular weight standards (Bio-Rad Lab. Richmond, CA) including myosin (200,000), β-galactosidase (116,250), phosphorylase (97,400), BSA (66,200) and ovalbumin (43,000).

N-terminal amino acid sequence determination

The protein was prepared by washing the purified endoxylanase with double-distilled water five times with a Centricon-30 (Amicon) filtration device to remove contaminating salts and buffer from the solution. The N-

terminal amino acid sequence was identified by a protein sequencer model 477A (Applied Biosystems, Foster City, CA) with an on-line phenylthiohydrantoin analyzer (Applied Biosystems) in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University.

Analysis of products of xylan hydrolysis by thin layer chromatography

The xylan and xylooligosaccharides were dissolved in 50mM sodium acetate buffer (pH 5.5) and incubated at 60°C with approximately 1.5 U of purified enzyme. Samples were withdrawn periodically and the hydrolysis products were analyzed by Whatman HP-K high performance silica gel plates (10 x 10 cm) using the following solvent system: butanol/acetic acid/distilled water (2:1:1, by vol.), and were visualized using an orcinol spray reagent (100 mg orcinol in 100 ml 20% sulphuric acid in methanol).

RESULTS

Construction of a genomic library and cloning of xylanolytic genes

In order to detect xylanolytic genes, a genomic library of *Thermoanaerobacter* B6A-RI was constructed in *E. coli* HB101 using the cosmid vector pHC79. Approximately 800 transformants were obtained. Xylanase activity was detected directly on the RBB-xylan plates. Two colonies produced clear halos, indicating the presence of xylanase activity. *E. coli* HB101 harboring pHC79, lacking cloned xylanolytic genes, did not clear RBB-xylan plates. When plasmid DNA was isolated from these positive clones and subjected to restriction enzyme analysis, both plasmids were found to contain a DNA insert of ca. 36 kbp with identical restriction patterns. This recombinant plasmid was designated as pZXA1 and its physical map was constructed (Fig. 1).

Genes involved in hemicellulose degradation have been found to be clustered in a number of bacteria. Due to the coordinate regulation during growth of *Thermoanaerobacter* B6A-RI on xylan and xylose (Lee, Lowe & Zeikus, manuscript in preparation), the hemicellulase genes may be closely situated on the chromosome. To investigate the xylanolytic activities expressed in the cosmid clone, *E. coli* HB101 containing the recombinant plasmid pZXA1 was grown on LB medium and assayed for various enzymatic activities. High levels of β-xylosidase and endoxylanase activity but relatively weak acetyl-esterase and arabinosidase activities were detected in the crude extract of the clone (data not shown).

The growth of *E. coli* HB101 harboring the recombinant plasmid pZXA1 was poor and some colonies lost the ability to give clear halos on RBB-xylan plates indicating that the cosmid clone was not stable. After several

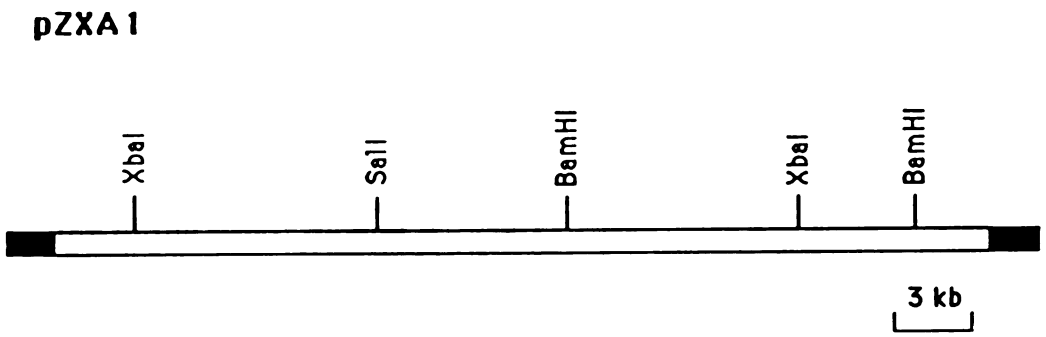


Figure 1. Linear restriction map of plasmid pZXA1 which carries the *Thermoanaerobacter* B6A-RI DNA insert expressing thermostable endoxylanase.

The open box represents *Thermoanaerobacter* chromosomal DNA insert and closed box represents cosmid vector pHC79.

transfers on LB medium supplemented with ampicillin, colonies were screened on RBB-xylan plates for endoxylanase activity and on LB agar plates containing methyl-umbelliferylxyloside and ampicillin for β-xylosidase activity in order to isolate stable clones having both activities. One colony which consistently gave both activities was isolated and the plasmid DNA obtained. This plasmid, designated as pXDM1 (Fig. 2), had a smaller size of DNA insert (28 kb) than the original clone indicating that the insert had undergone a partial deletion during propagation in the *E. coli* host.

Subcloning of the endoxylanase A gene

To localize the functional domain of the endoxylanase (*xynA*) and β-xylosidase (*xynB*) genes, several subcloning experiments were performed using plasmid pUC18. *E. coli* DH5α cells were transformed with subcloned plasmid DNA, and plated on RBB-xylan plates, and LB agar containing methyl-umbelliferylxyloside, to detect subclones expressing xylanase and β-xylosidase activity, respectively. Clones exhibiting endoxylanase activity were visualized directly as clear halos around the colonies due to the diffusion of the RBB dye released by cleavage. Clones exhibits β-xylosidase activity were visualized under ultraviolet as colonies having a blue-white fluorescence, due to the diffusion of the umbelliferone released by cleavage. The subcloning strategy and the resulting enzyme activities are summarized in Fig. 2. The two genes were clustered within a 20 kb DNA fragment, but not next to one another. The gene encoding for β-xylosidase (*xynB*) was located upstream of the endoxylanase (*xynA*) gene. Using restriction deletions, *xynB* was found to be a 2.5 kb fragment and *xynA* within a 5 kb fragment.

To confirm the chromosomal origin of the DNA insert in pZEP12 Southern hybridizartion was performed on chromosomal DNA from *E. coli*

Figure 2. Physical map of plasmid pXDM1 and functional mapping of the endoxylanase and β-xylosidase gene domain.

The 28 kb *Thermoanaerobacter* DNA insert was subjected to subcloning at various restriction sites (B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SalI; X, XbaI). Deleted areas are indicated with dotted lines. Solid lines indicate subcloned fragments. The hatched areas represent the location of the genes. The relative amounts of enzyme activities found in *E. coli* carrying the DNA fragments is denoted by + or -.

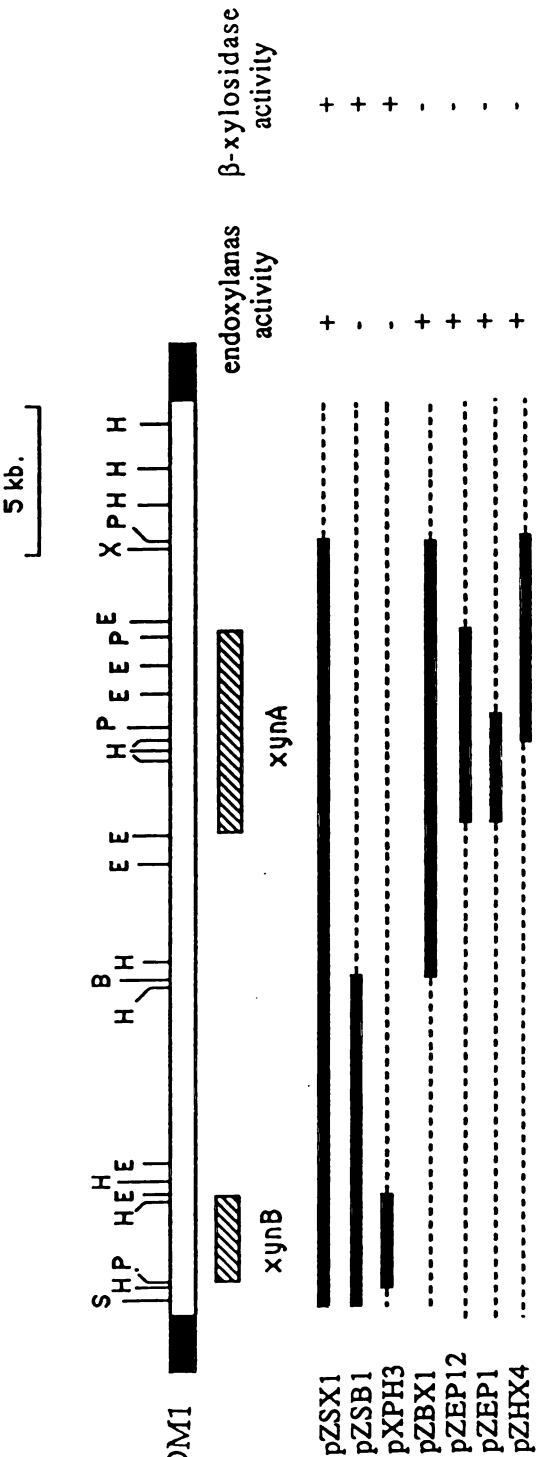
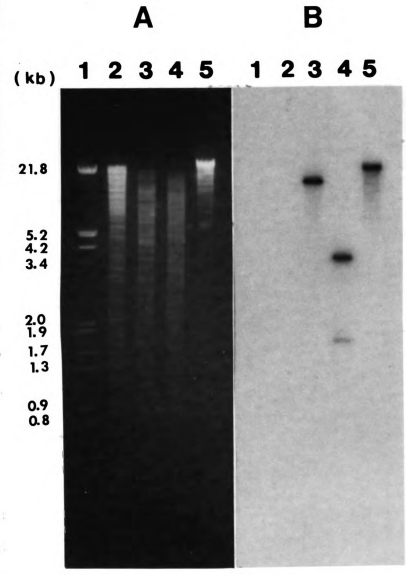

5 kb.
S H P H E H E E H B H E E H P E E P E X P H H H
pXDM1
xynB
xynA
endoxylanas activity
β-xylosidase activity
pZSX1 + +
pZSB1 - +
pXPH3 - +
pZBX1 + -
pZEP12 + -
pZEP1 + -
pZHX4 + -

Figure 3. Southern hybridization analysis of *Thermoanaerobacter* B6A-RI and *E. coli* genomic DNA digests with random-primed DNA synthesized from the 1.2 kb HincII-PstI fragment from pZEP12.

(A) Photograph of 0.8% agarose gel stained with ethidium bromide. (B) Autoradiogram of DNA transferred to membrane and hybridized with the ^{32}P-labeled insert fragment. Lane 1, molecular weight markers; lane 2, *E. coli* chromosomal DNA digested with PstI; lane 3, 4 & 5, *Thermoanaerobacter* B6A-RI chromosomal DNA digested with PstI, EcoRI and XbaI, respectively.


A
B
(kb)
1 2 3 4 5
1 2 3 4 5
21.8
5.2
4.2
3.4
2.0
1.9
1.7
1.3
0.9
0.8

and *Thermoanaerobacter* B6A-RI digested with EcoRI, PstI and XbaI. An internal 1.2 kb HincII-PstI fragment from pZEP12 was used as a probe for endoxylanase (*xynA*). The results are shown in Fig. 3. The fragment did not bind to *E. coli* chromosomal DNA but did hybridize to *Thermoanaerobacter* B6A-RI genomic DNA, indicating that the gene originated from *Thermoanaerobacter* B6A-RI.

xyn A sequence analysis

A deletion plasmid containing *xynA*, designated as pZEP12, was constructed from pXDM1, and the detailed physical map of *xynA* is shown in Fig. 4. The nucleotide sequence of a EcoRI-PstI fragment in pZEP12, determined by the dideoxy-chain termination method, is shown in Fig. 5. The sequence is consistent with the observed restriction map. An open reading frame (ORF) of 3471 bp, corresponding to 1157 amino acid residues, was found. The molecular weight of the enzyme calculated from the deduced amino acid sequence was 130 kDa. The N-terminal amino acid sequence of the endoxylanase, purified from *E. coli* (pZEP12), is shown as underlined in Fig. 5. The N-terminal sequence was preceded by a putative 33 amino acid signal peptide in *xynA* (boxed area in Fig. 5). The endoxylanase activity of *Thermoanaerobacter* B6A-RI was excretable (Lee, Lowe & Zeikus, manuscript prepared for publication), therefore it was expected that the endoxylanase had a signal peptide. Hydrophobicity analysis (data not shown) of the deduced amino acid sequence present in this putative signal peptide also supports the proposed function for this 33 amino acid sequence. The putative initiation codon, ATG, was found in two separate places upstream of the cleavage site of the signal peptide. The putative ribosome-binding sequence, 5'-AGGGAGG-3', was observed 5 bp upstream of the first intiation codon, ATG.

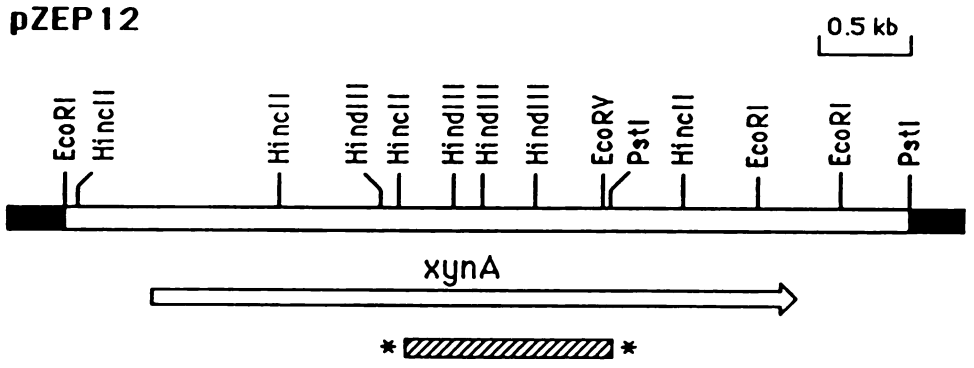


Figure 4. Physical and genetic map of the deletion plasmid pZEP12 containing *xyn*A contructed from pXDM1.

The cloned *Thermoanaerobacter* B6A-RI DNA insert is indicated by the open bar, and pUC18 by the closed bar. The location of the endoxylanase (*xynA*) and the location of transcription is shown by the arrow. Shaded bar indicates the DNA fragment used as a probe.

Figure 5. Nucleotide sequence and translated amino acid sequence for the endoxylanase A gene from *Thermoanaereobacter* B6A-RI.

