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DESCRIPTION OF A NEW SPECIES OF *CYTOPHAGA*

AND

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By

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ABSTRACT

DESCRIPTION OF A NEW SPECIES OF *CYTOPHAGA* AND CHARACTERIZATION OF ITS XYLAN-DEGRADING ENZYME SYSTEM

By

Sheridan Kidd Haack

Xylans are a class of cell wall polysaccharide widely distributed among both terrestrial and aquatic plants; however, our understanding of the microbiology and biochemistry of xylan degradation, especially in anaerobic habitats, is rather limited. Research described herein investigated the taxonomy and physiology of a new species of anaerobic bacterium in the genus *Cytophaga* and characterized the xylan-degrading enzyme system of the new isolate.

Isolates of gliding bacteria that attached in masses to, and dominated the fermentation of, xylan powder in methanogenic and sulfidogenic enrichments from freshwater sediments were all Gram negative, slender rods that formed no endospores, microcysts or fruiting bodies. Representative strain XM3 was a mesophilic, aeroduric anaerobe that grew by fermentation of mono-, di-, or polysaccharides (but not cellulose) in a mineral medium producing acetate, succinate, propionate, CO_2, and H_2. Strain XM3 possessed sulphonolipids and carotenoid, but not flexirubin, pigments. Its total cellular fatty acids were dominated by $C_{15:0}$ *anteiso* (75%), *n* (13%), and *iso* (2%) isomers. Strain XM3 had 45.5 mol% G + C in its DNA, and partial sequencing of its 16S rRNA placed XM3 within the *Bacteroides-Flavobacterium* phylogenetic group. Strain XM3 was proposed as the type strain of the new species *Cytophaga xylanolytica*.

Similar strains were also isolated from marine sediments.

The xylanase activity of *Cytophaga xylanolytica* strain XM3 was mainly (90-95%) cell-associated; the remainder was secreted into the culture fluid. Isoelectric focusing of Triton extracts of whole cells, followed by activity stains, suggested that xylose- and xylan-grown cells had common endoxylanase (pI 4.3), arabinofuranosidase (pI 5.85), and xylosidase (pI 4.6) (iso)enzymes. However, xylan-grown cells produced additional xylanase activity bands. Supernatants of both xylose- or xylan-grown cultures contained a similar arabinofuranosidase, but neither supernatant contained a xylosidase. Both supernatants also exhibited xylanase activity bands not found in the Triton extracts. Xylanase was fully induced only when cells were grown on xylan; however, cells retained low, constitutive levels of xylanase when grown on monosaccharide components of xylan, or on the glucose polymer lichenan.

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INTRODUCTION

Next to cellulose, hemicelluloses are the most abundant polysaccharides in nature (Wilkie 1983). Hemicelluloses occur in the primary (growing) and secondary (mature) cell walls of all terrestrial plants, as well as in the cell walls of algae and aquatic plants (Wilkie 1983, Wong et al. 1988). The structure of plant primary cell walls has been the subject of several recent reviews (Lamport 1986, Lamport and Catt 1981, McNeil et al. 1984, Wilson and Fry 1986). Up to 90% of the primary cell wall is polysaccharide variously substituted with acetyl esters and methyl and feruloyl esters and ethers (McNeil et al. 1984). Lignin is not a component of primary cell walls, but phenolic acids are common constituents of Gramineae (grasses) primary cell walls (Chesson et al. 1982). Approximately 10% of the primary plant cell wall is protein, which is comprised of hydroxyproline-rich, arabinose-substituted extensin, arabinogalactan proteins (the biological function of these two types of protein is unknown), and other proteins (frequently glycosylated) including malate dehydrogenase, peroxidase, phosphatases, glycosyl hydrolases and transferases, and endoglucanases (McNeil et al. 1984). In the current model, cellulose (β-1,4 linked D-glucan; 11-24% of the polysaccharide of the cell wall) fibrils are woven into an extensin matrix. The extensin helices are cross linked by isodityrosine dimers

resulting in an extremely insoluble protein mesh. The cellulose fibrils are "sheathed" by hemicellulose polymers. In dicots, the hemicellulose is primarily (20% of cell wall) xyloglucan, a β-1,4-linked D-glucose with some β-1,6-D-xylose side chains. In monocots, it is primarily (35% of cell wall) xylan