The boxed area represents the putative signal peptide. The arrow denotes the cleavage site of the signal peptide. The dashed region denotes the N-terminal amino acid sequence as determined from the purified recombinant endoxylanase. The numbers beneath the sequence denotes the amino acid number after cleavage of the signal peptide.

```
1   CACATTACAGTATGAATTGCAGAAAGTTTTGCAGTCAACACAGCAATATACACAGCAAGC   60

61  ATCATTTGATATGGGGCTAAAAAATAATTCTAATTCAGTTACACCTGAAAGCATAAGAGC   120

121 TGCTATGACAATAGTAGCAGTAGTTCCAATTGCAGAAATATTTTGTAAAGGGTTTGACAA   180

181 TTGGCAGCGTAAAAGGTTAGAATTTGTTATTTGGGTATTTGGAAATTATATGCGTGAAAG   240

241 GGGGATTAAAAATATTTATTTTTTAGATTAAGTCTTTTTGATTGTGAGGTGATAGTAAAT   300

301 AATTTTTCAATAATTAATTTTACACATGTTACACAGGGAGGTATTATGATGAAAAATAAT   360
                                  S.D.   MetMetLysAsnAsn

361 GTAGATAGGATTGTATCTATTGTTACAGCTTTAATAATGATTTTTGGAGCATCATTATTT   420
    ValAspArgIleValSerIleValThrAlaLeuIleMetIlePheGlyAlaSerLeuPhe

421 TCCCCGCCAATAAGAGTTTTTGCTGATGACACTAATATAAATCTGGTTTCTAATGGGGAC   480
    SerProProIleArgValPheAlaAspAspThrAsnIleAsnLeuValSerAsnGlyAsp
                              ↑
481 TTTGAATCAGGCACAATTGATGGCTGGATTAAGCAAGGTAATCCGACATTAGCAGTAACG   540
    PheGluSerGlyThrIleAspGlyTrpIleLysGlnGlyAsnProThrLeuAlaValThr
                                20

541 ACTGAGCAAGCAATTGGGCAATACAGTATGAAAGTTACTGGTAGAACACAGACATATGAA   600
    ThrGluGlnAlaIleGlyGlnTyrSerMetLysValThrGlyArgThrGlnThrTyrGlu
                                40

601 GGACCTGCATATAGCTTTTTAGGAAAAATGCAGAAAGGTGAATCATATAGTGTATCGCTT   660
    GlyProAlaTyrSerPheLeuGlyLysMetGlnLysGlyGluSerTyrSerValSerLeu
                                60

661 AAAGTTAGACTTGTTTCTGGACAAAATTCATCTAATCCTTTGATTACCGTGACTATGTTT   720
    LysValArgLeuValSerGlyGlnAsnSerSerAsnProLeuIleThrValThrMetPhe
                                80

721 AGAGAAGATGACAATGGCAAGCATTACGATACAATAGTTTGGCAAAAACAAGTTTCTGAA   780
    ArgGluAspAspAsnGlyLysHisTyrAspThrIleValTrpGlnLysGlnValSerGlu
                                100

781 GATTCATGGACTACTGTAAGCGGAACTTATACATTAGATTATATTGGAACATTGAAAACA   840
    AspSerTrpThrThrValSerGlyThrTyrThrLeuAspTyrIleGlyThrLeuLysThr
                                120

841 TTATATATGTATGTAGAATCACCCGATCCAACGCTGGAATACTATATTGATGATGTTGTA   900
    LeuTyrMetTyrValGluSerProAspProThrLeuGluTyrTyrIleAspAspValVal
                                140
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901  GTAACAACACAAAATCCAATTCAAGTAGGGAACGTAATTGCCAATGAAACTTTTGAAAAT  960
     ValThrThrGlnAsnProIleGlnValGlyAsnValIleAlaAsnGluThrPheGluAsn
                                160

961  GGAAATACTTCTGGATGGATTGGAACAGGTTCATCTGTTGTTAAGGCAGTGTATGGAGTG  1020
     GlyAsnThrSerGlyTrpIleGlyThrGlySerSerValValLysAlaValTyrGlyVal
                                180

1021 GCTCATAGCGGAGATTATAGCTTATTGACGACAGGGAGAACAGCTAATTGGAATGGTCCT  1080
     AlaHisSerGlyAspTyrSerLeuLeuThrThrGlyArgThrAlaAsnTrpAsnGlyPro
                                200

1081 AGCTATGATTTGACTGGCAAAATAGTGCCTGGTCAACAATACAATGTTGATTTTTGGGTG  1140
     SerTyrAspLeuThrGlyLysIleValProGlyGlnGlnTyrAsnValAspPheTrpVal
                                220

1141 AAATTTGTTAATGGCAACGATACAGAACAAATAAAGGCTACTGTTAAAGCGACTTCTGAC  1200
     LysPheValAsnGlyAsnAspThrGluGlnIleLysAlaThrValLysAlaThrSerAsp
                                240

1201 AAAGACAATTATATACAAGTTAATGATTTTGCAAATGTAAATAAAGGAGAATGGACAGAA  1260
     LysAspAsnTyrIleGlnValAsnAspPheAlaAsnValAsnLysGlyGluTrpThrGlu
                                260

1261 ATAAAAGGCAGTTTTACTTTACCTGTTGCAGATTACAGCGGTATTAGCATCTATGTAGAA  1320
     IleLysGlySerPheThrLeuProValAlaAspTyrSerGlyIleSerIleTyrValGlu
                                280

1321 TCTCAAAATCCTACTTTAGAGTTTTACATTGATGATTTTTCTGTAATAGGTGAAATTTCA  1380
     SerGlnAsnProThrLeuGluPheTyrIleAspAspPheSerValIleGlyGluIleSer
                                300

1381 AATAATCAGATTACGATACAAAATGACATTCCAGATTTATATTCAGTATTTAAAGATTAT  1440
     AsnAsnGlnIleThrIleGlnAsnAspIleProAspLeuTyrSerValPheLysAspTyr
                                320

1441 TTTCCTATAGGCGTTGCAGTTGATCCAAGTAGATTAAATGATGCTGATCCACATGCTCAA  1500
     PheProIleGlyValAlaValAspProSerArgLeuAsnAspAlaAspProHisAlaGln
                                340

1501 TTGACGGCTAAACATTTTAATATGCTTGTTGCAGAAAACGCCATGAAACCGGAAAGCTTG  1560
     LeuThrAlaLysHisPheAsnMetLeuValAlaGluAsnAlaMetLysProGluSerLeu
                                360

1561 CAACCTACAGAAGGGAACTTTACCTTCGATAATGCTGATAAAATTGTTGACTATGCAATA  1620
     GlnProThrGluGlyAsnPheThrPheAspAsnAlaAspLysIleValAspTyrAlaIle
                                380

1621 GCACATAATATGAAAATGAGAGGTCATACTTTACTTTGGCATAATCAAGTTCCAGATTGG  1680
     AlaHisAsnMetLysMetArgGlyHisThrLeuLeuTrpHisAsnGlnValProAspTrp
                                400
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1681  TTTTTCCAAGATCCATCTGACCCATCCAAGTCTGCTTCGAGAGATTTACTGCTTCAAAGA  1740
      PhePheGlnAspProSerAspProSerLysSerAlaSerArgAspLeuLeuGlnArg
                                420

1741  TTAAAAACGCATATAACAACTGTGTTAGACCATTTTAAAACAAAGTATGGTTCTCAAAAT  1800
      LeuLysThrHisIleThrThrValLeuAspHisPheLysThrLysTyrGlySerGlnAsn
                                440

1801  CCAATAATCGGATGGGATGTTGTAAATGAGGTTCTTGATGATAATGGCAATTTAAGAAAT  1860
      ProIleIleGlyTrpAspValValAsnGluValLeuAspAspAsnGlyAsnLeuArgAsn
                                460

1861  TCTAAGTGGTTGCAGATAATAGGACCTGATTATATAGAAAAAGCTTTTGAATATGCACAT  1920
      SerLysTrpLeuGlnIleIleGlyProAspTyrIleGluLysAlaPheGluTyrAlaHis
                                480

1921  GAAGCAGATCCATCTATGAAATTGTTTATTAATGACTATAACATTGAAAATAATGGTGTT  1980
      GluAlaAspProSerMetLysLeuPheIleAsnAspTyrAsnIleGluAsnAsnGlyVal
                                500

1981  AAGACGCAGGCTATGTATGACTTAGTGAAAAAATTAAAGAGTGAAGGTGTGCCAATAGAT  2040
      LysThrGlnAlaMetTyrAspLeuValLysLysLeuLysSerGluGlyValProIleAsp
                                520

2041  GGAATAGGCATGCAAATGCACATAAATATAAATTCAAATATTGACAATATAAAAGCTTCT  2100
      GlyIleGlyMetGlnMetHisIleAsnIleAsnSerAsnIleAspAsnIleLysAlaSer
                                540

2101  ATAGAAAAACTTGCTTCATTAGGTGTGGAAATACAAGTAACTGAATTAGATATGAACATG  2160
      IleGluLysLeuAlaSerLeuGlyValGluIleGlnValThrGluLeuAspMetAsnMet
                                560

2161  AACGGTAATATATCTAACGAAGCATTGCTTAAACAAGCTAGATTGTATAAGCAATTGTTT  2220
      AsnGlyAsnIleSerAsnGluAlaLeuLeuLysGlnAlaArgLeuTyrLysGlnLeuPhe
                                580

2221  GATTTATTTAAAGCTGAGAAACAGTATATAACTGCTGTAGTTTTTTGGGGAGTTTCAGAT  2280
      AspLeuPheLysAlaGluLysGlnTyrIleThrAlaValValPheTrpGlyValSerAsp
                                600

2281  GATGTAACTTGGCTTAGTAAGCCAAATGCTCCGCTGCTTTTTGATTCAAAATTGCAGGCG  2340
      AspValThrTrpLeuSerLysProAsnAlaProLeuLeuPheAspSerLysLeuGlnAla
                                620

2341  AAGCCAGCATTCTGGGCAGTAGTAGATCCAAGCAAAGCTATACCTGATATTCAATCTGCA  2400
      LysProAlaPheTrpAlaValValAspProSerLysAlaIleProAspIleGlnSerAla
                                640

2401  AAAGCTTTAGAAGGCTCACCGACGATTGGAGCAAATGTTGATAGTTCTTGGAAACTTGTA  2460
      LysAlaLeuGluGlySerProThrIleGlyAlaAsnValAspSerSerTrpLysLeuVal
                                660
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2461  AAACCATTGTATGTAAATACTTATGTAGAAGGAACGGTCGGAGCAACTGCTACTGTTAAG  2520
      LysProLeuTyrValAsnThrTyrValGluGlyThrValGlyAlaThrAlaThrValLys
                                680

2521  TCTATGTGGGATACTAAAAACTTGTATTTGTTAGTACAAGTTTCAGACAATACTCCATCT  2580
      SerMetTrpAspThrLysAsnLeuTyrLeuLeuValGlnValSerAspAsnThrProSer
                                700

2581  AATAATGATGGTATTGAGATTTTTGTAGATAAGAATGACGATAAATCTACTTCTTATGAA  2640
      AsnAsnAspGlyIleGluIlePheValAspLysAsnAspAspLysSerThrSerTyrGlu
                                720

2641  ACTGATGATGAACGTTATACAATTAAGAGGGATGGTACAGGGAGCTCAGATATTACCAAA  2700
      ThrAspAspGluArgTyrThrIleLysArgAspGlyThrGlySerSerAspIleThrLys
                                74

2701  TATGTGACATCTAATGCTGACGGATATGTAGCACAGCTAGCTATTCCAATTGAAGATATC  2760
      TyrValThrSerAsnAlaAspGlyTyrValAlaGlnLeuAlaIleProIleGluAspIle
                                760

2761  AGTCCTGCAGTTAATGATAAAATTGGATTTGACATTAGAATAAACGATGATAAAGGTAAT  2820
      SerProAlaValAsnAspLysIleGlyPheAspIleArgIleAsnAspAspLysGlyAsn
                                780

2821  GGTAAAATAGATGCAATAACGGTTTGGAATGATTATACCAACAGTCAAAATACTAATACA  2880
      GlyLysIleAspAlaIleThrValTrpAsnAspTyrThrAsnSerGlnAsnThrAsnThr
                                800

2881  TCGTATTTTGGAGATATTGTATTGTCAAAATCTGCACAAATCGCAACAGCTATATATGGC  2940
      SerTyrPheGlyAspIleValLeuSerLysSerAlaGlnIleAlaThrAlaIleTyrGly
                                820

2941  ACACCAGTTATTGATGGAAAAGTAGATGATATTTGGAATAATGTTGAACCTATTTCGACA  3000
      ThrProValIleAspGlyLysValAspAspIleTrpAsnAsnValGluProIleSerThr
                                840

3001  AATACATGGATTTTGGGTTCAAATGGTGCTACTGCGACACAGAAAATGATGTGGGACGAT  3060
      AsnThrTrpIleLeuGlySerAsnGlyAlaThrAlaThrGlnLysMetMetTrpAspAsp
                                860

3061  AAGTACCTTTATGTTTTGGCGGATGTTACAGATTCAAATCTGAACAAATCCAGTATTAAT  3120
      LysTyrLeuTyrValLeuAlaAspValThrAspSerAsnLeuAsnLysSerSerIleAsn
                                880

3121  CCATATGAACAAGATTCTGTAGAAGTTTTTGTAGATCAGAATAATGATAAGACAACTTAT  3180
      ProTyrGluGlnAspSerValGluValPheValAspGlnAsnAsnAspLysThrThrTyr
                                900

3181  TATGAGAATGATGACGGCCAATATAGAGTCAACTATGACAATGAACAGAGTTTTGGGGGA  3240
      TyrGluAsnAspAspGlyGlnTyrArgValAsnTyrAspAsnGluGlnSerPheGlyGly
                                920
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3241  AGCACCAATTCAAATGGGTTTAAGTCAGCAACAAGTCTTACACAAAGTGGATATATTGTA  3300
      SerThrAsnSerAsnGlyPheLysSerAlaThrSerLeuThrGlnSerGlyTyrIleVal
                                940

3301  GAAGAAGCCATTCCTTGGACGAGTATCACTCCGTCAAATGGCACTATCATAGGATTTGAC  3360
      GluGluAlaIleProTrpThrSerIleThrProSerAsnGlyThrIleIleGlyPheAsp
                                960

3361  TTGCAAGTTAATAATGCAGATGAAAATGGTAAGAGGACAGGTATTGTCACATGGTGCGAT  3420
      LeuGlnValAsnAsnAlaAspGluAsnGlyLysArgThrGlyIleValThrTrpCysAsp
                                980

3421  CCCAGCGGCAATTCATGGCAAGATACTTCTGGATTTGGCAATTTACTGCTTACAGGTAAG  3480
      ProSerGlyAsnSerTrpGlnAspThrSerGlyPheGlyAsnLeuLeuLeuThrGlyLys
                               1000

3481  CCATCTGGTGCCCTTAAAAAAGGTGTGACATTTGATGACATAAAGAATAGTTGGGCAAAA  3540
      ProSerGlyAlaLeuLysLysGlyValThrPheAspAspIleLysAsnSerTrpAlaLys
                               1020

3541  GACGCAATAGAAGTATTAGCATCAAGGCATATAGTAGAAGGAATGACAGACACTCAGTAT  3600
      AspAlaIleGluValLeuAlaSerArgHisIleValGluGlyMetThrAspThrGlnTyr
                               1040

3601  GAACCAAACAAGACAGTGACAAGAGCAGAATTCACAGCAATGATACTGAGGCTTTTAAAC  3660
      GluProAsnLysThrValThrArgAlaGluPheThrAlaMetIleLeuArgLeuLeuAsn
                               1060

3661  ATAAAAGAAGAGCAATACAGTGGAGAATTTAGCGATGTAAATAGTGGAGACTGGTATGCA  3720
      IleLysGluGluGlnTyrSerGlyGluPheSerAspValAsnSerGlyAspTrpTyrAla
                               1080

3721  AATGCAATAGAAGCAGCATATAAAGCGGGAATAATCGAAGGTGACGGAAAAAATGCAAGG  3780
      AsnAlaIleGluAlaAlaTyrLysAlaGlyIleIleGluGlyAspGlyLysAsnAlaArg
                               1100

3781  CCTAATGACAGCATAACAAGAGAAGAGATGACGCAATAGCCATGAGGGCATACGAGATGC  3840
      ProAsnAspSerIleThrArgGluGluMetThrGlnEnd
                               1120

3841  TGACACAGTACAAAGAAGAAAACATAGGTGCAACATCATTTAGCGATGACAAATCCATAA  3900

3901  GCGACTGGGCAAAGAATGTAGTGGCAAATGCGGCAAAACTAGGAATAGTAAATGGAGAGC  3960

3961  CAAATAACATGTTTGCACCTAAAGATATAGCTACGAGAGC  4000
```

Comparison of the deduced amino acid sequence of *xynA* from *Thermoanaerobacter* B6A-RI with GenBank and EMBL sequence libraries (see Table 1) indicated that *xynA* has a strong similarity to genes from family F β–glycanases, grouped by hydrophobic cluster analysis (12, 19).

Localization and biochemical characterization of the purified endoxylanase A.

To determine the location of the endoxylanase A produced in *E. coli*, cells were grown to early stationary phase and subjected to osmotic shock to isolate periplasmic enzymes, and sonicated to isolate intracellular enzymes (data not shown). The distribution of endoxylanase activity was approximately equal between the periplasm and cytoplasm in the clones containing *xynA*. These data suggest that the signal peptide of *Thermoanaerobacter* B6A-RI is recognized and processed by *E. coli* cells, because of the periplasmic location of the enzyme.

Thermoanaerobacter B6A-RI endoxylanase A expressed in *E. coli* was purified to homogeneity (Table 2). Heat treatment of cell extracts at 75°C for 10 min was a very efficient purification step for the enzyme from *E. coli* as most of the thermolabile *E. coli* proteins could be removed by this procedure. After heat treatment, the purification yield increased 7 fold, without loss of endoxylanase activity. After additional steps of purification by anion-exchange chromatography and affinity chromatography with xylan-coupled Sepharose CL-4B (Lee & Zeikus, manuscript prepared for publication), the enzyme was purified to homogeneity as demonstrated using SDS-polyacrylamide gel electrophoresis (Fig. 6). The purified enzyme consisted of a single band with a molecular weight of 130,000. The faint bands of lower molecular weight observed may be proteolytic digests of the enzyme, since they displayed endoxylanase activity. The apparent pH and temperature

Table 1. Comparison of the translated amino acid sequence of endoxylanase (*xynA*) from *Thermoanaerobacter* B6A-RI with other endoxylanases.

Organism	Gene	Similarity (%)	Identity (%)	Family
Bacillus circulans	*xynA*	44.1	22.7	G
Bacillus pumilus	*xynA*	49.7	24.6	G
Bacillus subtilis	*xynA*	42.6	22.5	G
Butyrivibrio fibrisolvens	*xynB*	53.5	30.2	F
Bacillus sp. C-125	*xynA*	68.4	47.6	F
Caldocellum saccharolyticum	*xynA*	66.2	42.8	F
Clostridium thermocellum	*xynZ*	53.8	30.1	F
Clostridium acetobutylicum	*xynB*	46.9	20.6	G
Pseudomonas fluorescens	*xynA*	51.6	29.7	F
Pseudomonas fluorescens	*xynB*	44.9	23.4	F

Table 2. Summary of purification of endoxylanase from *E. coli* (pZEP12)

Purification step	Total activity (units)	Total protein (mg)	Specific activity (Units/mg)	Purification (fold)	Yield (%)
Cell-free extract	1298	820	1.58	1.0	100
Heat treatment	1283	109	11.77	7.4	99
Q-Sepharose	651	41	15.88	10.0	50
Affinity chromatography	166	0.73	227.4	143.9	12.8

optimum for the activity of the cloned endoxylanase was 5.5 and 70°C respectively (Fig. 7 & 8). The cloned enzyme was stable at 75°C, with no detectable loss of activity over 60 min, but was less stable at higher temperatures with a half-life of approximately 35 min at 80°C. The specific activity of the purified endoxylanase A was 23 units/mg protein on oatspelt xylan.

Hydrolysis of xylan and xylooligosaccharides

The time course of hydrolysis of the soluble fraction of oat spelt xylan and xylooligosaccharides by the recombinant endoxylanase is shown in Fig. 9. Several different sizes of xylooligosaccharides were released from oat spelt xylan. After 30 min of incubation an increase in xylobiose and xylotriose was observed with a concommitant decrease in intermediary products of hydrolysis such as xylotetrose and xylopentose. When the incubation was prolonged to 5 hr, the main products were xylobiose and xylotriose with small amounts of xylose and xylopentose. Endoxylanase A was not active on xylobiose. Xylotriose was cleaved to xylobiose and xylose, and xylopentose was initially cleaved to xylotriose and xylobiose, but on prolonged incubation a small amount of xylose was formed. These results indicate that the cloned xylanase A was an endo-acting enzyme, able to cleave xylan at random, and having no activity on xylobiose.

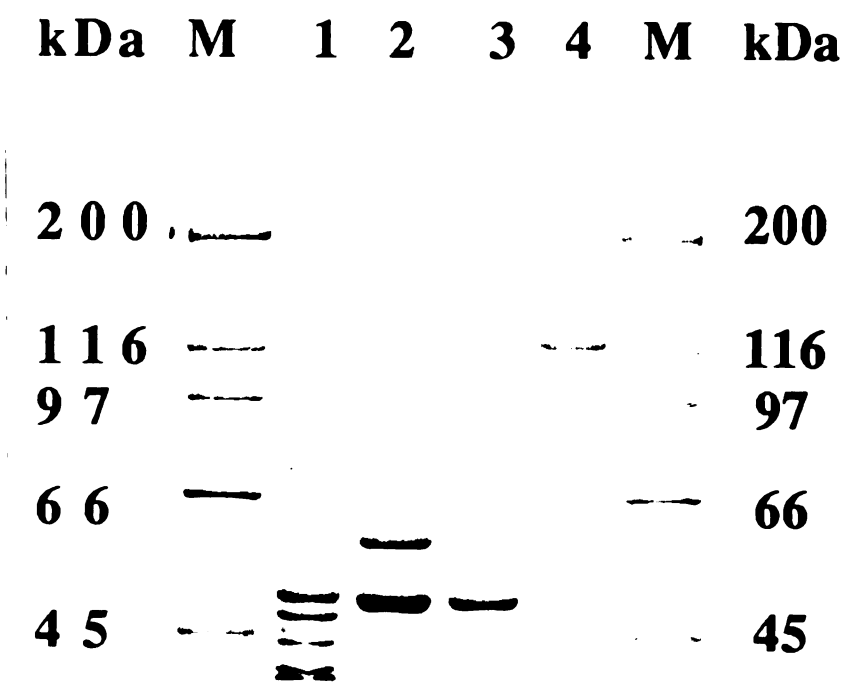


Figure 6. SDS-PAGE analysis of recombinant endoxylanase purification from *E. coli* (pZEP12).

Lane M, molecular weight markers; lane 1, crude *E. coli* (pZEP12) extract (20 μg protein); lane 2, following heat treatment (10 μg protein); lane 3, after anion exchange chromatography (5 μg protein) and lane 4, after affinity chromatography (3 μg protein).

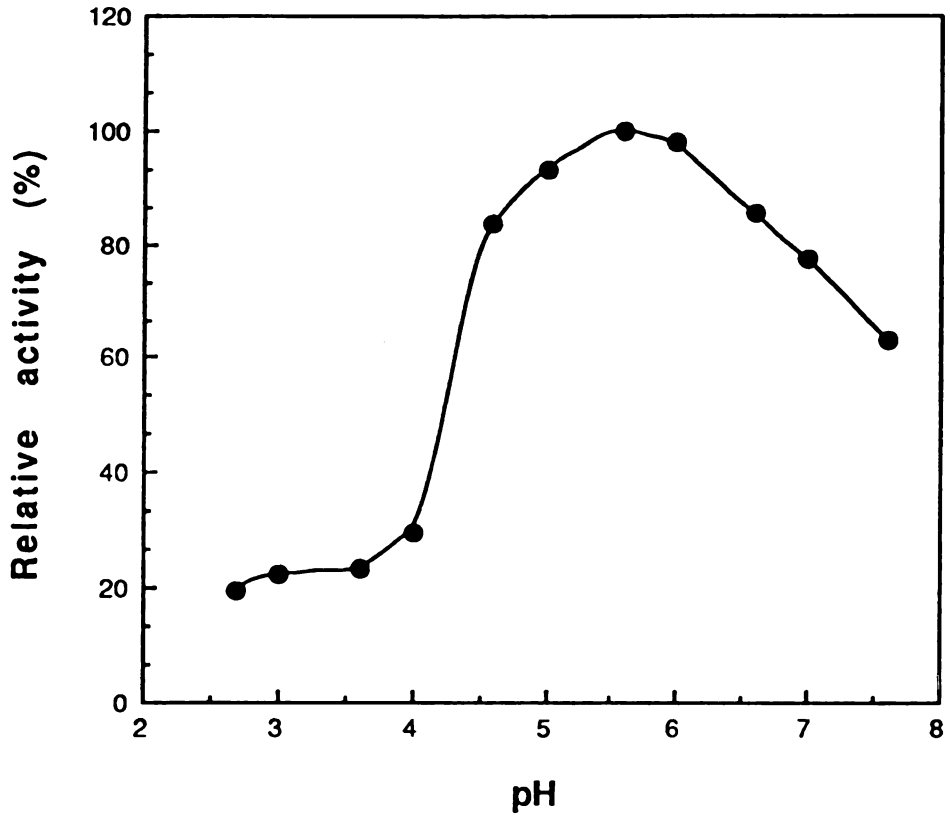


Figure 7. The effect of pH on the activity of recombinant endoxylanase from *E. coli* (pZEP12).

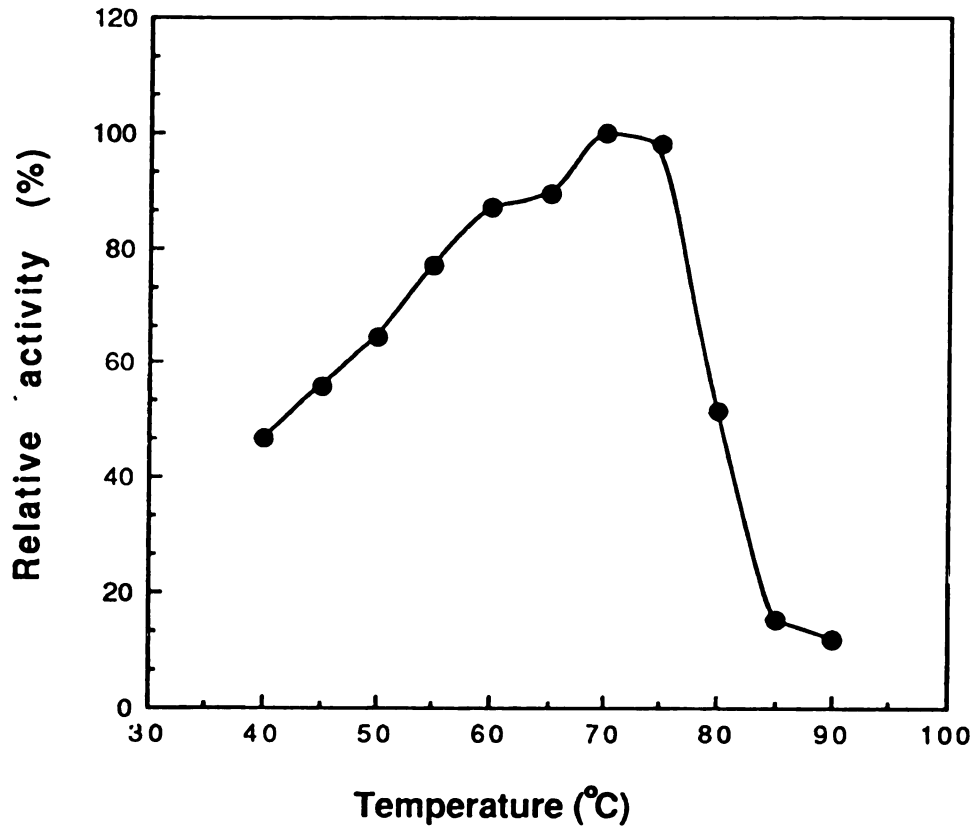
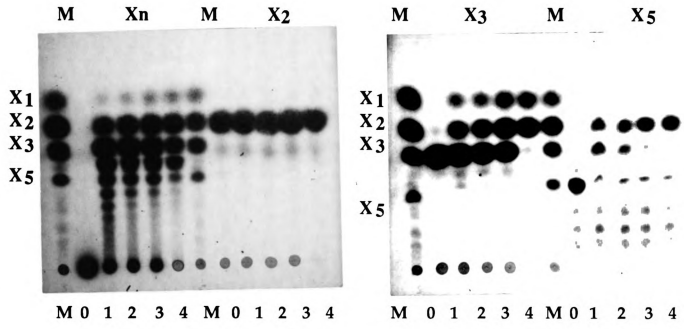


Figure 8. The effect of temperature on the activity of recombinant endoxylanase from *E. coli* (pZEP12).

Figure 9. Time course of hydrolysis of the soluble fraction of oat spelt xylan and xylooligsaccharides by the recombinant endoxylanase.

Oligosaccharides of xylose (xylobiose, xylotriose and xylopentose) and oat spelt xylan were incubated with purified endoxylanase and analyzed using thin layer chromatography. M = size markers of xylose (X_1), xylobiose (X_2), xylotriose (X_3) and xylopentose (X_5). Samples were taken at time 0 (0), 15 min (1), 30 min (2), 1 hour (3) and 5 hours (4). Hydrolysis of xylan (Xn), xylobiose (X_2), xylotriose (X_3) and xylopentose (X_5) were determined.


M
Xn
M
X2
M
X3
M
X5
X1
X2
X3
X5
M 0 1 2 3 4 M 0 1 2 3 4
M 0 1 2 3 4 M 0 1 2 3 4
Incubation time

DISCUSSION

Previous studies in our laboratory have shown that endoxylanase and β-xylosidase appear to be coordinately regulated during growth of *Thermoanaerobacter* B6A-RI on xylan and xylose (Lee, Lowe & Zeikus, manuscript prepared for publication) and the findings here demonstrate that the genes encoding for these two enzymes are closely situated on the chromosome, thus, facilitating coordinate expression and production of enzymes for efficient hydrolysis of xylan.

Initially we intended to clone the gene encoding endoxylanase since it cleaves the β-1,4-linkage of β-1,4-xylan, which is a major component of the hemicellulose. However, conversion of xylan to D-xylose requires at least two enzymes; the endoxylanase converts xylan to its small molecular weight oligosaccharide form, then β-xylosidase is used to convert the xylooligosaccharides to D-xylose. It has been demonstrated that genes encoding xylanolytic enzymes are clustered on the chromosome in many bacteria including *Bacteroides ovatus* (40), *Bacillus pumilus* (29) and *Caldocellum saccharolyticum* (24). The genes encoding endoxylanase and β-xylosidase are the first two genes to be cloned from *Thermoanaerobacter* B6A-RI. They were isolated together from the genomic library of this organism by virtue of their close proximity on the chromosome and expression as active enzymes in *E. coli*.

Most enzymes of family F have xylanase activity. For example, the specific activity of *C. thermocellum xynZ* towards carboxymethyl cellulose (CMC) is less than 0.5% of the activity towards xylan (15). However, *Cellulomonas fimi* exoglucanase in spite of having high xylanase activity, has an even higher activity towards CMC (13). In this regard, it is not too

surprising that *xynA* from *Thermoanaerobacter* B6A-RI should show strong similarity in its catalytic domain to enzymes within this group.

The similarity of *xynA* with other genes in family F seemed to have little bearing on the similarity of the organisms carrying the genes. *xyn*A was most similar to the endoxylanase (*xynA*) from the aerobic mesophile *Bacillus* sp. strain C-125 (18), and *xynA* from the anaerobic thermophile *Caldocellum saccharolyticum* (24). Slightly lower homology was observed between *xynZ* from *C. thermocellum* (16), *xyn*A from *Butyrivibrio fibrisolvens* (25) and *xyn*A from *Pseudomonas flourescens* (17). Interestingly, with the exception of endoxylanase A from *Pseudomonas flourescens,* and *xynA* from *Butyrivibrio fibrisolvens* for which data was not available, all of the genes showing high homology with *xynA* from *Thermoanaerobacter* B6A-RI encode for thermostable endoxylanases.

Multiple xylanases have been reported in several microorganisms (1, 30, 35, 40, 45), and *Thermoanaerobacter* B6A-RI is also known to have several endoxylanases of different molecular weight (Lee, Lowe & Zeikus, manuscript prepared for publication). Southern hybridization analysis, using the cloned *xynA* as a probe indicated that any additional endoxylanase genes, if present in *Thermoanaerobacter* B6A-RI, must have limited homology with the cloned endoxylanase gene, as hybridization of the probe did not occur with other fragments of the chromosomal DNA.

The endoxylanase activity from *Thermoanaerobacter* B6A-RI was excretable, with increasing levels becoming extracellular under xylan limitation (Lee, Lowe & Zeikus, manuscript prepared for publication). The distribution of endoxylanase activity was approximately equal between the periplasm and cytoplasm in *E. coli* containing the intact endoxylanase gene.

This finding suggests that the putative signal peptide identified in the gene is recognized and the protein is processed by *E. coli* cells.

The mode of action of endoxylanase A from *Thermoanaerobacter* B6A-RI was similar to other described endoxylanases from *Cryptococcus albidus* (2, 3), *Aspergillus niger* (14) and *C. stercorarium* (1). These enzymes cleave xylotriose, therefore requiring at least three xylose residues for catalytic activity. Xylan is hydrolyzed randomly, giving xylobiose and xylotriose, and these enzymes have no activity against xylobiose. The endoxylanase A was similar in its mode of action to these enzymes.

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Chapter V

Genetic organization, sequence and biochemical characterization of recombinant β–xylosidase from *Thermoanaerobacter* strain B6A-RI

ABSTRACT

A deletion plasmid, pXPH3, was constructed from cosmid clone pXDM1 which contained both endoxylanase (*xynA*) and β-xylosidase (*xynB*) genes. The nucleotide sequence of a PstI-HindIII fragment in pXPH3 that contained *xynB* was determined. An open reading frame (ORF) of 1,500 bp, corresponding to 500 amino acid residues, was found. The molecular weight of the enzyme deduced from the DNA sequence was 55 kDa. Another open reading frame (ORF1) of unknown function was found 21 bp downstream from the first stop codon of *xynB*. *xynB*, ORF1 and *xynA* had the same direction of transcription. Southern blots confirmed the chromosomal origin of the DNA insert in pXPH3 and that xyn*A* and xyn*B* were closely situated on the chromosome of *Thermoanaerobacter* B6A-RI. The *xynB* from *Thermoanaerobacter* B6A-RI exhibited 45% amino acid similarity with 18% amino acid identity to *xynA* of *Thermoanaerobacter* B6A-RI and 61% similarity and 37% identity with β-xylosidase gene from *Caldocellum saccharolyticum*. Recombinant β-xylosidase was purified from *E. coli* (pXPH3) cells. The enzyme was a monomer with a molecular mass of 55 kDa. The specific activity and pH and temperature optima for hydrolysis of p-nitrophenyl-β-D-xylopyranoside (PNPX) was 5.53 U/mg, 5.5 and 70°C, respectively. The β-xylosidase was stable at 65°C, but lost activity at 85°C. The purified enzyme had hydrolytic activity towards xylopentose, xylotriose, xylobiose and PNPX, but had no activity toward xylan. The activity of the purified enzyme was also tested on a number of different sunstrates.

INTRODUCTION

Xylan is the second most abundant renewable polysaccharide in nature and is comprised of a β–1,4-linked D-xylose polymer with arabinofuranose, glucuronic acid and methylglucuronic acid side groups (27). Complete breakdown of xylan requires the action of several hydrolytic enzymes of which endoxylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) are the most important (3). Endoxylanase randomly cleaves the β-1,4 bonds in the xylan backbone to yield oligosaccharides of varying chain length. The resulting xylooligosaccharides are attacked by β-xylosidase from the non-reducing end (29).

Microbial β-xylosidases occur in both bacteria and fungi, with the latter group being the most studied. Relatively little work has been done on β-xylosidases from thermophilic bacteria (1, 9, 18). β-xylosidase genes from *Bacillus pumilus* (19, 30), *Bacillus polymixa* (23), *Bacillus subtilis* (2), *Butyrivibrio fibrisolvens* (25), and *Caldocellum saccharolyticum* (14) have been cloned into *Escherichia coli.*

Thermoanaerobacter strain B6A-RI, a thermophilic anaerobic bacterium isolated from Yellowstone National Park, grows actively on xylan as a sole carbon source and does not have cellulase activity (13). In this organism the degradation of xylan to xylooligosaccharides is catalyzed by excreted or cell-bound endoxylanases followed by hydrolysis to xylose by intracellular β-xylosidase. Both enzymes were regulated together as their production were induced by xylan or xylose but not glucose (Lee, Lowe & Zeikus, manuscript prepared for publication).

To isolate genes encoding for xylanolytic enzymes a genomic library of *Thermoanaerobacter* strain B6A-RI was constructed using the cosmid vector

pHC79. One cosmid clone, pZXA1, which has a 36 Kbp DNA insertion was found to have both endoxylanase and β-xylosidase activities. The endoxylanase (*xynA*) and β-xylosidase (*xynB*) genes were closely linked within 20 kb on the chromosome and the *xyn*A has been subcloned and its complete nucleotide sequence determined (Lee & Zeikus, manuscript prepared for submission). The purpose of the present report is to describe the complete nucleotide sequence of the *xynB* gene, its genetic organization with xylanase A (*xynA*) and the biochemical properties of the *Thermoanaerobacter* β-xylosidase expressed in *E. coli*.

MATERIALS AND METHODS

Bacterial strains

Thermoanaerobacter strain B6A-RI was isolated from Yellowstone National Park as described previously (13). *Escherichia coli* DH5α (F- ϕ80d*lac*ZΔM15 Δ[*lac*ZYA-*arg*F]U169 *deo*R *rec*A1 *end*A1 *hsd*R17[r_K^-, m_K^+] *sup*E44 l- *thi*-1 *gyr*A96 *rel*A1) was used as the subcloning host and was purchased from BRL (Life Technologies, Inc., Gaithersburg, MD).

Chemicals, media and culture conditions

All chemicals were of reagent grade and were of the highest purity available. Xylose, xylobiose, PNPX, and nitrophenyl derivatives of sugars were purchased from Sigma Chemical Co. (St. Louis, Mo). Xylotriose and xylopentose were kindly given by Dr. P. Reilly at Iowa State University. Restriction enzymes, T4 DNA ligase and calf intestinal alkaline phosphatase were obtained from BRL or Boehringer Mannheim Biochemicals (BMB, Indianapolis, IN). *Thermoanaerobacter* strain B6A-RI was grown in TYE medium (31) containing 0.5% xylose at 60°C. LB medium (10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl per liter) suplemented with ampicillin (50 μg/ml) was used to grow *E. coli* strains harboring recombinant plasmids.

Preparation of chromosomal DNA from *Thermoanaerobacter* B6A-RI

Chromosomal DNA of *Thermoanaerobacter* strain B6A-RI was prepared from cells grown in TYE medium containing 0.5% xylose at 60°C by the method of Marmur (16). Plasmid DNA harbored in *E. coli* cells was prepared from cleared lysates by the procedure described by Clewell (6). For

screening of recombinant DNA in transformants, the rapid method using the alkaline extraction procedure of Birnboim and Doly (4) was used.

Subcloning procedure

The basic procedure was performed according to the method of Sambrook et al. (22). Plasmid pXDM1 (Lee & Zeikus, manuscript prepared for submission) which contained both *xynA* and *xynB* was digested with the restriction endonucleases PstI and HindIII. A 2.0 kb PstI-HindIII fragment which contain *xyn*B was isolated by electroelution, using the Elutrap (Schleicher & Schell Inc., Keene, NH). This fragment was ligated into pUC18 digested with PstI and HindIII, and the resulting recombinant plasmid was named pXHP3. Transformation of *E. coli* DH5α was performed by the Hanahan method as described by Perbal (20). The transformed colonies were replica plated onto LB agar medium containing ampicillin and screened on LB agar plates containing 10 μg/ml of the fluorogenic substrate 4-methylumbelliferyl-β-D-xylopyranoside (Sigma), by incubating the colonies overnight at 37°C and then transferring to 60°C. The plates were examined under ultraviolet light and the positive clones identified by a blue-white fluorescence due to the diffusion of the umbelliferone released by cleavage of the substrate.

Southern hybridization

Chromosomal DNA from *Thermoanaerobacter* strain B6A-RI and *E. coli* HB101 were digested completely with restriction enzymes, electrophoresed through agarose gels and stained with ethidium bromide. For hybridization with radiolabed probes, the DNA was transferred to a Zeta-Probe blotting membrane (Bio-Rad Laboratories, Richmond, CA) by the

method of Southern (26). Gel-purified DNA fragments were labeled with a random prime DNA-labeling kit (Boehringer Mannheim, Indianapolis, IN) and α-^{32}P dATP (3,000 Ci/mmol, New England Nuclear, Wilmington, DE) according to the manufacturers instructions. Hybridization of the DNA was by the method of Sambrook et al. (22).

DNA sequencing

Double-stranded DNA was sequenced in both directions by using the dideoxy-chain termination method (24) and Sequenase (United States Biochemical Co. Cleveland, OH) according to the manufacturer's instructions. Additional sequencing primers were synthesized by the Michigan State University Macromolecular facility. DNA sequences were analyzed using the GENEPRO software package (Hoefer Scientific Instrument, San Francisco, CA) and the University of Wisconsin Genetics Computer Group GCG package (Version 7) (8).

Enzyme assays

The activity of β-xylosidase was measured with p-nitrophenyl β-D-xylopyranoside (PNPX) in 50 mM sodium acetate buffer, pH 5.5. The reaction mixture, composed of 1 mM PNPX, 50 mM sodium acetate (pH 5.5), and diluted enzyme in 0.4 ml reaction volume, was incubated at 60°C for 10 min. The reaction was stopped by the addition of 0.8 ml of 0.5 M Na_2CO_3 and the absorbance of the nitrophenol released was measured at 410 nm. 1 unit of enzyme was defined as the amount of enzyme capable of releasing 1 μmol nitrophenol in 1 min. Other nitrophenol derivatives of sugars were also tested as substrates under the same conditions.

Purification of β-xylosidase

Unless otherwise stated, all purification steps were carried out at room temperature.

(i) Preparation of cell extract: β–xylosidase was purified from *E. coli* DH5α harboring plasmid pXPH3. Cells grown on 1 L LB medium supplemented with ampicillin (50 μg/ml) were suspended in 40 ml of 50 mM sodium acetate pH 5.5, and disrupted by passage through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 20,000 lb/in^2. The supernatant obtained by centrifugation (12,000 x g, 20 min) was used as the crude enzyme preparation.

(ii) Heat treatment: The extract of *E. coli* was heat treated at 75°C for 15 min in a shaking water bath, and chilled on ice. The denatured proteins were removed by centrifugation at 12,000 x g for 20 min.

(iii) Anion exchange column chromatography: The supernatant from the heat treatment step was loaded onto a column (2.5 cm x 18 cm) of Q-Sepharose (Pharmacia, Piscataway, NJ) equilibrated with 50 mM sodium acetate buffer (pH 5.5). The enzyme was eluted with a linear gradient (0 - 1.0 M) of sodium chloride in a total volume of 500 ml. The active fractions were pooled and concentrated by ultrafiltration with a YM 30 membrane (Amicon Co. Ltd., Danvers, MA).

(iv) Gel filtration chromatography: The concentrated sample was further purified using a Waters system FPLC (Division of Millipore, Milford, MA) with a Superose-12 column (Pharmacia) equilibrated with 50 mM sodium acetate buffer (pH 5.5) containing 50 mM sodium chloride.

N-terminal amino acid sequence determination of β-xylosidase

The protein was prepared by washing the purified β-xylosidase with double-distilled water five times with a Centricon-30 (Amicon) filtration device to remove contaminating salts and buffer from the solution. The samples were hydrolyzed in a gas phase for 24 h using 5.7 M-HCl. Amino acid composition analysis was performed with a Pico-Tag amino acid analyzer (Waters Associates, Milford, MA). The N-terminal amino acid sequence was identified by a protein sequencer model 477A (Applied Biosystems, Foster City, CA) with an on-line phenylthiohydrantoin analyzer (Applied Biosystems) in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University.

Protein measurement and polyacrylamide gel electrophoresis

Protein concentration was estimated by the method of Bradford (5) using Protein assay reagent from Bio-Rad with bovine serum albumin (Sigma) as a standard. SDS-PAGE was performed as described previously (11) with 12% acrylamide. Proteins bands were visualized by Coomassie brilliant Blue G-250 staining. The molecular weight of the denatured protein was estimated by SDS-PAGE with low-range molecular weight standards (Bio-Rad) including phosphorylase (97,000), BSA (66 000), ovalbumin (43 000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).

Analysis of products of xylan hydrolysis

The xylan and xylooligosaccharides were dissolved in 50 mM sodium acetate buffer (pH 5.5) and incubated at 60°C with approximately 1U of purified enzyme. Samples were withdrawn periodically and the hydrolysis

products were analyzed by Whatman HP-K high performance silica gel plates (10 x 10 cm) using the following solvent system: butanol/acetic acid/distilled water (2:1:1, by vol.), and were visualized using an orcinol spray reagent (100 mg orcinol in 100 ml 20% sulphuric acid in methanol).

RESULTS

Subcloning and DNA sequencing

A deleted cosmid clone, pXDM1, consisting of the 28 kb chromosomal DNA of *Thermoanaerobacter* strain B6A-RI and pHC79, containing the endoxylanase (*xynA*) and β-xylosidase (*xynB*) genes is shown in Fig. 1. The complete nucleotide sequence of *xynA* and its flanking regions have been determined previously (Lee & Zeikus, manuscript prepared for submission). A deletion plasmid, pXPH3, containing *xynB*, was constructed from pXDM1 and the physical map of *xynB* is shown in Fig. 2.

The nucleotide sequence of a PstI-HindIII fragment in pXPH3, determined by the dideoxy-chain termination method, is shown in Fig. 3. The sequence is consistent with the observed restriction map. An open reading frame (ORF) of 1,500 bp, corresponding to 500 amino acid residues, was found. The molecular weight of the enzyme deduced from the DNA sequence was 55 kDa.

The putative ribosome-binding sequence, 5'-GGGGGG-3', was observed 7 bp upstream of the intiation codon, ATG. Another open reading frame (ORF1) of unknown function was found 21 bp downstream from the first stop codon of *xynB*. Interestingly *xynB*, ORF1 and *xynA* have the same direction of transcription (Fig. 1).

Southern hybridization analysis

To confirm the chromosomal origin of the DNA insert in pXPH3 and to determine that *xynA* and *xynB* were closely situated on the chromosome of *Thermoanaerobacter* B6A-RI, rather than occurring together as a result of rearrangement during the cloning, Southern blots were performed on

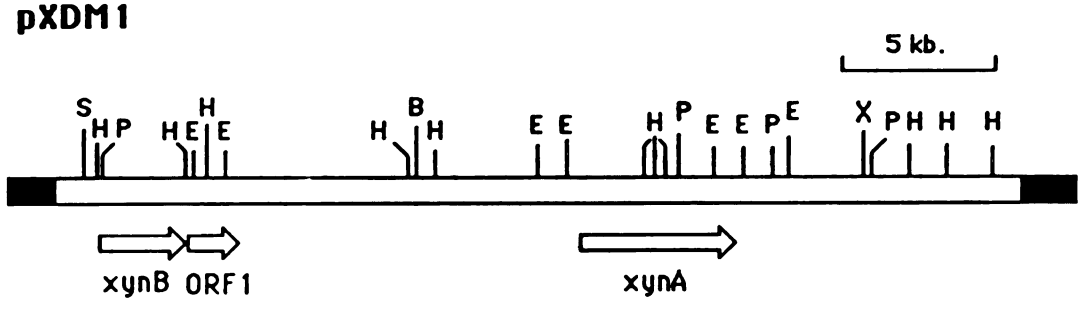


Figure 1. Physical map of cosmid clone pXDM1 which contains the 28 kb chromosomal DNA of *Thermoanaerobacter* strain B6A-RI expressing the endoxylanase (*xynA*) and β-xylosidase (*xynB*) genes.

The open area denotes the 28kb insert and the closed area represents the pHC79. The DNA was cut at various restriction sites (B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SalI; X, XbaI). Arrows indicate the location and transcriptional orientation of the genes.

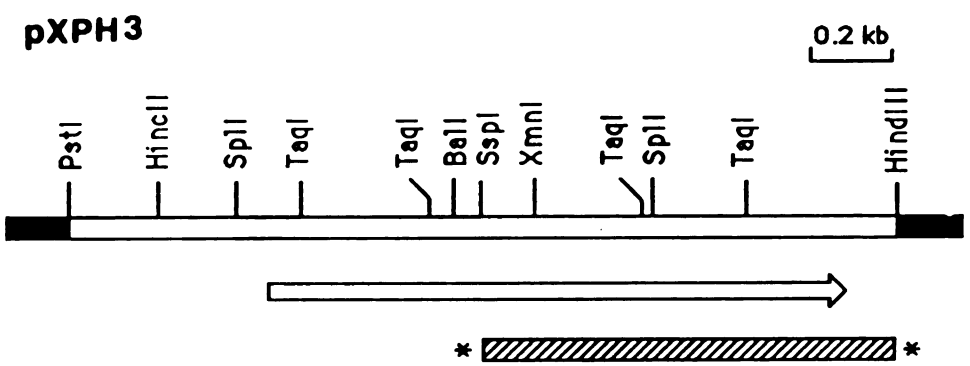


Figure 2. Physical and genetic map of the deletion plasmid pXPH3 containing the β–xylosidase (*xynB*) from *Thermoanaerobacter* B6A-RI.

The location and transcriptional orientation of xynB is indicated by arrow. The shaded bar represents the DNA fragment used as aprobe for Southern hybridization.

Figure 3. The nucleotide sequence and deduced amino acid sequence of β-xylosidase (*xynB*) from *Thermoanaerobacter* B6A-RI and its flanking region.

The under lined region denotes the N-terminal amino acids determined from the purified enzyme and the start sites of correct reading frame for xyn*B* and ORF1 are indicated by arrows.

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1   CTGCAGTATTTGAGAATGGCAACACATCAAGGATAATACCTGCTGTAGAAGCATTGGTAT   60

61  ATCCATATGTTGTAGGATATACTGACTTTGTAAGTGAAGATGGTGTGTTTGGTGAGCTTA  120

121 TAAAAGCCTTAAAGAAGCATGTAATGACGATCATGAAGCCTGGTATATGCATAGATGAAG  180

181 TATCTGGCGGTTGGAAACTTTCGTCAACCAGCAAGAATACATGGAATAGCAAAATTTTCT  240

241 TGTGTCAATATGTGATAAAAGATGTGCTAAATATAGATTTTGGAGAAAAAGAAGGCGAAT  300

301 GGGACAAAGTACATGCCACATGGCAACAGGTGTCTTGCAGTGAAGATTGTGCTACAGATC  360

361 AGGTAAACAGCGATACAGGTACGCCAAGAGGAAGCCGCTTGTATCCTAGGCTTGTAACCA  420

                                                  pXPH3
421 GCATACTGTGGATGAAATAGACTTTGGGGGGTTTAACATGATTAAAGTAAGAGTGCCAGA  480
                    S. D.          MetIleLysValArgValProAs

481 TTTTTCCGATAAGAAGTTTTCTGATAGATGGAGATATTGTGTCGGAACAGGCAGACTTGG  540
    pPheSerAspLysLysPheSerAspArgTrpArgTyrCysValGlyThrGlyArgLeuGl

541 CCTTGCGCTTCAAAAGGAATACATCGAGACATTAAAATATGTGAAAGAAAATATAGACTT  600
    yLeuAlaLeuGlnLysGluTyrIleGluThrLeuLysTyrValLysGluAsnIleAspPh

601 TAAGTATATAAGAGGACATGGCCTTTTGTGTGACGATGTAGGCATATACAGAGAAGATGT  660
    eLysTyrIleArgGlyHisGlyLeuLeuCysAspAspValGlyIleTyrArgGluAspVa

661 GGTAGGAGATGAGGTAAAGCCTTTTTACAATTTTACCTATATTGACAGGATTTTTGACTC  720
    lValGlyAspGluValLysProPheTyrAsnPheThrTyrIleAspArgIlePheAspSe

721 ATTTTTAGAAATCGGAATAAGGCCATTTGTGGAAATCGGATTTATGCCTAAAAAATTAGC  780
    rPheLeuGluIleGlyIleArgProPheValGluIleGlyPheMetProLysLysLeuAl

781 ATCTGGTACACAGACAGTATTTTATTGGGAGGGGAATGTCACTCCTCCCAAGGATTATGA  840
    aSerGlyThrGlnThrValPheTyrTrpGluGlyAsnValThrProProLysAspTyrGl

841 AAAGTGGAGCGATCTTGTCAAAGCGGTTTTGCATCACTTTATTTCTCGATATGGGATTGA  900
    uLysTrpSerAspLeuValLysAlaValLeuHisHisPheIleSerArgTyrGlyIleGl
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901  AGAAGTCTTGAAGTGGCCATTTGAGATATGGAATGAACCAAACTTAAAAGAGTTTTGGAA  960
     uGluValLeuLysTrpProPheGluIleTrpAsnGluProAsnLeuLysGluPheTrpLy

961  AGATGCTGATGAGAAAGAATATTTTAAGCTGTACAAGGTTACTGCAAAGGCTATAAAGGA  1020
     sAspAlaAspGluLysGluTyrPheLysLeuTyrLysValThrAlaLysAlaIleLysGl

1021 AGTAAATGAAAATTTGAAGGTAGGAGGGCCTGCTATATGCGGTGGTGCTGACTACTGGAT  1080
     uValAsnGluAsnLeuLysValGlyGlyProAlaIleCysGlyGlyAlaAspTyrTrpIl

1081 AGAAGATTTTTTGAATTTCTGCTATGAAGAAAATGTTCCTGTAGATTTTGTATCGCGACA  1140
     eGluAspPheLeuAsnPheCysTyrGluGluAsnValProValAspPheValSerArgHi

1141 CGCTACCACATCTAAGCAAGGTGAATATACGCCACATCTCATATACCAGGAGATTATGCC  1200
     sAlaThrThrSerLysGlnGlyGluTyrThrProHisLeuIleTyrGlnGluIleMetPr

1201 ATCTGAATACATGCTAAACGAATTTAAAACAGTGAGAGAGATCATAAAAAACTCACATTT  1260
     oSerGluTyrMetLeuAsnGluPheLysThrValArgGluIleIleLysAsnSerHisPh

1261 TCCGAACCTTCCGTTTCATATAACTGAGTACAATACTTCATATAGTCCTCAAAATCCTGT  1320
     eProAsnLeuProPheHisIleThrGluTyrAsnThrSerTyrSerProGlnAsnProVa

1321 ACACGATACGCCATTTAATGCTGCCTATATTGCCAGGATTTTAAGCGAAGGCGGAGATTA  1380
     lHisAspThrProPheAsnAlaAlaTyrIleAlaArgIleLeuSerGluGlyGlyAspTy

1381 TGTTGATTCATTTTCTTACTGGACGTTTAGCGACGTTTTCGAAGAAAGAGATGTGCCGCG  1440
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1441 ATCCCAATTCCATGGAGGATTTGGACTTGTGGCATTGAATATGATACCAAAGCCTACCTT  1500
     gSerGlnPheHisGlyGlyPheGlyLeuValAlaLeuAsnMetIleProLysProThrPh

1501 TTACACATTTAAATTCTTTAATGCTATGGGAGAGGAAATGCTTTATAGAGATGAGCACAT  1560
     eTyrThrPheLysPhePheAsnAlaMetGlyGluGluMetLeuTyrArgAspGluHisMe

1561 GCTTGTGACGAGGAGAGATGATGGCTCTGTTGCACTCATAGCTTGGAATGAAGTTATGGA  1620
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1621 TAAGACTGAAAATCCAGATGAAGATTATGAAGTCGAGATACCAGTTAGATTCAGAGATGT  1680
     pLysThrGluAsnProAspGluAspTyrGluValGluIleProValArgPheArgAspVa
```

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1681  GTTTATTAAAAGACAATTGATTGATGAAGAACATGGCAATCCATGGGGAACGTGGATACA  1740
      lPheIleLysArgGlnLeuIleAspGluGluHisGlyAsnProTrpGlyThrTrpIleHi

1741  CATGGGAAGGCCGAGGTATCCTAGCAAAGAACAGGTAAATACATTGAGAGAAGTTGCAAA  1800
      sMetGlyArgProArgTyrProSerLysGluGlnValAsnThrLeuArgGluValAlaLy

1801  GCCAGAGATTATGACAAGTCAGCCTGTTGCGAATGACGGATACTTAAATCTAAAGTTTAA  1860
      sProGluIleMetThrSerGlnProValAlaAsnAspGlyTyrLeuAsnLeuLysPheLy

1861  ATTAGGTAAAAATGCAGTTGTATTGTATGAATTGACTGAGAGAATTGATGAATCAAGCAC  1920
      sLeuGlyLysAsnAlaValValLeuTyrGluLeuThrGluArgIleAspGluSerSerTh

1921  ATATATAGGACTTGATGATAGCAAGATAAATGGATATTGATGCACATTAGGAGGGATTGA  1980
      rTyrIleGlyLeuAspAspSerLysIleAsnGlyTyrEnd

      ┌──► ORF1
1981  TATGGGACTTTTTGACATGCCACTGCAAAAGCTTAGAGAATACACTGGTACAAATCCATG  2040
       MetGlyLeuPheAspMetProLeuGlnLysLeuArgGluTyrThrGlyThrAsnProCy

2041  CCCTGAAGATTTCGATGAGTATTGGGATAGGGCTTTAGATGAGATGAGGTCAGTTGATCC  2100
      sProGluAspPheAspGluTyrTrpAspArgAlaLeuAspGluMetArgSerValAspPr

2101  CAAAATTAAAATGAAAAAAAGTAGCTTTCAAGTGCCTTTTGCAGAGTGCTACGATTTGTA  2160
      oLysIleLysMetLysLysSerSerPheGlnValProPheAlaGluCysTyrAspLeuTy

2161  CTTTACAGGTGTTCGTGGTGCCAGAATTCATGCAAAGTATATAAGACCTAAGACAGAAGG  2220
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2221  GAAACATCCAGCGTTGATAAGATTTCATGGATATTCGTCAAATTCAGGCGACTGGAACGA  2280
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2281  CAAATTAAATTACGTAGCGGCAGGCTTTACCGTTGTGGCTATGGATGCAAGAGGTCAAGG  2340
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2341  AGGGCAGTCTCAAGATGTTGGCGGTGTAAATGGGAACACTTTAAATGGGCATATTATAAG  2400
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2941 ATAAGGAGATGCAAAGTGCATCTCTTAACTGTATTCTTTATTAATTTTTTATATTCGCTT 3000
rEnd

3001 ACGTTACGTTTAAGCCTGTGTATTTTTTAAAACACGATGAAAAATAATTTGGGTCTTGTA 3060

3061 TGCCTACAGACTTGGCGATTTCAAATGACTTCATGTTTTTTTCTTTAATAAGGCTTATGG 3120

3121 CTTATGGCTTTTTCCATTCGCTTATTTGTCAGGTATTCTATGAAGTTTATGCCTGTCTCC 3180

3181 TTTTTGAATGTTGG 3194

chromosomal DNA from *E. coli* and *Thermoanaerobacter* B6A-RI digested with EcoRI, PstI and XbaI. An internal 1.2 kb HincII-PstI fragment from pZEP12 and a 0.9 kb SspI-HindIII fragment from pXPH3 were used as a probe for endoxylanase (*xynA*) and β-xylosidase (*xynB*), respectively. The results are shown in Fig. 4. Both fragments did not bind to *E. coli* chromosomal DNA, but did hybridize to *Thermoanaerobacter* B6A-RI genomic DNA, indicating that these genes originated from *Thermoanaerobacter* B6A-RI. Both probes hybridized to identical size bands of *Thermoanaerobacter* B6A-RI DNA digested with PstI (lane 3) and XbaI (lane 5). This indicates that these two genes (*xynA* and *xynB*) are clustered on the chromosomal DNA of *Thermoanaerobacter* B6A-RI and no apparent rearrangement occurred during the cloning procedure.

Comparison of *xynB* with other genes

Comparison of the deduced amino acid sequence of *xynB* from *Thermoanaerobacter* B6A-RI to *xynA* from the same organism, and β-xylosidases from *B. pumillus* IPO (30), *Butyrivibrio fibrisolvens* (25) and *Caldocellum saccharolyticum* (14) are presented in Table 1. The *xynA* and *xynB* from *Thermoanaerobacter* B6A-RI exhibited 45% amino acid similarity to each other with 18% amino acid identity (Table 1). Comparison of *xynB* with GenBank and EMBL sequence libraries identified the β-xylosidase gene from *Caldocellum saccharolyticum* as being most similar, with 61% similarity and 37% identity. The translated sequence for *xynB* did not share a strong identity with other endoxylanases. No genes were found which showed a high amino acid similarity to the *Thermoanaerobacter* B6A-RI ORF1.

Figure 4. Southern hybridization analysis of *Thermoanaerobacter* B6A-RI and *E. coli* genomic DNA digests with random-primed DNA synthesized from the 1.2 kb HincII-PstI fragment from pZEP12 and 0.9 kb SspI-HindIII fragment from pXPH3.

(A) Photograph of 0.8% agarose gel stained with ethidium bromide. (B) and (C) Autoradiogram of DNA transferred to membrane andhybridized with the ^{32}P-labeled insert fragment from pZEP12 and pXPH3, respectively. Lane 1, molecular weight markers; lane 2, *E. coli* chromosomal DNA digested with PstI; lane 3, 4 & 5, *Thermoanaerobacter* B6A-RI chromosomal DNA digested with PstI, EcoRI and XbaI, respectively.

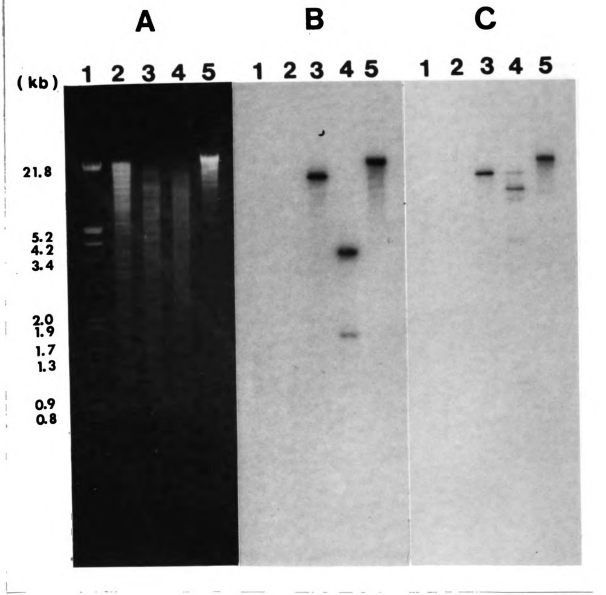

A
B
C
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
(kb)
21.8
5.2
4.2
3.4
2.0
1.9
1.7
1.3
0.9
0.8

Table 1. Relative homology between β-xylosidase from *Thermoanaerobacter* B6A-RI and other β-xylosidases.

Strain	% Similarity	% Identity
Bacillus pumilus IPO	40.9	16.0
Butyrivibrio fibrisolvens	45.9	15.5
Caldocellum saccharolyticum	60.5	36.5
Thermoanaerobacter B6A-RI (*xynA*)	44.7	17.5

Purification and characterization of recombinant β-xylosidase

Recombinant β-xylosidase was purified from *E. coli* (pXPH3) cells to determine its physicochemical properties. A summary of the purification procedure is shown in Table 2. Heat treatment was a particularly effective step, resulting in a 5-fold purification and 81% yield. SDS-PAGE analysis (Fig. 5) revealed that the enzyme was monomeric with an approximate size of 55 kDa. This is identical to the value (55 kDa) calculated from the deduced amino acid sequence. The molecular mass of the recombinant enzyme was estimated to be 60 kDa by FPLC. The N-terminal amino acid sequence of the recombinant β-xylosidase was Met-Ile-Lys-Val-Arg-Val-Pro-Asp-Phe-Ser-Asp. This was identical to the N-terminal amino acid sequence deduced from the DNA sequence.

The pH optimum for hydrolysis of PNPX was approximately 5.5 with the enzyme showing high activity from pH 5.0 to 6.6 (Fig. 6). The optimum temperature for hydrolase activity was 70°C, with activity decreasing rapidly at higher temperatures (Fig. 7). To examine the thermostability of the enzyme, β-xylosidase was incubated in 50 mM sodium acetate buffer, pH 5.5, at various temperatures for up to 60 min, and the residual activities were assayed and compared with the untreated sample. β-xylosidase was stable at 65°C, but lost activity at 85°C. The half-life of the enzyme at 75°C was 55 min (data not shown).

The purified β-xylosidase had hydrolytic activity towards xylopentose, xylotriose, xylobiose and PNPX, but had no activity toward xylan. The activity of the purified β-xylosidase with various para- and ortho-nitrophenyl-glycosidic substrates was tested (Table 3). The β–xylosidase was highly specific, as it was only active on p-nitrophenyl-β-D-xylopyranoside and o-nitrophenyl-β-D-xylopyranoside. The low level of activity observed with p-

Table 2. Summary of purification of β-xylosidase from *E. coli* (pXPH3)

Purification step	Total activity (units)	Total protein (mg)	Specific activity (Units/mg)	Purification (fold)	Yield (%)
Cell-free extract	165	391	0.422	1.0	100
Heat treatment	134	67	2.6	4.7	81.2
Q-Sepharose	101	21	4.81	11.4	61.2
Gel filtration	83	15	5.53	13.1	50.3

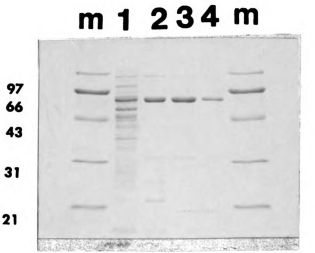


Figure 5. SDS-PAGE analysis of the purification of recombinant β-xylosidase from *E. coli* (pXPH3) cells.

Lane M, molecular weight markers; lane 1, crude *E. coli* (pXPH3) extract (20 μg protein); lane 2, following heat treatment (10 μg protein); lane 3, after anion exchange chromatography (5 μg protein) and lane 4, after gel filtration chromatography (3 μg protein).

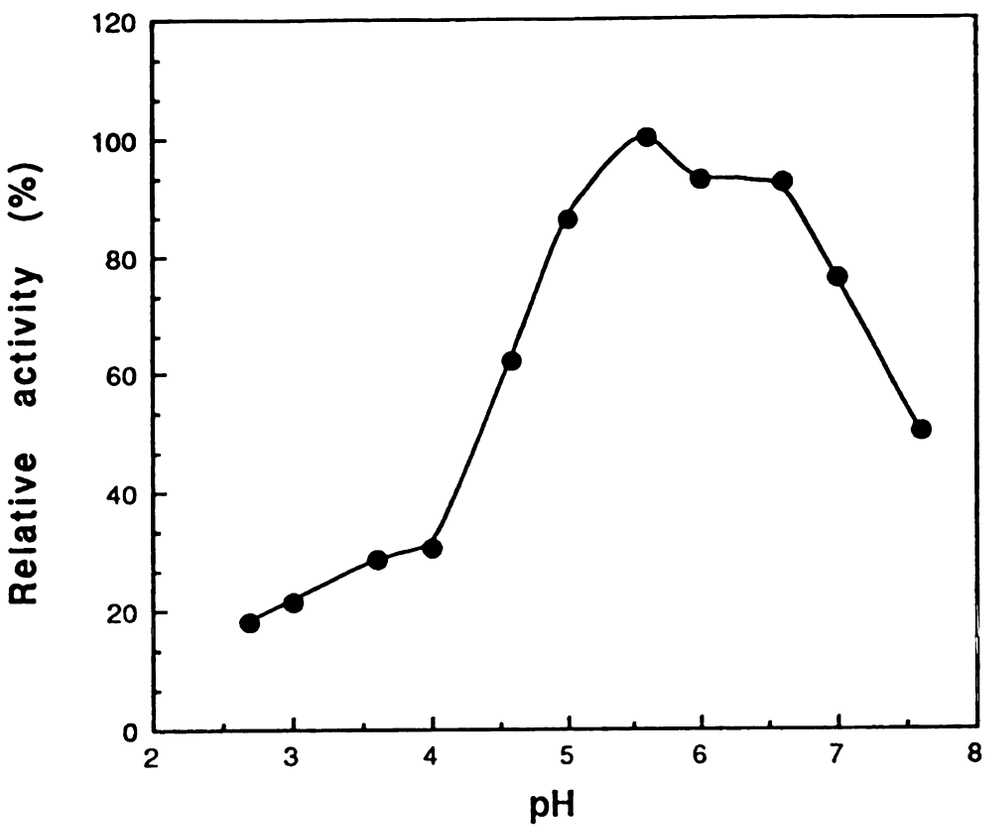


Figure 6. The pH profile of recombinant β-xylosidase

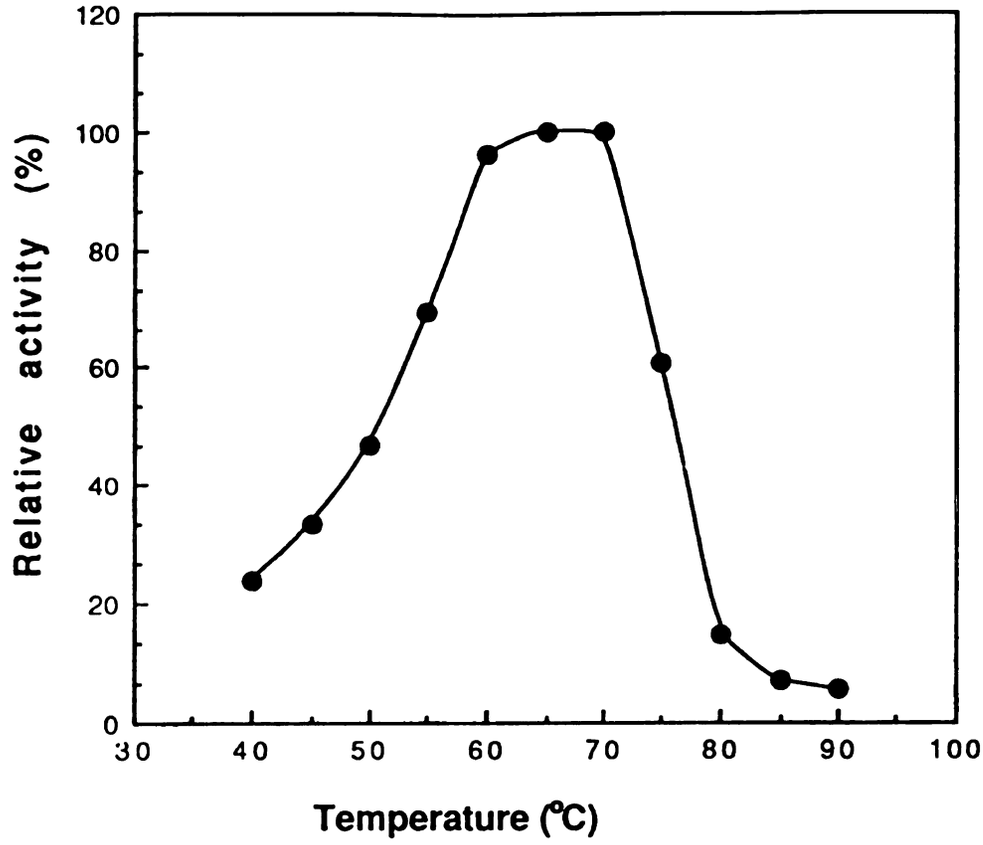


Figure 7. The temperature profile of recombinant β-xylosidase

Table 3. Hydrolysis of various nitrophenyl-glycosides by the purified β-xylosidase

Substrate	Specific activity (U/mg)*
p-Nitrophenyl-β-D-xylopyranoside	5.529
o-Nitrophenyl-β-D-xylopyranoside	4.696
p-Nitrophenyl-β-D-fucopyranoside	0.045
p-Nitrophenyl-α-D-glucopyranoside	0.000
p-Nitrophenyl-β-D-glucopyranoside	0.141
p-Nitrophenyl-α-L-arabinofuranoside	0.073
o-Nitrophenyl-β-D-galactopyranoside	0.000
p-Nitrophenyl-β-D-lactopyranoside	0.000

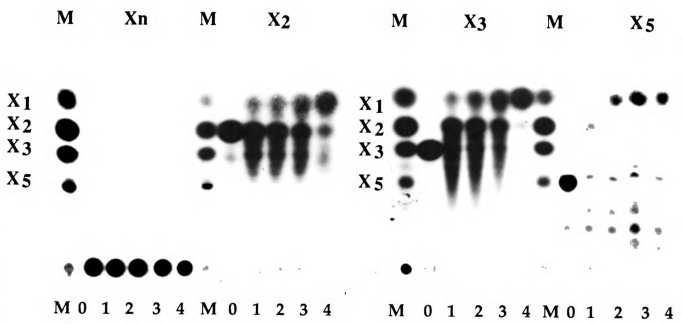
*These values represent the mean values of three independent experiments.

nitrophenyl-β-D-fucopyranoside, p-nitrophenyl-α-L-arabinofuranoside and p-nitrophenyl-β–D-glucopyranoside may not be significant, and no activity was detected with the other substrates tested.

Oligosaccharides of xylose (xylobiose, xylotriose and xylopentose) and oat spelt xylan were incubated with purified β–xylosidase and analyzed using thin-layer chromatography (Fig. 8). All of the xylooligosaccharides (polymers of two to five xylose residues) were degraded to xylose. Transferase activity was also detected during the early stages of hydrolysis. No activity could be detected using oat spelt xylan as the substrate. No β-xylosidase activity was detected in control extracts from *E. coli*.

Figure 8. Time course of hydrolysis of the soluble fraction of oat spelt xylan and xylooligsaccharides by the recombinant β-xylosidase.

Oligosaccharides of xylose (xylobiose, xylotriose and xylopentose) and oat spelt xylan were incubated with purified β-xylosidase and analyzed using thin layer chromatography. M = size markers of xylose (X_1), xylobiose (X_2), xylotriose (X_3) and xylopentose (X_5). Samples were taken at time 0 (0), 15 min (1), 30 min (2), 1 hour (3) and 5 hours (4). Hydrolysis of xylan (Xn), xylobiose (X_2), xylotriose (X_3) and xylopentose (X_5) were determined.


M
Xn
M
X2
M
X3
M
X5
X1
X2
X3
X5
X1
X2
X3
X5
M 0 1 2 3 4 M 0 1 2 3 4
M 0 1 2 3 4 M 0 1 2 3 4
Incubation time

DISCUSSION

The complete nucleotide sequence of the β-xylosidase gene (*xynB*) of *Thermoanaerobacter* B6A-RI was established together with its flanking regions. In our other studies, the endoxylanase and β-xylosidase activity in *Thermoanaerobacter* B6A-RI have been shown to be under coordinate control, induced by xylose or xylan and the endoxylanase gene (*xynA*) was cloned and sequenced (Lee & Zeikus, manuscript prepared for submission).

In the cloned chromosomal DNA from *Thermoanaerobacter* B6A-RI, *xynA* and *xynB* genes were clustered within the 20 kb region, and had the same transcriptional orientation. The proximity of *xynB*, ORF1 and *xynA* on the cloned DNA and their same direction of transcription suggests the possibility of their being organized together as an operon. This is further substantiated bt the finding that no terminator or prominent stem-loop was identified between *xynB* and ORF1 and the downstream region of ORF1 by sequence analysis. However, it still remains to be shown that *xynA* and *xynB* are part of the same operon in *Thermoanaerobacter* B6A-RI. Studies using RNA, such as Northern blot analysis and primer extension experiments would answer this question. Recent studies with *Bacteroides ovatus* (28), *Bacillus pumilus* (17) and *Caldocellum saccharolyticum* (14) have reported the clustering of genes involved in hemicellulose degradation in these organisms.

Confirmation that the cloned *xynB* is from the *Thermoanaerobacter* B6A-RI chromosomal DNA was obtained by Southern blot analysis. A single PstI and XbaI fragment was hybridized to β-xylosidase probe suggests that only one copy of the cloned gene is present in the chromosome. However, the possibility that *Thermoanaerobacter* B6A-RI has more than one β-xylosidase

cannot be ruled out, since multiple β–xylosidases have been reported in *B. pumulis* (19) and *Caldocellum saccharolyticum* (14).

Localization studies of the cloned β-xylosidase revealed that the enzyme was located within the *E. coli* cell (data not shown), and this finding is in accordance with the findings of others that bacterial β-xylosidases are generally intracellular (12, 18). Most of the β-xyosidase activity from *Thermoanaerobacter* B6A-RI was intracellular (data not shown).

The deduced amino acid sequence of thermostable *Thermoanaerobacter* B6A-RI β-xylosidase exhibited a higher degree of homology to thermostable β–xylosidase from *C. saccharolyticum* (36% identity) (14) than to those of less thermostable β-xylosidases from *Bacillus* (30) and *Butyrivibrio* (25) (less than 16% identity).

The cloned β-xylosidase had a monomeric molecular weight of 55 kDa which was similar to the size of β-xylosidase from *B. fibrisolvens* (25) and *B. pumulis* (19) each comprising of a single subunit of 60,000 and 62,600 molecular weight, respectively. The β-xylosidase from *C. acetobutyricum* (12) is composed of two different subunits with molecular weights of 85,000 and 63,000. Fungal β-xylosidases have relatively larger molecular weight subunits of 116,000 to 118,00 (10, 15).

The substrate specificity of β–xylosidase from *Thermoanaerobacter* B6A-RI for xylooligosaccharides was similar to other bacterial β-xylosidases characterized (7, 12, 19, 25). Most β–xylosidases have little or no activity against xylan, and this was also found with β-xylosidase from *Thermoanaerobacter* B6A-RI. These enzymes degrade xylooligosaccharides to xylose and also have transferase activity, which are considered to be the properties of a β-xylosidase (21).

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Chapter VI

Cloning, sequencing and biochemical characterization of xylose isomerase from *Thermoanaerobacter* strain B6A-RI

ABSTRACT

The xylose isomerase gene from *Thermoanaerobacter* strain B6A-RI was cloned by complementation using *Escherichia coli xyl*-5 mutant strain HB101. One positive clone was detected and the recombinant plasmid, pZXI6, was isolated. The clone contained vector pUC18 and an insert fragment of 4.5 kilobases. The cloned xylose isomerase gene (*xylA*) was expressed constitutively in *E. coli*. The gene contained one open reading frame (ORF) of 1317 base pairs in length which corresponds to 439 amino acid residues. The predicted molecular weight of 50,474, was calculated from the deduced amino acid sequence. A putative promotor region (Pribnow box), TATAATATATAAT, which repeated twice at the -10 region in *E. coli*, was found 25 bp upstream of the ribosomal binding site. The deduced amino acid sequence of *Thermoanaerobacter* B6A-RI xylose isomerase exhibited very high homology to those from *C. thermosulfurogenes* and *C. thermohydrosulfuricum*. Codon usage in *xyn*A, *xyn*B and *xyl*A showed a clear propensity of AT-containing isocodons. The native molecular mass of the purified recombinant thermostable xylose isomerase was 200kDa, and the enzyme was a tetramer comprised of identical subunits. The apparent temperature and pH optimum for the activity of the cloned xylose isomerase was 80°C and 7.0 to 7.5 respectively.

INTRODUCTION

Thermoanaerobacter strain B6A-RI is a thermophilic anaerobic bacteria capable of actively degrading xylan. Endoxylanase and β-xylosidase degrade the polymer to D-xylose, which is isomerized to D-xylulose and further catabolized in the cell. The isomerization of D-xylose is catalyzed by D-xylose isomerase (EC 5.3.1.5) which is also used industrially to convert D-glucose to D-fructose syrups (2, 6, 7).

In *Bacillus subtilis*, the enzymes encoded by the xylose regulon consist of genes for xylan and xylose utilization, which cooperate in the utilization of hemicelluloses (14). These genes are negatively regulated by the repressor (*xylR*) and are induced by xylose (16). In *Thermoanaerobacter* strain B6A-RI, the endoxylanase and β-xylosidase activities were induced during growth on xylan and xylose (Lee, Lowe & Zeikus, manuscript prepared for publication). The synthesis of xylose isomerase from *Thermoanaerobacter* was also induced by either xylose or xylan (18).

We have isolated genes encoding endoxylanase (*xynA*) and β-xylosidase (*xynB*) from the genomic library of *Thermoanaerobacter* strain B6A-RI, which were found to be organized in a cluster on the chromosome. To date there is no data regarding the genetic organization of the xylose isomerase (*xyl*) genes in *Thermoanaerobacter*. In this report, we describe the cloning, sequencing of the *xyl* gene from *Thermoanaerobacter* strain B6A-RI and discuss it s relationships to endoxylanase and β-xylosidase.

MATERIALS AND METHODS

Bacterial strains and plasmids

Thermoanaerobacter strain B6A-RI (22) was used as the source of the xylose isomerase gene. *E. coli* xyl$^-$ mutant strain HB101 (F$^-$*mcr*B *mrr* *hsd*S20[$r_B^-m_B^-$] *rec*A13 *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20[Sm^r] *xyl*5 λ^- *leu* *mtl*1) (3) were used as a host strain for cloning by complementation. Plasmid pUC18 was used as the cloning vector.

Chemicals, media and growth conditions

All chemicals were of reagent grade and were of the highest purity available. *Thermoanaerobacter* strain B6A-RI was grown under anaerobic conditions at 60°C in TYE medium (37) containing 0.5% xylose as a carbon source. For growth of *E. coli* strains, Luria broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) was used for liquid culture, and solid medium contained 1.5% agar (Difco Laboratories, Detroit, MI). Ampicillin (50μg/ml) was supplemented for the selection of transformants. MacConkey agar (Difco, Ann Arbor, MI) plates containing 1% xylose were used to detect xylose isomerase positive clones.

DNA preparation and cloning procedure

Chromosomal DNA of *Thermoanaerobacter* strain B6A-RI was isolated by a modification of the Marmur method as described previously (19). Plasmid DNA was purified in large scale by the procedure described by Clewell (8), followed by centrifugation in a cesium chloride-ethidium bromide density gradient. The alkaline denaturation method of Birnboim & Doly (4) was used for rapid extraction of plasmid DNA.

Chromosomal DNA from *Thermoanaerobacter* B6A-RI was partially digested with restriction endonuclease AccI and 4 to 10 kbp DNA fragments were isolated from an agarose gel by electroelution, using the elutrap (Scheicher & Schuell Inc., Keene, NH). Plasmid vector pUC18 was completely digested with AccI and dephosphorylated with Calf intestinal alkaline phosphatase. Ligation was performed by the procedure of Sambrook et al. (28) and transformed into *E. coli* HB101 competent cells prepared by the Hanahan method as described by Perbal (26). Transformants were selected on MacConkey agar plates supplemented with 1% xylose and ampicillin.

Sequence determination of the *xylA* gene

The recombinant plasmid was denatured by the method of Zhang et al. (38), and the nucleotide sequence was determined by the dideoxy chain termination method (29), using a Sequenase version 2.0 kit (United States Biochemical Co. Cleveland, OH). The sequence information was analyzed using the GENEPRO software package (Hoefer Sceientific Instrument, San Francisco, CA) and the University of Wisconsin Genetics Computer Group GCG package (11).

Enzyme assay

Cell extracts prepared by sonication and purified preparations were used as enzyme sources. Glucose isomerase activity was measured by incubating a reaction mixture that contained 10 mM $MgSO_4$, 1 mM $CoCl_2$, 0.8 M glucose, and the enzyme in 100mM MOPS buffer (pH 7.0) at 65°C. The xylose isomerase activity assay was performed at 65°C with a reaction mixture contained 70 mM xylose, 10 mM $MnSO_4$ and enzyme in the same buffer. The amount of fructose or xylulose formed was estimated by the cystein-carbazole-

sulfuric acid method (12). One unit of activity was defined as the amount of enzyme which released 1 μmol of ketose per min under the assay conditions described.

Purification of xylose isomerase

Unless otherwise stated all operations were performed at room temperature and used the method of Lee et al. (21).

(i) Preparation of cell extract: *E. coli* (pZXI6) cells from a 1 L overnight culture were suspended in 40 ml of 50 mM MOPS buffer (pH 7.0) containing $MgSO_4$ (10 mM) and $CoCl_2$ (1 mM). The cells were broken by two passages through a French pressure cell at 16,000 lb/in^2. The cell debris were removed by centrifugation at 12,000 X g for 20 min, and the supernatant used as the crude enzyme preparation.

(ii) Heat treatment: The cell extract obtained was heated at 85°C for 15 min and chilled in ice. The precipitated protein was removed by centrifugation at 12,000 X g for 20 min, and the soluble fraction used for xylose isomerase purification.

(iii) Anion exchange chromatography: The soluble fractions from the heated cell extracts were loaded onto a DEAE-Sepharose CL-6B (Pharmacia, Piscataway, NJ) column equilibrated with 50 mM MOPS buffer (pH 7.0) containing 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$. The enzyme was eluted with a linear NaCl gradient (0 to 1.0 M) in the same buffer. Active fractions were pooled and concentrated by ultrafiltration using a YM 30 membrane with a molecular weight cut-off of 30 kDa (Amicon Co. Danvers, MA).

(iv) Gel filtration chromatography: The concentrated xylose isomerase sample was applied to a Superose 12 HR 10/30 gel filtration column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) with a Waters fast-

protein liquid chromatography (FPLC) system (Division of Millipore, Milford, MA)

Protein determination and SDS-PAGE

The protein amount was determined by the Bradford method using the Bio-Rad protein reagent (Bio-Rad Laboratories Ltd., Richmond, CA) with bovine serum albumin (Sigma Chemical Co.) as the standard. Enzyme fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels in a Mini-PROTEAN II system (Bio-Rad). Protein bands were visualized by Coomassie brilliant blue staining.

N-terminal amino acid sequence determination of xylose isomerase

The protein was prepared by washing the purified xylose isomersae with double-distilled water five times with a Centricon-30 (Amicon) filtration device to remove contaminating salts and buffer from the solution. The samples were hydrolyzed in a gas phase for 24 h using 5.7 M-HCl. Amino acid composition analysis was performed with a Pico-Tag amino acid analyzer (Waters Associates, Milford, MA, U.S.A.). The N-terminal amino acid sequence was identified by a protein sequencer model 477A (Applied Biosystems, Foster City, CA) with an on-line phenylthiohydrantoin analyzer (Applied Biosystems) in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University.

RESULTS

Cloning of the xylose isomerase gene

The xylose isomerase gene from *Thermoanaerobacter* strain B6A-RI was cloned by complementation using *E. coli xyl*-5 mutant strain HB101. Transformants were screened on MacConkey agar plates supplemented with 1% xylose and ampicillin. One positive clone which gave a red color on this plate was detected and the recombinant plasmid, pZXI6, was isolated. The clone contained vector pUC18 and an insert fragment of 4.5 kilobases (Fig. 1). The orientation of the fragment of *Thermoanaerobacter* B6A-RI chromosomal DNA in the vector, did not affect the level of xylose isomerase activity (data not shown) suggesting that the cloned *xylA* gene from *Thermoanaerobacter* B6A-RI was expressed in *E. coli* by its own promoter.

In *Thermoanaerobacter* B6A-RI xyose isomerase activity was induced by D-xylose, whereas in *E. coli* the cloned *xylA* was expressed constitutively and addition of D-xylose to the medium did not increase the production (data not shown).

Nucleotide sequence of the *xyl* gene

The nucleotide sequence and deduced amino acid sequences are shown in Fig. 2. The computer analysis of the nucleotides sequenced revealed one open reading frame (ORF) of 1317 base pairs in length which corresponds to 439 amino acid residues. This was confirmed as the correct reading frame for the *Thermoanaerobacter* B6A-RI *xylA* gene by comparison of the amino-terminal amino acid sequence (underlined in Fig. 2), determined from the cloned xylose isomerase purified from *E. coli* (pZXI6).

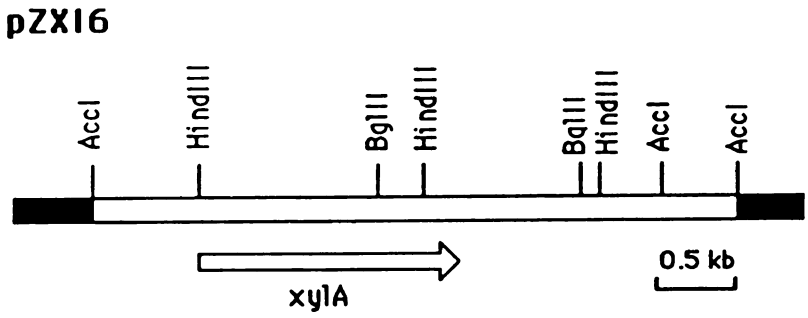


Figure 1. The physical map of pZXI6 containing vector pUC18 with the insert fragment containing the xylose isomerase gene.

The xylose isomerase gene from *Thermoanaerobacter* strain B6A-RI was cloned by complementation using *E. coli xyl*-5 mutant strain HB101. Transformants were screened on MacConkey agar plates supplemented with 1% xylose and ampicillin.

Figure 2. The nucleotide sequence and deduced amino acid sequences of the cloned xylose isomerase.

The computer analysis of the nucleotides sequenced revealed one open reading frame (ORF) of 1317 base pairs in length which corresponds to 439 amino acid residues. The underlined region denotes the correct reading frame for the *Thermoanaerobacter* B6A-RI *xylA* gene by comparison of the amino-terminal amino acid sequence determined from the xylose isomerase purified from *Thermoanaerobacter* B6A (Lee & Zeikus, 1991).

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1   TATTTTAGTATCTTCCCAGATGTTGGCTAATAGTTGGTTTGACAGGGTCGGTTATTTTTT  60

61  TTAAAATTTTTTTATTATGAAGAAGGATTTTTTAAATTTGTGTAGAATATATAATATATA  120

121 ATGAAAGAAGGACAGACAAACGAATAGAAGGAGGAAGCTTTATGAATAAATATTTTGAGA  180
                          S. D.                 MetAsnLysTyrPheGluA

181 ACGTATCTAAAATAAAATATGAAGGACCAAAATCAAATAATCCTTATTCCTTTAAATTTT  240
    snValSerLysIleLysTyrGluGlyProLysSerAsnAsnProTyrSerPheLysPheT

241 ACAATCCAGAGGAAGTAATCGATGGCAAGACGATGGAGGAGCATCTCCGCTTTTCTATAG  300
    yrAsnProGluGluValIleAspGlyLysThrMetGluGluHisLeuArgPheSerIleA

301 CTTATTGGCACACTTTTACTGCTGATGGAACAGATCAATTTGGCAAGGCTACTATGCAAA  360
    laTyrTrpHisThrPheThrAlaAspGlyThrAspGlnPheGlyLysAlaThrMetGlnA

361 GACCATGGAACCACTACACAGATCCTATGGATATAGCGAAACGAAGGGTAGAAGCAGCAT  420
    rgProTrpAsnHisTyrThrAspProMetAspIleAlaLysArgArgValGluAlaAlaP

421 TTGAGTTTTTTGATAAGATAAATGCACCTTTCTTCTGCTTCCATGATAGGGATATTGCCC  480
    heGluPhePheAspLysIleAsnAlaProPhePheCysPheHisAspArgAspIleAlaP

481 CTGAAGGAGATACTCTTAGAGAGACAAACAAAAACTTAGATACAATAGTTGCTATGATAA  540
    roGluGlyAspThrLeuArgGluThrAsnLysAsnLeuAspThrIleValAlaMetIleL

541 AGGATTACTTAAAGACCAGCAAGACAAAAGTTTTGTGGGGTACCGCAAATCTTTTCTCCA  600
    ysAspTyrLeuLysThrSerLysThrLysValLeuTrpGlyThrAlaAsnLeuPheSerA

601 ATCCGAGATTTGTACATGGTGCATCAACATCCTGCAATGCTGACGTTTTTGCATATTCTG  660
    snProArgPheValHisGlyAlaSerThrSerCysAsnAlaAspValPheAlaTyrSerA

661 CAGCGCAAGTCAAAAAAGCCCTTGAGATTACTAAGGAGCTTGGCCGCGAAAACTACGTAT  720
    laAlaGlnValLysLysAlaLeuGluIleThrLysGluLeuGlyArgGluAsnTyrValP

721 TTTGGGGTGGAAGAGAAGGGTACGAGACGCTTCTCAATACAGATATGGAGTTAGAGCTTG  780
    heTrpGlyGlyArgGluGlyTyrGluThrLeuLeuAsnThrAspMetGluLeuGluLeuA

781 ATAACTTTGCAAGATTTTTGCACATGGCTGTTGACTATGCAAAGGAAATCGGCTTTGAAG  840
    spAsnPheAlaArgPheLeuHisMetAlaValAspTyrAlaLysGluIleGlyPheGluG
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841  GTCAGTTCTTGATTGAGCCGAAGCCAAAGGAGCCTACAAAACATCAATACGACTTTGACG  900
     lyGlnPheLeuIleGluProLysProLysGluProThrLysHisGlnTyrAspPheAspV

901  TGGCAAATGTATTGGCATTCTTGAGAAAATACGACCTTGACAAATATTTCAAAGTAAATA  960
     alAlaAsnValLeuAlaPheLeuArgLysTyrAspLeuAspLysTyrPheLysValAsnI

961  TCGAAGCAAACCATGCGACATTGGCATTCCACGACTTCCAACATGAGCTAAGATACGCCA  1020
     leGluAlaAsnHisAlaThrLeuAlaPheHisAspPheGlnHisGluLeuArgTyrAlaA

1021 GAATAAACGGTGTATTAGGATCAATTGACGCAAATACAGGCGACATGCTTTTGGGATGGG  1080
     rgIleAsnGlyValLeuGlySerIleAspAlaAsnThrGlyAspMetLeuLeuGlyTrpA

1081 ATACGGACCAGTTCCCTACAGATATACGCATGACAACGCTTGCTATGTATGAAGTCATAA  1140
     spThrAspGlnPheProThrAspIleArgMetThrThrLeuAlaMetTyrGluValIleL

1141 AGATGGGTGGATTTGACAAAGGTGGCCTTAACTTTGATGCAAAAGTAAGACGTGCTTCAT  1200
     ysMetGlyGlyPheAspLysGlyGlyLeuAsnPheAspAlaLysValArgArgAlaSerP

1201 TTGAGCCAGAAGATCTTTTCTTAGGTCACATAGCAGGAATGGATGCTTTTGCAAAAGGCT  1260
     heGluProGluAspLeuPheLeuGlyHisIleAlaGlyMetAspAlaPheAlaLysGlyP

1261 TTAAAGTTGCTTACAAGCTTGTGAAAGATGGCGTATTTGACAAGTTCATCGAAGAAAGAT  1320
     heLysValAlaTyrLysLeuValLysAspGlyValPheAspLysPheIleGluGluArgT

1321 ACGCAAGCTACAAAGAAGGCATTGGCGCTGATATTGTAAGCGGTAAAGCTGACTTCAAGA  1380
     yrAlaSerTyrLysGluGlyIleGlyAlaAspIleValSerGlyLysAlaAspPheLysS

1381 GCCTTGAAAAGTATGCATTAGAGCACAGCCAGATTGTAAACAAATCAGGCAGACAAGAGC  1440
     erLeuGluLysTyrAlaLeuGluHisSerGlnIleValAsnLysSerGlyArgGlnGluL

1441 TATTAGAATCAATCCTAAATCAGTATTTGTTTGCAGAATAATGAAACATGAGGGCGGCTT  1500
     euLeuGluSerIleLeuAsnGlnTyrLeuPheAlaGluEndEnd

1501 CATGCTTCATTAAGGCTGCCCTCAACAAAAATCATGGAGGTAAATGTATGTATTTTTTAG  1560

1561 GGATAGATTTAGGGACATCATCAGTTAAGATAATACTGATG  1601
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The ATG initiation codon is preceded with a spacing of 7 base pairs, by a putative ribosomal binding sequence AAGGAGG which is complementary to the 3' end of both the *E. coli* and *B. subtilis* 16S ribosomal RNA (25, 33). A putative -10 promotor region (Pribnow box), TATAATATATAAT, which repeated twice at the -10 region in *E. coli*, was found 25 bp upstream of the ribosomal binding site.

Comparison of amino acid sequences

The deduced amino acid sequence of xylose isomerase from *Thermoanaerobacter* B6A-RI was compared to those of *Clostridium thermosulfurogenes* (20), *Clostridium thermohydrosulfuricum* (9), *E. coli* (30), *Bacillus subtilis* (34), *Streptomyces violaceoniger* (13), *Streptomyces rubiginosus* (36) and *Thermus thermophilus* (10) and the results are shown in Table 1.

The length of the polypeptide chain encoding for the *xylA* gene in *Thermoanaerobacter* B6A-RI was identical to that of *C. thermosulfurogenes* 4B (439 amino acid residues) and was similar to those of *C. thermohydrosulfuricum* 39E (438 residues), *E. coli* and *B. subtilis* enzymes (440 residues), whereas the enzymes from *S. violaceoniger*, *Ampullariella*, and *Arthrobacter* have polypeptides which are shorter by 47 amino acid residues at the NH_2-terminal end. The deduced amino acid sequence of *Thermoanaerobacter* B6A-RI xylose isomerase exhibits very high homology to those from *C. thermosulfurogenes* and *C. thermohydrosulfuricum* (98% and 89% identity, respectively) and their sequence alignment is shown in Fig. 3.

Table 1. Relative homology between xylose isomerase from *Thermoanaerobacter* B6A-RI and other xylose isomerases.

Strain	% Similarity	% Identity
B. subtilis	82.5 %	69.6 %
E. coli	64.0 %	45.9 %
T. thermophilus	51.1 %	30.5 %
S. rubiginosus	49.9 %	26.5 %
S. violaceoniger	48.3 %	26.4 %
C. thermosulfurogenes	99.1 %	98.2 %
C. thermohydrosulfuricum	N.D	90.4 %

Figure 3. Comparison of the xylose isomerase amino acid sequence of: [1] *Thermoanaertobacter* B6A-RI, [2] *Clostridium thermosulfurogenes* 4B, [3] *Clostridium thermohydrosulfuricum* 39E. Stars under the sequence indicate amino acids which are different in all three sequences compared.

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B6A [1] MNKYFENVSKIKYEGPKSNNPYSFKFYNPEEVIDGKTMEEHLRFSIAYWH   50
 4B [2] MNKYFENVSKIKYEGPKSNNPYSFKFYNPEEVIDGKTMEEHLRFSIAYWH   50
39E [3] -MEYFKNVPQIKYEGPKSNNPYAFKFYNPDEIIDGKPLKEHLRFSVAYWH   49
        ***  *  **             *              ***

    [1] TFTADGTDQFGKATMQRPWNHYTDPMDIAKRRVEAAFEFFDKINAPFFCF  100
    [2] TFTADGTDQFGKATMQRPWNHYTDPMDIAKARVEAAFEFFDKINAPYFCF  100
    [3] TFTANGTDPFGAPTMQRPWDHFTDPMDIAKARVEAAFELFEKLDVPFFCF   99
            *   *  **       *           *       *    **

    [1] HDRDIAPEGDTLRETNKNLDTIVAMIKDYLKTSKTKVLWGTANLFSNPRF  150
    [2] HDRDIAPEGDTLRETNKNLDTIVAMIKDYLKTSKTKVLWGTANLFSNPRF  150
    [3] HDRDIAPEGETLRETNKNLDTIVAMIKDYLKTSKYKVLWGTANLFSNPRF  149

    [1] VHGASTSCNADVFAYSAAQVKKALEITKELGRENYVFWGGREGYETLLNT  200
    [2] VHGASTSCNADVFAYSAAQVKKALEITKELGGENYVFWGGREGYETLLNT  200
    [3] VHGAATSCNADVFAYAAAQVKKALEITKELGGQNYVFWGGREGYETLLNT  199
            *           *                **

    [1] DMELELDNFARFLHMAVDYAKEIGFEGQFLIEPKPKEPTKHQYDFDVANV  250
    [2] DMEFELDNFARFLHMAVDYAKEIGKEGQFLIEPKPKEPTKHQYDFDVANV  250
    [3] DMELELDNLARFLHMAVEYAQEIGFEGQFLIEPKPKEPTKHQYDFDAASV  249
           *     *             *                      * *

    [1] LAFLRKYDLDKYFKVNIEANHATLAFHDFQHELRYARINGVLGSIDANTG  300
    [2] LAFLRKYDLDKYFKVNIEANHATLAFHDFQHELRYARINGVLGSIDANTG  300
    [3] HAFLKKYDLDKYFKLNIEANHATLAGHDFQHELRYARINNMLGSIDANMG  299
                                    *             **      *

    [1] DMLLGWDTDQFPTDIRMTTLAMYEVIKMGGFDKGGLNFDAKVRRASFEPE  350
    [2] DMLLGWDTDQFPTDIRMTTLAMYEVIKMGGFDKGGLNFHAKVRRASFEPE  350
    [3] DMLLGWDTDQYPTDIRMTTLAMYEVIKMGGFNKGGLNFDAKVRRASFEPE  349
                                          *      *

    [1] DLFLGHIAGMDAFAKGFKVAYKLVKDGVFDKFIEERYASYKEGIGADIVS  400
    [2] DLFLGHIAGMDAFAKGFKVAYKLVKDRVFDKFIEERYASYKDGIGADIVS  400
    [3] DLFLGHIAGMDAFAKGFKVAYKLVKDGVFDRFIEERYKSYREGIGAEIVS  399
                                       *          *

    [1] GKADFKSLEKYALEHSQIVNKSGRQELLESILNQYLFAE             439
    [2] GKADFKSLEKYALERSQIVNKSGRQELLESILNQYLFAE             439
    [3] GKANFKTLEEYALNNPKIENKSGKQELLESILNQYLFSE             438
           *     *    **** *                  *
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Codon usage in *xynA*, *xynB* and *xylA*

Table 2 summarizes codon usage in the three genes from *Thermoanaerobacter* B6A-RI which are involved in xylan degradation. *xynA* and *xynB* were closely linked on a chromosome of *Thermoanaerobacter* B6A-RI (Lee & Zeikus, manuscript prepared for publication). Averaged codon usage for *E. coli* (1) is included for comparison. The patterns of codon usage among the three genes were very similar to each other. There is a clear propensity of AT-containing isocodons, and this probably results from the low G+C content (36 mol%) of this organism.

Biochemical characterization of cloned xylose isomerase

The thermostable *Thermoanaerobacter* B6A-RI xylose isomerase expressed in *E. coli* was purified to homogeneity (Fig. 4). Heat treatment of cell extracts at 85°C for 15 min was a very efficient purification step for the enzyme from *E. coli* as approximately 90% of *E. coli* proteins could be removed by this procedure. After heat treatment, the purification yield increased 18 fold. After additional steps of purification by anion-exchange chromatography and gel filtration. Preparations of homogeneous recombinant xylose isomerase were obtained and its purity was demonstrated on SDS-polyacrylamide gel electrophoresis (Fig. 4). The purified enzyme consisted of one type of subunit with a molecular mass of 50 kDa. The molecular mass of the purified recombinant enzyme was 200kDa, indicating that the enzyme is a tetramer comprised of identical subunits.

The apparent temperature and pH optimum for the activity of the cloned xylose isomerase was 80°C and 7.0 to 7.5 respectively. The enzyme was stable at 80°C for 60 min, and 0.5 mM Co^{2+} and 5 mM Mg^{2+} were required for optimum enzyme activity and thermostability.

Table 2. A comparison of the codon usage frequency in the three genes from *Thermoanaerobacter* B6A-RI which are involved in xylan degradation.

Amino acid	Codon	Frequency (mol%) of codon usage			
		Thermoanaerobacter B6A-RI			E. coli
		xynA	xynB	xylA	
Phe	TTT	2.8	5.6	5.2	1.3
	TTC	0.4	1.0	3.0	2.2
Leu	TTA	2.1	1.4	1.6	0.7
	TTG	1.7	2.0	1.8	0.9
	CTT	1.4	2.0	3.0	0.8
	CTC	0	0.4	0.5	0.8
	CTA	0.1	0.4	0.7	0.2
	CTG	0.6	0.2	0	6.8
Ile	ATT	3.3	2.4	1.6	2.2
	ATC	0.6	0.8	1.1	3.7
	ATA	3.4	3.4	2.3	0.2
Met	ATG	1.8	2.2	2.7	2.8
Val	GTT	3.1	2.2	1.1	2.9
	GTC	0.3	1.0	0.5	1.2
	GTA	2.8	2.4	2.7	1.8
	GTG	1.0	1.8	0.5	2.2
Ser	TCT	2.4	1.4	0.7	1.3
	TCC	0.2	0.4	0.7	1.5
	TCA	2.0	1.0	1.4	0.4
	TCG	0.4	0.2	0	0.6
	AGT	1.5	0.4	0	0.3
	AGC	1.2	1.2	1.1	1.4
Pro	CCT	1.2	2.0	1.4	0.5
	CCC	0.2	0.2	0	0.3
	CCA	1.9	2.4	1.1	0.7
	CCG	0.4	0.8	0.5	2.5
Thr	ACT	2.9	1.2	1.1	1.1
	ACC	0.4	0.6	0.5	2.4
	ACA	3.6	2.0	2.7	0.3
	ACG	1.1	1.0	0.9	0.8
Ala	GCT	2.0	1.6	2.7	2.6
	GCC	0.4	0.4	0.7	2.2

Table 2. A comparison of the codon usage frequency in the three genes from *Thermoanaerobacter* B6A-RI which are involved in xylan degradation (cont.).

Amino acid	Codon	Frequency (mol%) of codon usage			
		Thermoanaerobacter B6A-RI			E. coli
		xynA	xynB	xylA	
Ala	GCA	3.1	1.2	4.6	2.3
	GCG	0.4	0.6	0.7	3.2
Tyr	TAT	3.8	3.8	2.3	1.0
	TAC	0.7	2.2	2.5	1.5
His	CAT	1.0	1.4	1.4	0.7
	CAC	0.1	1.0	1.4	1.2
Gln	CAA	3.1	1.0	1.4	1.0
	CAG	1.1	0.8	0.9	3.2
Asn	AAT	6.8	4.2	3.0	1.0
	AAC	1.3	0.8	2.3	2.8
Lys	AAA	4.4	3.2	5.0	4.1
	AAG	2.0	3.8	3.4	1.3
Asp	GAT	6.3	5.4	4.6	2.5
	GAC	2.3	1.4	3.0	3.0
Glu	GAA	4.2	6.4	3.9	4.9
	GAG	0.7	2.8	3.9	1.8
Cys	TGT	0	0.4	0	0.4
	TGC	0.1	0.4	0.5	0.5
Trp	TGG	2.2	2.2	1.1	0.7
Arg	CGT	0.1	0	0.2	3.1
	CGC	0	0	0.7	2.0
	CGA	0	0.6	0.2	0.2
	CGG	0	0	0	0.2
	AGA	1.2	3.0	2.5	0.1
	AGG	0.4	1.2	0.5	0.1
Gly	GGT	2.3	1.0	2.0	3.8
	GGC	1.3	1.4	2.5	3.1
	GGA	2.8	3.2	1.8	0.4
	GGG	0.7	0.6	0.2	0.6

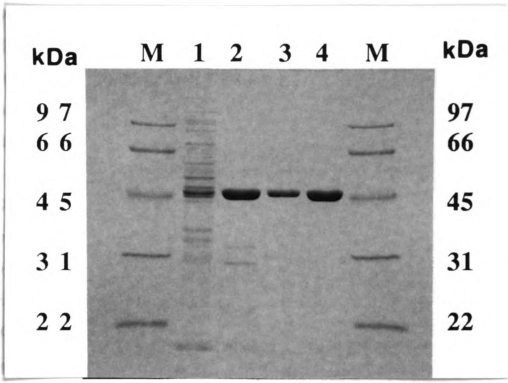


Figure 4. SDS-PAGE analysis of the thermostable *Thermoanaerobacter* B6A-RI xylose isomerase expressed in *E. coli* (pZXI6).

Lane M, molecular weight markers; lane 1, crude extract; lane 2, after heat treatment; lane 3, after anion exchange chromatography; lane 4, after gel filtration.

DISCUSSION

Xylose isomerase is an important industrial enzyme (2, 6, 7) because of its ability to catalyze the conversion of glucose to fructose. Xylose isomerases from thermophilic bacteria have high temperature optima and are thermostable, indicating their potential use for the commercial conversion of starch to high fructose corn syrup at high temperature with increased fructose yield.

Xylose isomerases has been isolated and studied from many microorganisms. *xyl* genes from *E. coli* (30), *B. subtilis* (34, 35), *T. thermophilus* (10), *S. violaceoniger* (24), *C. thermohydrosulfuricum* (10) and *C. thermosulfurogenes* (19) have been cloned and sequenced.

Comparison of the primary structure of xylose isomerases from three thermophilic anaerobic bacteria, *Thermoanaerobacter* B6A-RI, *C. thermosulfurogenes* and *C. thermohydrosulfuricum,* indicate extensive similarities between this enzyme from these organisms. Because these strains have evolved in thermal hotsprings in Yellowstone National Park, (22, 31) these organisms might have a common phylogenic origin with conservation of xylose isomerase, or alternatively gene transfer might have occurred between these strains.

In several bacteria in which the genetic organization of the *xyl* genes has been examined, the xylose isomerase and xylulose kinase genes were found to be part of one operon (15, 17, 23, 32, 34). Another open reading frame with the same transcriptional orientation was found downstream of *xylA* from the cloned DNA fragment (data not shown) and this may be a xylulose kinase gene (*xylB*).

The predicted molecular weight of 50,474, calculated from the deduced amino acid sequence, is in good agreement with the subunit molecular weight of 50,000 reported for xylose isomerase purified from *Thermoanaerobacter* (21). The recombinant protein was purified to homogeneity and its physical and biochemical properties were determined. The molecular mass of the purified recombinant xylose isomerase was 200 kDa, indicating that the enzyme is a tetramer composed of identical subunits. This result is consistent with the native xylose isomerase from *Thermoanaerobacter* B6A (21). The xylose isomerase expressed in *E. coli* displayed identical pH and temperature optimum, thermostability, and metal ion requirements with the xylose isomerase from *Thermoanaerobacter* (21).

The base composition of the coding region of *xylA* is 39.7 mol% G+C, a value that is close to the G+C content (36 mol %) of this organism (22). The low G+C content of *Thermoanaerobacter* B6A-RI *xylA* results in an extremely biased usage of synonymous codons, A or T, which were present in the third position of all dominant codons examined in this organism.

The high thermostability of *Thermoanaerobacter* B6A-RI xylose isomerase made it possible to use heat treatment as one of the most efficient purification steps of the cloned enzyme produced in the mesophilic host *E. coli*. The presence of metal ions (Mg^{2+} and Co^{2+}) and a high protein concentration in cell extracts were essential for optimal recovery of the enzyme during heat treatment. The thermostable xylose isomerase produced by the recombinant plasmid pZXI6 was one of the most abundant protein in *E. coli*.

The overall biochemical and physico-chemical properties of the recombinant xylose isomerase purified from *E. coli* (pZXI6) were identical to the native enzyme purified from *Thermoanaerobacter* B6A (21). The N-

terminal amino acid sequences in the recombinant and native xylose isomerase were identical (21).

The catalytic mechanism for xylose isomerase was originally believed to involve histidine-directed general base catalysis (27). Lee et al. (20) identified the active site histidine residues of xylose isomerase from *C. thermosulfurogenes* by site-directed mutagenesis, and found that His^{101} was the only essential histidine residue involved directly in catalysis. The rate limiting step in the isomerization reaction was found to be hydrogen transfer and not substrate ring opening.

At present the molecular mechanism for high thermophilicity (i.e. temperature optimum for activity at 80°C and thermostability at 85°C) of this enzyme is not clear. This could be addressed through the use of nested deletion mutants in order to identify regions on the gene which are responsible for activity and thermostability.

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Chapter VII

Characterization of the active site and thermostability regions of endoxylanase from *Thermoanaerobacter*

ABSTRACT

Deletion mutants were constructed from pZEP12 which contained the intact *Thermoanaerobacter* endoxylanase gene (*xynA*). Deletion of 2 kb from N-terminal end of *xynA* resulted in a mutant enzyme that retained activity but lost thermostability. Deletion of 1.5 kb from the C-terminus did not alter thermostability or activity. The deduced amino acid sequence of *Thermoanaerobacter* B6A-RI endoxylanase *xynA* was aligned with five other enzymes in family F β-glycanases by using the PILEUP program of the GCG package. This multiple alignment of amino acids sequences revealed 6 highly conserved motifs which included the consensus sequence ITELD in the catalytic domain. Endoxylanase was inhibited by EDAC suggesting that Asp and/or Glu were involved in catalysis. Three aspartic acids, two glutamic acids, and one histidine were conserved in all six enzymes aligned and they were targeted for analysis by site-specific mutagenesis. Substitution of Asp-612 by Asn, Glu-508 by Gln, and His-539 by Asn had little effect on the enzyme activity, whereas substitution of Asp-504 and Asp-569 by Asn and Glu-567 by Gln completely destroyed endoxylanase activity and implicated their role in general acid catalysis mechanism.

INTRODUCTION

β-1,4-xylan is a major component of hemicellulose and has a backbone of β-1,4-linked D-xylopyranoside residues substituted with acetyl, arabinosyl and uronyl side chains (3). After cellulose, xylan is the most abundant renewable polysaccharide in nature, accounting for 20 to 30% of the dry weight of woody tissue (39).

Complete breakdown of branched acetyl xylan requires the action of several hydrolytic enzymes (3, 28, 41). The most important microbial enzymes involved in xylan degradation are endo-1,4-β-D-xylanase (EC 3.2.1.8) and β-D-xylosidase (EC 3.2.1.37). Endo-1,4-β-D-xylanase acts on chains of xylans, arabinoxylans and 4-MeO-glucuronoxylans. β-xylosidase breaks down short oligosaccharides from the nonreducing end to xylose, and has substantial transferase activity (28).

These xylan degrading enzymes are produced by a wide variety of microorganisms including aerobic and anaerobic mesophiles and thermophiles (3, 38). The majority of the studies have been carried out in aerobic, mesophilic fungi and bacteria (3, 28, 38, 41).

Recently β-glycanases were grouped into 9 different structural paradigms (families A to families I) according to hydrophobic cluster analysis with the finding that most xylanases belong to either family F or family G (40). Generally family F xylanases are larger in size (269 to 1,011 amino acid residues) than family G xylanases which have a small molecular weight (182 to 234 amino acid residues). Family F includes *Bacillus* sp. strain C-125 *xynA* (14), *Butyrivibrio fibrisolvens xynA* (18), *Caldocellum saccharolyticum CelB, xynA* and ORF4 (19, 32), *Cellulomonas fimi Cex* (25), *Clostridium thermocellum xynZ* (12), *Cryptococcus albidus Xyn* (5), *Pseudomonas*

fluorescens subsp. *cellulosa* *xynA* and *xynB* (13, 17), and *Thermoascus aurantiacus* *xyn* (33).

Despite the interest in the xylanases for the bioconversion of renewable plant cell materials and for their industrial applications, little is known about their mechanism of catalysis. Xylanases and cellulases, like other glycosidases, have been suggested to act by general acid catalysis involving amino acid carboxy groups (1, 6, 7, 9, 16, 21, 23, 24, 26, 35, 42).

In the absence of X-ray crystallography data, some informations about the amino acid residues involved in the active site can be obtained by using labeled substrates and chemical modification. These approaches are limited as it is not possible to identify the specific amino acid residues in the active site. Identification of conserved amino acid residues through alignment of amino acid sequences from similar enzymes can be a useful method to predict essential amino acids for catalysis, and this prediction can be confirmed by site-directed mutagenesis.

We have recently cloned and sequenced the endoxylanase A gene (*xynA*) and showed it belongs to family F (Lee & Zeikus, manuscript prepared for submission). The purpose of the present report is to identify the catalytic and thermostability domains of endoxylanase A of *Thermoanaerobacter* and to provide evidence of the specific Glu and Asp residues involved in catalysis.

MATERIALS AND METHODS

Strains, enzymes, and chemicals

E. coli strain SDM (hsdR17, *mcr*AB, *rec*A1, *sup*E44, *Tet*^{r}, Δ (lac-proAB), [F' *tra*D36, *pro* AB$^+$, *lac*I^{q}ZΔM15]) was used as a host strain for site-directed mutagenesis. Restriction endonucleases and T4 DNA ligase used for subcloning experiments were obtained from Bethesda Research Laboratories (Gaithersburg, MD) and Boehringer Mannheim Biochemicals (Indianapolis, IN). [α-^{35}S]dATP (12.5 mCi/ml) was purchased from DuPont-New England Nuclear (Bannockburn, IL). 1-(3-Dimethylaminopropenyl)-3-ethylcarbodiimide hydrochloride (EDAC) was obtained from Aldrich (Milwaukee, WI). All other chemicals were reagent grade.

General DNA procedures

Plasmid DNA preparation, electrophoresis and DNA fragment isolation were performed using standard procedures (30). The alkaline denaturation method (4) was used for rapid extraction of plasmid DNA. Enzymes were used as recommended by the manufacturers. Bacterial transformation was performed by the Hanahan method as described by Perbal (27).

Subcloning experiments

Plasmid pZEP12 was the parent of all the plasmids generated throughout this work (Figure 1). It carries the *xynA* gene on a 4.5 kb EcoRI-PstI restriction fragment inserted at the multicloning site of the pUC18 vector. Deletion mutants were derived from pZEP12 by subcloning as described previously Lee et al., manuscript prepared for submission).

A 4.5 kb EcoRI-PstI DNA fragment, containing the intact *xynA* gene, was isolated from pZEP12 by partial digestion and inserted at the EcoRI-PstI sites of the phagemid vector pUC119 (37). The resulting recombinant plasmid pSDM12 was transformed into *E. coli* strain SDM and used for the isolation of single-stranded DNA for site-directed mutagenesis.

Computer analysis

The amino acid sequences of six family F xylanases and cellulases were aligned with the deduced amino acid sequence of *Thermoanaerobacter xynA* using the Sequence Analysis Software Package of the Genetics Computer Group (GCG), version 7 (University of Wisconsin) (10).

Chemical modification

The inhibitory action of EDAC on xylanase activity was studied by varying EDAC concentrations in the reaction mixture from 10 to 100 mM. The reaction mixture contained EDAC with enzyme (500 μg) with 50 mM glycine methyl ester with 50 mM imidazole (pH 6.0) and was incubated at 45°C. After 1 h a 10 μl sample of the each reaction mixture was withdrawn and added to 90 μl of 100 mM sodium acetate buffer, pH 5.5, to quench the residual reagent. The remaining endoxylanase activity of the modified enzymes were determined by the 3,5-dinitrosalicylic acid assay (Miller, 1959) using oat spelt xylan (1%) as a substrate. To assess the ability of the soluble xylan to protect xylanase from modification with EDAC, the enzyme (500 μg) was incubated with 100 mM EDAC in the presence of soluble xylan (1%), and the remaining activity measured.

Site-directed mutagenesis and nucleotide sequencing

Plasmid pSDM12 was used for site-directed mutagenesis. Based on the sequence alignment, the mutagenic oligonucleotides were designed to be complementary to the single-stranded template DNA and to contain appropriate mismatches as indicated in Table 1. The oligonucleotide primers were synthesized by the Michigan State University Macromolecular facility using a automated DNA synthesizer (Applied BioSystems, Foster City, CA). Synthesis of mutant genes and selection was performed by the method of Vandeyar et al. (36), using a kit of T7-GEN™ *In Vitro* Mutagenesis kit (United State Biochemical Co., Cleveland, OH). Mutant strains were cultured in the presence of M13 helper phage M13KO7 (37) and single stranded DNA was prepared from the culture supernatants as described previously (30). Nucleotide sequences of the isolated single-stranded DNA were determined by the dideoxy chain termination method (31), using a Sequenase version 2.0 kit (U. S. Biochemical Co.).

Table 1. Synthetic oligonucleotides used to isolate mutant endoxylanases

Mutant	Amino acid change	Codon change	Nucleotide primer*
D504N	$Asp^{504} \rightarrow Asn^{504}$	GAC → AAC	GTTTATTAAT<u>A</u>ACTATAACATTG
E508Q	$Glu^{508} \rightarrow Gln^{508}$	GAA → CAA	ACTATAACATT<u>C</u>AAAATAATGGT
H539N	$His^{539} \rightarrow Asn^{539}$	CAC → AAC	AGGCATGCAAATG<u>A</u>ACATAAATA
E567Q	$Glu^{567} \rightarrow Gln^{567}$	GAA → CAA	TACAAGTAACT<u>C</u>AATTAGATATG
D569N	$Asp^{569} \rightarrow Asn^{569}$	GAT → AAT	TAACTGAATTA<u>A</u>ATATGAACA
D612N	$Asp^{612} \rightarrow Asn^{612}$	GAT → AAT	GGAGTTTCA<u>A</u>ATGATGTAACTTG

* underline indicates the base change

RESULTS

Subcloning and characterization of *xyn*A thermostability domain

The physical map of the *Thermoanaerobacter* B6A-RI *xynA* gene and its deletion mutants are shown in Fig. 1. Deletion of 2 kb from N-termial end of *xynA* using deletion mutant resulted in a reduction of thermostability and thermophilicity (Table 2). Although the deletion mutant was still active at high temperature, the optimum temperature for activity was 65°C versus 75°C for wild type enzyme. Deletion of 1.5 kb from C-terminal end of xyn*A* did not affect thermostability or thermophilicity.

Comparison of the deduced amino acid sequences of xylanase A with the family F β-1,4-glycanases.

The complete nucleotide sequence of the endoxylanase gene (*xynA*) from *Thermoanaerobacter* B6A-RI was determined previously (Lee & Zeikus, manuscript prepared for submission). It has an open reading frame of 3471 bp encoding 1157 amino acid residues. The deduced amino acid sequence was also compared to those of other proteins deposited in the data base. Most proteins showing strong homology to *xynA* were xylanases and cellulases of family F β-1,4-glycanases. This is also confirmed by hydrophobic cluster analysis which grouped β-glycanases into 9 families based on the sequence analysis of conserved sequences found in discrete domains (11).

The consensus sequence, ITELD, is found in the catalytic domain of the family F glycanases, which currently comprises 3 glucanases and 11 xylanases (2, 11, 15, 35). Alignment of the deduced amino acid sequences of endoxylanase from *Thermoanaerobacter* B6A-RI with those of family F β-

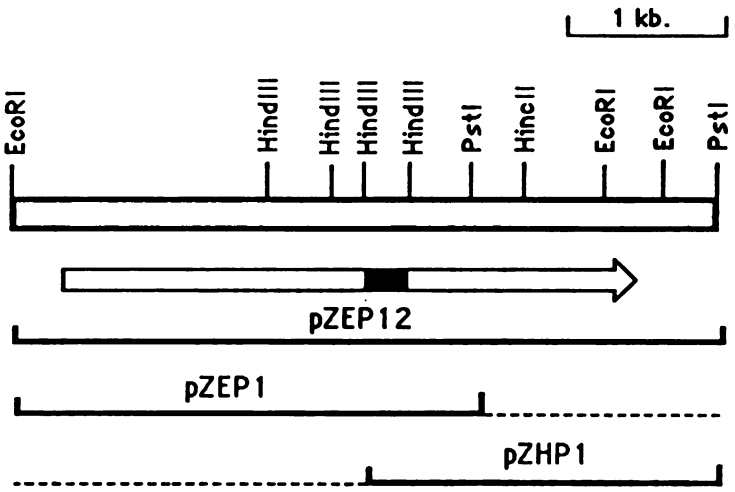


Figure 1. The physical map of the DNA fragment containing the *Thermoanaerobacter* B6A-RI *xynA* gene and the deletion mutants.

The black box indicates the location of consensus sequence in the putative catalytic domain. Dashed line represents deleted fragment.

Table 2. Comparison of temperature optimum and thermostability of endoxylanase with its deletion derivatives.

Mutant plasmid	Size deleted	Deleted region	Temp optimum	Thermostability[a]
pZEP12	none	none	70°C	75°C
pZEP1	1.5 kb	C-terminus	70°C	75°C
pZHP1	2.0 kb	N-terminus	65°C	65°C

[a] The activity was not decreased at this temperature at least 1 h.

glycanases reveals that *Thermoanaerobacter* B6A-RI xylanase also contains the conserved sequence, ITELD, in its putative catalytic domain (Fig. 2).

Thermoanaerobacter B6A-RI endoxylanase A has 68% similarity to the *Bacillus* sp. strain C-125 endoxylanase and 43 to 66% similarity to other family F β-glycanases. For more detailed analyses the deduced amino acid squence of the *Thermoanaerobacter* B6A-RI endoxylanase *xynA* was aligned with those of the other five enzymes in family F β-glycanases by using the PILEUP program of the GCG package (University of Wisconsin) (Fig. 3). This multiple alignment of amino acids sequences revealed 6 highly conserved motifs shown in the box of Fig. 3 which includes the consensus sequence ITELD.

Role of Glu and Asp in catalysis

In order to confirm that amino acid carboxy groups are essential for endoxylanase activity, we investigated the inhibition effect of EDAC on the endoxylanase activity (Table 3). Incubation of the enzyme in the presence of 100 mM of EDAC for 1 h resulted in total loss of activity and addition of xylan before EDAC treatment prevented the loss of activity. This result indicated that Aspartic acid and/or Glutamic acid are involved in the active site of the endoxylanase.

The predicted importance of aspartic and glutamic acids in catalysis of the endoxylanase were tested by altering the conserved amino acids found in the sequence alignment. Three aspartic acids, two glutamic acids, and one histidine were conserved in all six enzymes aligned and these six amino acids were targeted for site-specific mutagenesis. Table 1 shows the oligomers used and the position of the mutations introduced. The conserved aspartic acid residues at position 504, 569 and 612 were changed to asparagine. The

Thermoanaerobacter B6A-RI	*xynA*	560	G	V	E	I	Q	V	T	E	L	D	M
Clostridium thermocellum	*xynZ*	747	G	V	I	V	S	F	T	E	L	D	I
Caldocellum saccharolyticum	*xynA*	245	G	L	E	I	H	I	T	E	L	D	I
Caldocellum saccharolyticum	*celB*	278	G	I	E	I	H	I	T	E	L	D	M
Caldocellum saccharolyticum	ORF4	209	G	L	Q	I	H	I	T	E	L	N	F
Cellulomonas fimi	*cex*	267	G	V	D	V	R	I	T	E	L	D	I
Pseudomonas fluorescence	*xynA*	499	T	L	K	I	K	I	T	E	L	D	V
Pseudomonas fluorescence	*xynB*	523	G	K	P	I	Y	I	S	E	Y	D	I
Bacillus sp. strain 125	*xynA*	294	G	L	D	N	Q	V	T	E	L	D	M
Butyrivibrio fibrisolvens	*xynA*	304	G	F	E	V	Q	I	T	E	L	D	I
Cryptococcus albidus	*xyn*	207	G	L	E	V	P	M	T	E	L	D	V

Figure 2. Alignment of the deduced amino acid sequences of endoxylanase from *Thermoanaerobacter* B6A-RI with those of family F β-glycanases.

Box indicates the consensus sequence.

Figure 3. Amino acid squence alignment of the endoxylanase (*xynA*) from the *Thermoanaerobacter* B6A-RI with those of the other five enzymes in family F β-glycanases.

The amino acids conserved in all enzymes are shown in bold and the highly conserved motifs are shown in the box. Small circles above the sequence indicate the amino acids targeted for site specific mutagenesis.


TSxynA 472 NSKWL-QIIGPDYIEKAFEYAHE-ADPSMKLFINDYNIENNGVKTQAMYD
BAxynA 205 ESEWY-QITGTDYIKVAFETARKYGGEEAKLYINDYNTE-VPSKRDDLYN
CSxynA 156 ESNWR-KIIGDDYIKIAFEIAREYAG-DAKLFYNDYNNE-MPYKLEKTYK
CScelB 189 RSTWY-EICGPEYTEKAFIWAHE-ADPNAKLFVNDYNTE-ISKKRDFIYN
CFcex 179 DSAFQ-QKLGNGYIETAFRAARA-ADPTAKLCINDYNVEGINAKSNSLYD
PFxynA 407 QSVFYRQFGGPFYIDEAFRRAPR-ADPTAELYYNDFNTEENGAKTTALVN
TSxynA 520 LVKKLKSEGVPIDGIGMQMHINI--NSNIDNIKASIEKLASL--GVEIQV
BAxynA 253 LVKDLLEQGVPIDGVGHQSHIQI-GWPSIEDTRASFEKFTSL--GLDNQV
CSxynA 203 VLKELLERGTPIDGIGIQAHWNIWDKNLVSNLKKAIEVYASL--GIEIHI
CScelB 236 MVKNLKSKGIPIHGIGMQCHINV-NWPSVSEIENSIKLFSSI-PGIEIHI
CFcex 227 LVKDFKARGVPIDCVGFQSHLIVGQVP--GDFRQNLQRFADL--GVDVRI
PFxynA 456 LVQRLLNNGVPIDGVGFQMHV-MNDYPSIANIRQAMQKIVALSPTLKIKI
TSxynA 566 TELDMNMNG----------N-----ISNEALIKQARLYKQLFDLFKAE-
BAxynA 300 TELDMSLYGWPPTGAYTSYDD-----IPAELLQAQADRYDQLFELYEEL-
CSxynA 251 TELDISVFEF--EDKRTDLFE-----PTPEMLELQAKVYEDVFAVFREY-
CScelB 284 TELDMSLYNY---GSSENYST-----PPQDLLQKQSQKYKEIFTMLKKY-
CFcex 273 TELDIRMRT----------P-----SDATKLATQAADYKKVVQACMQV-
PFxynA 505 TELDVRLNNPYDGNSSNDYTNRNDCAVSCAGLDRQKARYKEIVQAYLEVV
TSxynA 599 -KQYITAVVFWGVSDDVTWL----------SKPNRPLLFDSKLQRSQHSG
BAxynA 344 -AADISSVTFWGIADNHTWLDGRAREYNNGVGIDAPFVFDHNYRVKPAYW
CSxynA 293 -KDVITSVTLWGIDDRHTWKD----NFPVKGRKDWPLLFDVNGKPKEALY
CScelB 325 -KNVVKSVTFWGIKDDVSWLRSFY------GKNDWPLLFFEDYSAKPAYW
CFcex 306 -TRC-QGVTVWGITDKYSWVPDVF------PGEGAALVWDASYAKKPAYA
PFxynA 555 PPGRRGGITVWGIADPDSWL------YTHQNLPDWPLLFNDNLQPKPAYQ

Table 3. Effect of EDAC on recombinant endoxylanase A activity.[a]

EDAC conc. (mM)	Residual activity (%)
0	100
5	73
10	48
20	29
30	17
50	12.5
100	1.7
100 (+ 1% xylan)	92

[a] Purified enzymes (500 μg) in 50 mM imidazole buffer (pH 6.0) were incubated with various EDAC concentration at 45°C for 1 h. The residual endoxylanase activity was assayed as described in Materials & Methods and expressed as percentage of specific activity found in the control without EDAC.

conserved glutamic acids at position 508 and 567 were changed to glutamine. The conserved histidine at position 539 was changed to asparagine. The mutations were confirmed by nucleotide sequencing.

Table 4 shows that substitution of Asp-504 and Asp-569 by Asn and Glu-567 by Gln completely destroyed endoxylanase activity whereas the other amino acid substitutions had little effect on enzyme activity.

Table 4. Specific activity of mutated endoxylanases

Enzyme	Mutation	Specific activity, U/ml (%)[a]
Wild type	None	3.01 ± 0.057 (100.0)
D504N	$Asp^{504} \longrightarrow Asn$	0.03 ± 0.008 (1.0)
E508Q	$Glu^{508} \longrightarrow Gln$	1.97 ± 0.045 (65.4)
H539N	$His^{539} \longrightarrow Asn$	2.23 ± 0.055 (74.1)
E567Q	$Glu^{567} \longrightarrow Gln$	0.065 ± 0.011 (2.1)
D569N	$Asp^{569} \longrightarrow Asn$	0.056 ± 0.003 (1.9)
D612N	$Asp^{612} \longrightarrow Asn$	3.68 ± 0.364 (122.2)

[a] The enzyme activity present in crude extract was determined and percent of specific activity relative to wild-type enzyme is given in parenthese.

DISCUSSION

To date only active site domains and substrate binding domains have been characterized in glycanases including xylanases. Essentially nothing is known about thermostability domains in thermophilic enzymes. The N-terminal end of *xyn*A appear to confer thermostability and thermophilicity on endoxylanase A from *Thermoanaerobacter* B6A-RI. Further deletion from the N-terminal end may result in complete loss of activity, demonstrating a region responsible for activity and stability at high temperature.

Despite the structural differences in the substrates cellulose and xylan, cellulases and xylanases share a high degree of homology to each other. The highly conserved regions were suggested to be part of the catalytic and substrate binding site. It is assumed that β-1,4-glycanases, like other enzymes which hydrolyze glycosidic bonds, use a general acid-base catalytic mechanism, promoted by aspartate and/or glutamate residues. Stabilization of the carbonium intermediate is acheived by a residue of the active site with a negatively charged group (Asp, Glu) or by a histidine residue. As active site residues are usually highly conserved during evolution, the catalytic domains of cellulases and xylanases have been analyzed for conserved aspartates and glutamates in an attempt to target catalytic residues (15).

Hydrophobic cluster analysis has been proven to be an appropriate method for the comparison of cellulases and xylanases (15), which were found to comprise 9 different families. Sequence alignment and hydrophobic cluster analysis data indicated that *Thermoanaerobacter* B6A-RI endoxylanase (*xynA)* belongs to family F β-glycanases. A search for Asp and Glu residues occurring at the same position in all members of family F may identify the

putative catalytic amino acids. A conserved motif, ITELD, was found in all family F β–glycanases and was assumed to be involved in catalytic site.

In *Cellulomonas fimi* which is a member of family F, the active site nucleophile has been identified as glutamic acid residue 274 by using a tritium-labeled inactivator and sequencing the radiolabeled peptide (35). This glutamic acid residue is completely conserved in the family F enzymes, occurring in all of these enzymes. Using chemical modification studies, evidence was provided for the importance of carboxy groups for catalysis in the active site of the *Schizophyllum commune* xylanase (6). The importance of Glu residues had been suggested by chemical modification of various cellulases (2), including endo-β-1,4-glucanase from *Schizophyllum commune* (34), and cellobiohydrolase I from *Trichoderma reesei* (9). Using site-directed mutagenesis Glu-194 and Glu-169 were changed to the isosteric glutamine form in endo-β-1,4-glucanase from *Bacillus polymyxa* and *Bacillus subtilis* respectively, resulting in a dramatic loss of carboxymethyl cellulase activity which could be restored by reverse mutation (1). The involvement of the carboxy group is consistent with the view that cleavage of a β-1,4-glycosidic linkage proceeds through a mechanism based on general acid catalysis.

To date only two small molecular weight xylanases, a xylanase from *Bacillus pumilus* IPO (22, 29) and a xylanase from *Trichoderma harzianum* (29), were crystallized and analyzed by X-ray crystallography. In the absence of a known three dimensional structure, sequence comparisons among enzymes with similar function have proven useful for defining minimum functional size, and binding and catalytic domains. We used multiple sequence alignment among family F β–glycanases to predict essential, functional residues whose catalytic role was tested by site-directed mutagenesis. Six conserved amino acids were found in the putative catalytic domains of these

enzymes, and within these regions two glutamic acids, three aspartic acids and one histidine are conserved in all of the sequences aligned. Changing Asp-504 to Asn, Glu-567 to Gln and Asp-569 to Asn resulted in a dramatic loss of endoxylanase activity, suggesting that these carboxylic residues in the enzyme could act by general acid catalysis as has been shown for other hydrolytic enzymes such as lysozyme (8). The loss of activity which follows the relatively conservative change of a charged residue to an isosteric, neutral but polar residue, is expected if this Asp-504, Glu-567 or Asp-569 is responsible for donating the proton during hydrolysis. Alternatively, loss of a charged residue might alter protein folding enough to affect either the stability of the enzyme or the ability of this enzyme to bind to the substrate and this may be the case for Glu-508 and His-539 as their substitution resulted in partial loss of activity.

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Chapter VIII

Evidence for xylanosomes on the cell surface of *Thermoanaerobacter* B6A-RI

ABSTRACT

Negatively charged surface structures were visualized on cells of *Thermoanaerobacter* strain B6A-RI grown on xylan or xylose, using cationized ferritin and gold labelling with scanning and transmission electron microscopy. These structures were absent on glucose grown cells that were non-xylanolytic. The presence of these putative xylanosome structures coincided with production of cell bound endoxylanases and the ability of the cells to bind tightly to xylan. The xylanosomes appeared to be specific for cellular adhesion to xylan, as cells would not bind to cellulose. These findings extend the surface structures known to exist on the bacterial cell wall, and provide the first example of a cell surface structure specific for cellular adhesion to and degradation of xylan. These hydrolysome structures further demonstrate the ability of microorganisms to effectively form a glycoprotein complex to efficiently degrade insoluble substrates by facilitating both the concerted action of hydrolytic enzymes and the transfer of degradation products into the cell.

INTRODUCTION

Hemicelluloses are heteropolymers of various pentoses and hexoses and contain mainly xylan which is a β-1,4-linked polymer of D-xylose with D-glucuronic acid, L-arabinose and other sugar substituents. The complete hydrolysis of this rather complex substrate to monosaccharides requires the action of many hydrolytic enzymes such as endoxylanase and β-xylosidase (5, 21). Many xylanolytic microorganisms produce multiple xylanases (6, 27). This multiplicity could be as a result of degradation of one protein, post-translational modification of one enzyme, or the products of different genes (27).

C. thermocellum is one of the most actively studied cellulolytic microorganisms from which at least 15 genes encoding endoglucanases and genes encoding endoxylanases were cloned (4, 9). The various components of the cellulase system of *C. thermocellum* form an extracellular multienzyme complex with an Mr of about 2.1 million. The complex, termed the cellulosome, comprises 14-18 different polypeptides, has a strong affinity for cellulose and is responsible for the strong activity toward cellulose (12, 15). The cellulosome is present both in the culture supernatant and at the surface of the cells, where it is found in mutimeric aggregates which form protuberances mediating adhesion of the organism to the substrate (2, 3, 14). Several of the endoglucanases purified from *C. thermocellum* are glycoproteins (20).

The ability of microorganisms to adhere to the surface of insoluble substrates rather than to excrete soluble enzymes might be critical for their survival in the environment, especially in hot water, not only because of

intense competition for limited energy sources but also the diffusion of the polymer hydrolysis products.

Little is known about the microbial transformation of hemicellulose in thermoanaerobic habitats. A number of thermophilic, anaerobic, saccharolytic bacteria, including *Thermoanaerobacter ethanolicus*, *Thermobacteroides acetoethylicus*, *Thermoanaerobium brockii*, and *Clostridium thermocellum* ferment xylan albeit at a very slow rate (25, 26). Certain *Thermoanaerobacter* strains readily degrade xylan (16, 23). *Thermoanaerobacter* strain B6A-RI (17) degrade xylan but not cellulose and is a model organism to understand the mechanisms thermoanaerobes employ to efficiently degrade hemicellulose. We have previously demonstrated that *Thermoanaerobacter* B6A-RI possesses multiple endoxylanases that induced by growth on xylan or xylose (Lee et al., manuscript prepared for submission). In addition, this organism produced multiple endoxylanases which were excreted when growth on limiting amount of xylan.

Because of the structural complexity of xylan, xylanolytic organisms produce several different kinds of hydrolytic enzymes for xylan degradation which might be organized in a discrete complex on the cell surface with a function analogues to the cellulosome. Although thermoanaerobes have been studied with respect to their growth on xylan, little is known about the enzymes involved in the degradation of this insoluble substrate and currently nothing is known about cell surface structures involved in adhesion and degradation of xylan.

The purpose of the present paper is to provide evidence for the presence of xylanosome structures on the cell surface of *Thermoanaerobacter* which enables the cell to succesfully bind and degrade insoluble xylan.

MATERIALS AND METHODS

Chemicals and gases

All chemicals were reagent grade or better and were obtained from Sigma Chemcal Co., St. Louis, Mo., or Mallinckrodt, Inc., Paris, Ky. All gases used were at least 99.9% pure and were passed over copper-filled Vycor furnaces (Sargent Welch Scientific Co., Skokie, Ill.) to remove oxygen.

Organism and culture conditions

Thermoanaerobacter strain B6A-RI was grown on TYE medium at 60^{o}C in anaerobic tubes containing 10 ml of medium wih either glucose, xylose or xylan as the substrate as described previously (16, 17). The organism was subcultured at least three times in medium containing the same substrate prior to visualization of cell surface structures, to avoid any substrate carry over.

Morphological studies

Bacterial morphology and cellular adhesion to xylan was investigated using an Olympus phase-contrast microscope model BHS.

Affinity cytochemistry

Thermoanaerobacter strain B6A-RI was grown on glucose, xylose or xylan to midexponential phase (30 ml of culture were grown on each substrate). Cells were treated with cationized ferritin as described previously (3). The cells were washed twice with 0.9%(w/v) NaCl and resuspended in 5 ml of the same solution with 2 ml of 2.5%(v/v) glutaraldehyde in 0.1 M Na_2HPO_4-KH_2PO_4 (pH 7.2). After 20 mins at room temperature the cells were

washed three times with saline and then resuspended in 3 ml of saline. To 1.5 ml of this suspension was added 5 ml of the glutaraldehyde-phosphate buffer, and the cells were stored at 4°C. To the remaining 1.5 ml of cell suspension, was added 0.25 ml cationized ferritin (Sigma), and the solution incubated at room temperature for 1 hour before being centrifuged. The cells were washed twice with saline and then resuspended in glutaraldehyde-phosphate buffer, and stored at 4°C overnight before being processed for electron microscopy.

Scanning electron microscopy

Cells were processed in the absence and presence of cationized ferritin as described above and collected on to a 13 mm diameter polyester membrane, pore size 4 μm (Nucleopore, Pleasanton, CA) by suction and placed in a solution of cold 2.5% glutaraldehyde in 0.1 M Na_2HPO_4-KH_2PO_4 (pH 7.2) and then prepared for scanning electron microscopy as described previously (11).

Transmission electron microscopy

Cells were fixed overnight to 2 days in cold 2.5% glutaraldehyde in 0.1 M Na_2HPO_4-KH_2PO_4 (pH 7.2). The cells were embedded in agar and, after a buffer wash, post-fixed for 1 h at room temperature in 1% OsO_4 in the same buffer as above. They were then dehydrated through an ethanol series, treated with propylene oxide, and embedded in either Poly/Bed 812 (Polysciences Inc., Warrington, Pa.) or VCD/HXSA (Ladd Research Industries, Inc., Burlington, Vt.) epoxy resin. Thin sections were cut with a diamond knife mounted on an Ultratome III (LKB Instruments, Inc., Rockville, Md),

stained with uranyl acetate and lead citrate, and examined with an electron microscope (CM-10; Philips Electronic Instruments, Co., Mahwah, NJ).

RESULTS

Adhesion of cells to xylan

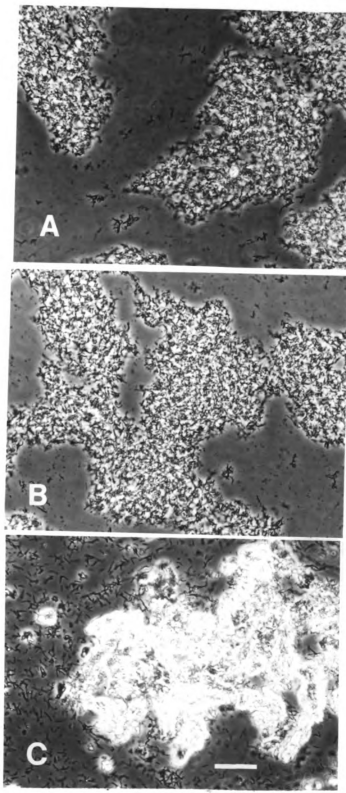
When cells of *Thermoanaerobacter* strain B6A-RI were mixed with xylan, the cells attached to xylan and were sedimented leaving a clear supernatant devoid cells. Using light microscopy, cells of *Thermoanaerobacter* strain B6A-RI cultured on xylan were observed to closely bind to the insoluble substrate (Fig. 1A). To determine the importance of the growth substrate on cellular adhesion to xylan, cultures were grown on xylose or glucose and incubated with xylan for up to 1 h. Samples were taken at intervals and examined using light microscopy. Cells grown on xylose or xylan closely adhered to xylan (Fig. 1B), whereas cells grown on glucose did not adhere to the substrate and tended to either associate together or remain free in solution (Fig. 1C). To determine if the substrate played a role in cellular adhesion of *Thermoanaerobacter* B6A-RI, cells were grown on xylose and then incubated in the presence of another insoluble substrate, cellulose. Examination of these cells using light microscopy, showed that the cells did not bind to the cellulose, but rather remained free in the medium (data not shown).

The presence of cell surface structures

As both the cellular location of the endoxylanases and adhesion of cells to xylan varied depending on the growth conditions, cells were examined for the presence of cell surface structures using cationized ferritin and gold labelling to identify negatively charged structures with scanning and transmission electron microscopy. In the absence of cationized ferritin, surface structures were not evident on cells of *Thermoanaerobacter* strain

Figure 1. Light microscopy of cells of *Thermoanaerobacter* strain B6A-RI grown on (A) xylan, (B) xylose and (C) glucose.

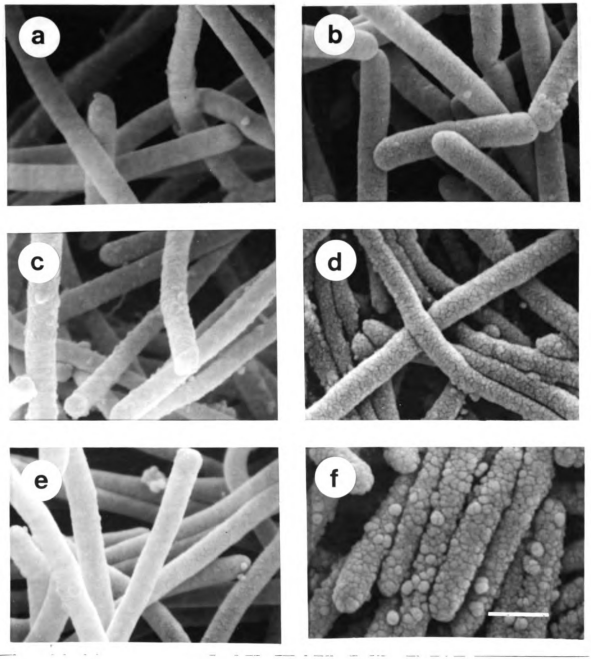
Cells were cultured on the different substrates and incubated in the presence of xylan for one hour and then examined. Scale bar represents 30 μm.


A
B
C

B6A-RI grown on glucose, xylose or xylan (Fig. 2). Glucose grown cultures treated with cationized ferritin appeared similar to non treated cells, with the cell surface remaining smooth in appearance (Fig. 2A & B). This differed from cultures grown on xylose or xylan. Treatment of these cells of *Thermoanaerobacter* strain B6A-RI with cationized ferritin revealed the presence of numerous protruberances covering the cell surface (Fig. 2D & F).

To investigate these cell surface structures in more detail, transmission electron microscopy was performed on cells grown on xylan. This study revealed the presence of distinct particulate structures surrounding the cell that appear as differentiation of the outer wall S-layer to which cationized ferritin bound (Fig. 3). These structures varied in size, being either closely associated with the cell, stretching out away from the cell or appearing as a matrix linking the cells together.

Figure 2. Scanning electron microscopy of gold labelled cells of *Thermoanaerobacter* strain B6A-RI grown on glucose (a, b), xylose (c, d), and xylan (e, f). Figures a, c, and e are of cells in the absence of cationized ferritin, and figures b, d, and f, are cells treated with cationized ferritin (d). Scale bar represents 20μm.


a
b
c
d
e
f

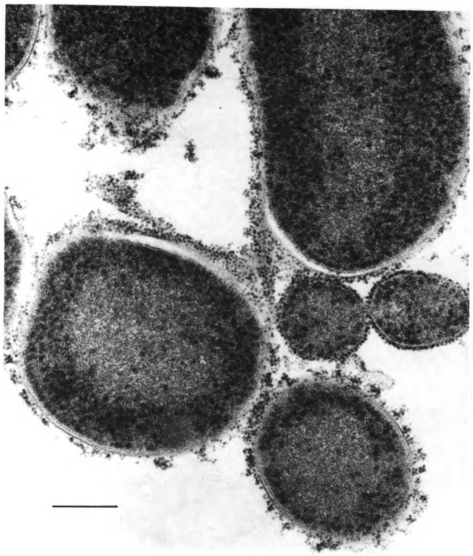


Figure 3. Transmission electron microscopy of cells of *Thermoanaerobacter* strain B6A-RI grown on xylan and treated with gold and cationized ferritin.

Scale bar represents 0.2 μm.

DISCUSSION

This report provides the first ultrastructural study of the cell surface components of a hemicellulolytic bacterium, and demonstrates the presence of cell surface structures which appear to be involved in cellular adhesion to xylan and coincide with the production of cell bound endoxylanases necessary for substrate hydrolysis. This provides evidence for a xylanosome that is induced by xylan and which maybe be analogous to cellulosome demonstrated in cellulolytic anaerobes.

In *Clostridium thermocellum* a protein complex, termed the cellulosome, containing enzymes necessary for the degradation of cellulose has been demonstrated. This complex exists in cell surface bound and cell-free forms, and has been shown to be responsible for cellular adherence to cellulose and for the degradation of cellulose to cellobiose by the intact organism (1, 14, 15). The cellulosome constitutes the majority of the endoglucanase activity (~70%) and about one third of the total extracellular protein, and possesses the major proteins so far reported for the entire cellulolytic apparatus in this organism (12). Recently, xylanase activity has also been found to be localized in the cellulosome (19).

The ultrastructure of cellulosomes located on the surfaces of *C. thermocellum* have been studied by transmission electron microscopy using specific labels (3), immunocytochemical techniques (1), and by scanning electron microscopy using cationized ferritin (13).

Many anaerobic cellulolytic bacteria possess high molecular weight, multisubunit cellulases which are often found associated with the cell surface (28) or sedimentable membranous fragments (10). *Bacteroides succinogenes* produces CMCase, β-glucosidase, xylanase and β-xylosidase

during growth on cellulose, and approximately 50% of each of the released enzymes was associated with sedimentable subcellular membrane vesicles which adhered to cellulose (7). In these cellulolytic anaerobes enzyme complexes analogous to the cellulosome may be present.

In *Thermoanaerobacter* strain B6A-RI, the finding of predominantly cell bound endoxylanase and other xylanolytic activity, a strong affinity of the cells for the substrate and the presence of cell surface protuberances suggests the presence of a xylanosome which functions similar to the cellulosome in hydrolysis of insoluble polymer. Moreover the xylanosome appears to be specific for cellular adhesion to xylan, as cells of *Thermoanaerobacter* strain B6A-RI would not bind to cellulose. These findings mark a distinct difference between *Thermoanaerobacter* strain B6A-RI and *Thermoanaerobacter* strain B6A, as the former organism binds very tightly to xylan whereas there was a lack of extensive attachment of the latter organism to hemicellulose substrates (23).

There existed some similarities between the cell surface complex in *Thermoanaerobacter* B6A-RI and the cellulosome in *C. thermocellum*. The cell surface structures as visualized using SEM were very similar in size and abundance when compared to cells of *C. thermocellum* LQRI but differed from other strains of *C. thermocellum* (13). During the same study Lamed et al. (13) demonstrated the presence of similar structures on cells of anumber of bacteria, including *Acetovibrio cellulolyticus, Bacteroides cellulovorans, Clostridium cellulovorans* and *Clostridium cellobioparum*. In all of the mesophilic, anaerobic, cellulolytic bacteria, cell surface structures similar but not identical to those found on *Thermoanaerobacter* B6A-RI were found, thus, it would seem that the exact topology of these structures varies between organisms and strains.

Transmission electron microscopy of the cationized ferritin labeled cells of C. *thermocellum* YS showed labeling over the entire cell surface, fibrous structures which sometimes connected adjacent cells, and distinct evenly shaped protuberances (1). Cells of *Thermoanaerobacter* B6A-RI grown on xylan had a similar ultrastructure, and the less defined and more diffuse nature of the cell surface protruberances may be due to differences between the two organisms, and unrelated to cellular function.

Most Eubacteria and Archaebacteria possess layered assemblies of polymers and heteropolymers external to the plasma membrane. The upper most layer is referred to as the S-layer and is composed of protein or glycoprotein (22). The finding that endoxylanase from *Thermoanaerobacter* B6A-RI was glycosylated and associated with the cell surface, and the presence of a thin dark layer on the surface of cells of *Thermoanaerobacter* B6A-RI (as visualized with transmission electron microscopy), suggests that the xylanolytic complex may be part of the S-layer. This is further substantiated by a recent study in which a novel linkage of O-linked carbohydrates was found in the crystalline surface layer glycoprotein of *Clostridium thermohydrosulfuricum* (18). A similar type of linkage was found in the glycan chains of the cellulosome of C. thermocellum (8), further substantiating the interrelationship between the S-layer and catalytic cell surface complexes.

From this study we propose that the majority of the xylanolytic activity is associated with the cell, and that *Thermoanaerobacter* strain B6A-RI binds xylan on to the cell surface to facilitate the concerted action of xylanasesand to enhance the transfer of the degradation products into the organism. The genes encoding the major endoxylanase and β-xylosidase activities in *Thermoanaerobacter* strain B6A-RI have been cloned (Lee & Zeikus,

manuscripts in preparation), and the endoxylanase gene contains a leader sequence whereas the β-xylosidase gene does not, confirming the findings from our previous study of endoxylanase activity being cell bound to the surface or excreted and β-xylosidase being almost completely intracellular.

These findings extend the cell surface structures known to exist on the bacterial cell, and provide the first example of a cell surface structure specific for cellular adhesion to and degradation of xylan. Such structures further demonstrate the ability of microorganisms to effectively form a hydrolytic complex of enzymes to efficiently degrade insoluble substrates.

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Chapter IX

Conclusions and recommendations for future research

Until recently only one thermoanaerobic strain, *Thermoaerobacter* strain B6A, that actively grows on xylan has been reported. Our group has isolated several xylanolytic strains from thermal spring ecosystems in Yellowstone National Park. New isolates *Thermoanaerobacter* strain B6A-RI and LX-11 expressed high xylanase activities and grew readily on xylan.

In order to define the genetic relationship among xylanolytic thermoanaerobes and to facilitate the establishment of species names, we have compared DNA homologies of these strains by DNA-DNA hybridization. This technique has been useful for taxonomic studies of other species and a variation of this procedure that uses free-solution hybridization and S1 nuclease treatment of the hybrids was employed here. The results indicate the existence of three groups among the xylanolytic thermoanaerobes known to date: Group I includes one species *Clostridium thermocellum* since this organism exhibits less than 14% homology to other species studied; Group II is composed of *Thermoanaerobacter* strains B6A-RI, LX-11 and *Clostridium thermosulfurogenes* 4B that showed more than 40% of homology among themselves; Group III comprised of *Clostridium thermohydrosulfuricum* 39E and E100-69, *Thermoanaerobium brockii* HTD4 and *Thermoanaerobacter ethanolicus* JW200 as they exhibited homologies between each other's DNA ranging from 57% to 97%.

From these findings three different groups emerged and new taxonomic assignments were proposed. *C. thermocellum* LQRI was placed in group I retaining its original taxonomic assignment without change. *C. thermosulfurogenes* strain 4B and the new isolates B6A-RI and LX-11 are closely related and fell into group II for which a genus name *Thermoanaerobacterium* gen. nov. was given. Group III was termed

Thermoanaerobacter and includes *T. ethanolicus* strain JW200, *C. thermohydrosulfuricum* strain 39E and strain E100-69, and *Thermoanaerobium brockii* strain HTD4. *Thermoanaerobacter* B6A-RI was selected as a model organism for further studies, because of its high xylanolytic activity and lack of cellulases.

During growth on xylan and xylose *Thermoanaerobacter* strain B6A-RI produced endoxylanase, β-xylosidase, arabinofuranosidase and acetylesterase and these activities appeared to be produced coordinately. Under nonlimiting xylan conditions these enzyme activities were predominantly cell associated, however, during growth on limiting concentrations of xylan the majority of endoxylanase activity was extracellular rather than cell associated. Endoxylanase, β-xylosidase and arabinofuranosidase activities were induced by xylan, xylose and arabinose respectively. Acetylesterase activity was constitutive and endoxylanase activity was catabolite repressed by glucose. For purification of endoxylanases an affinity chromatography method was developed by coupling of oat spelt xylan to Sepharose CL-4B and the enzymes bound at >45°C and were eluted using soluble xylan. Activity staining of SDS-PAGE indicated that *Thermoanaerobacter* B6A-RI possesses multiple forms of endoxylanse. At present it is not clear whether they represent the products of different genes, multiple molecular forms of the same subunit polypeptides, or forms with different degrees of post-translational modification such as glycosylation or proteolysis. Evidence for these possibilities can be obtained using antibodies prepared from the cloned endoxylanase, by deglycosylation studies or by cloning more endoxylanase genes.

To isolate the genes involved in xylan degradation and to study their genetic organization in *Thermoanaerobacter* B6A-RI, a genomic library was

constructed using a cosmid vector pHC79 and a cosmid clone coding for endoxylanase and β-xylosidase activities was isolated. A 28 kilobase fragment of *Thermoanaerobacter* genomic DNA insert was subjected to a series of subclonings to identify the location of the genes. The DNA sequence of this endoxylanase was determined using the dideoxy sequencing method. One open reading frame (ORF) of 3471 bp which encodes a xylanase of 1157 amino acid residues (Mr 130 kDa) was found. The distribution of endoxylanase activity was approximately equal between the periplasm and cytoplasm in a subclone containing the endoxylanase gene. This finding suggests that the signal peptide is recognized and the protein is processed by *Escherichia coli* cells. The N-terminus of the processed enzyme in *E. coli* was determined by amino acid sequencing and a putative 33 amino acid signal peptide was found from the deduced amino acid sequence. The cloned endoxylanase was purified and its physico-chemical properties were determined. The pH optimum was 5.5 and the optimum temperature was 70°C. The cloned endoxylanase hydrolyzed xylan to mainly xylobiose and xylotriose, and xylobiose was not cleaved.

The location of a gene encoding β-xylosidase activity was identified in a cosmid clone and subsequently subcloned into pUC18. The complete nucleotide sequence of the β-xylosidase gene and its flanking regions were established. A 1500-bp open reading frame for β-xylosidase and another open reading frame of unknown function were observed. The amino acid sequence of the N-terminal region and the molecular weight (55 kDa) of the β–xylosidase, deduced from the DNA sequence, agreed with the result obtained for the purified enzyme. The endoxylanase gene of the same strain was 12 kbp downstream of the 3' end of the β–xylosidase gene and they had the same orientation. The cloned β–xylosidase was purified using heat

treatment, ion-exchange and gel-filtration chromatography. The pH optimum was 5.5, and the optimum temperature was 65°C. The cloned β-xylosidase cleaved both xylotriose and xylobiose to xylose and also had transferase activity. No activity was found on xylan.

Xylose isomerase activity is responsible for the conversion of xylose to xylulose prior to xylolysis. The gene encoding for xylose isomerase was cloned and its nucleotide sequence determined. The amino acid sequence deduced from the coding sequence of the gene exhibited considerable homology to the sequences of other xylose isomerases studied to date including *C. thermosulfurogenes* strain 4B, *C. thermohydrosulfuricum* strain 39E, *E. coli* and *B. subtilis*. The xylose isomerases from three thermoanaerobes, *Thermoanaerobacter* strain B6A-RI, *C. thermosulfurogenes* strain 4B and *C. thermohydrosulfuricum* strain 39E, shared a higher homology among themselves than with *E. coli* or *Bacillus subtilis*. Codon usage analysis of three genes from *Thermoanaerobacter* strain B6A-RI showed a bias for A or T for the third base, and is reflected by the low G+C mol% content of 36 found in this organism.

In order to identify the putative catalytic sites on the endoxylanase gene more precisely, multiple sequence analysis was performed using sequence data available through the Wisconsin-GCG Sequence analysis program. There were very significant homologies between endoxylanase from *Thermoanaerobacter* strain B6A-RI and the family F β-glycanases. This grouping of β–glycanases in family F is based on the hydrophobic cluster analysis program, which was used to confirm that endoxylanase from *Thermoanaerobacter* strain B6A-RI should be placed in this family.

The catalytic domains in endoxylanase from *Thermoanaerobacter* B6A-RI were investigated. The catalytic mechanism of endoxylanase has been

modeled after the acid-base catalytic mechanism proposed for lysozyme. Chemical modification of the endoxylanase from *Thermoanaerobacter* strain B6A-RI with group specific reagents indicated that either aspartate or glutamate was involved in catalysis. Based on sequence alignment, hydrophobic cluster analysis and chemical modification, 6 amino acids were chosen and mutated by site-specific mutagenesis. Changing Glu-567 to Gln and Asp-504 and Asp-569 to Asn resulted in a dramatic loss of xylanase activity, suggesting that endoxylanase from B6A-RI, like other hydrolytic enzymes, could act by general acid catalysis involving carboxylic residues.

Ultrstructural studies were performed to characterize cell topological features involved in xylan adhesion and hydrolysis. It was observed that in the exponential phase of growth *Thermoanaerobacter* B6A-RI cells grown on xylan or xylose would bind tightly to particles of insoluble xylan, whereas the cells grown on glucose remained unattached. Negatively charged surface structures were visualized on cells of *Thermoanaerobacter* strain B6A-RI grown on xylan, using cationized ferritin and gold labelling with scanning and transmission electron microscopy. These structures were absent on cells grown on glucose. The presence of these structures (i.e. xylanosome) coincides with production of cell associated endoxylanases and the ability of the cells to bind tightly to xylan.

It has been shown that cellulolytic enzymes of *C. thermocellum* were organized into distinct multi-subunit complexes called cellulosomes. The xylanosomes of *Thermoanaerobacter* strain B6A-RI may represent a putative cell surface enzyme complex enabling rapid hydrolysis and absorption of the xylan degrading products by the cell. Using antibodies prepared from the purified cloned enzymes, endoxylanase, β-xylosidase and xylose isomerase, immunogold labelling experiments will be performed to demonstrate their

cellular location and possible juxtapositio of different xylan degrading enzymes.

The following studies should be continued to reveal the molecular nature of *Thermoanaerobacter* xylanase system: (1) isolate and identify the individual components which exist in the xylanosome; (2) establish the role of the xylanosome components, and the basis for organization; (3) elucidate the synergistic interactions between xylanolytic enzymes, and their potential juxtapositions in the xylanosome; (4) isolate and characterize the putative xylan-binding factor; (5) elucidate the mechanism for thermophilicity in the endoxylanase; (6) chemically characterize the glycoconjugate of rndoxylanase; (7) characterize the S-layer relationship to xylanosome components. Finally, it would be worth continuing studies on establishing process uses for thermophilic xylanases in the manufacturing of foods, feeds, paper and fibers.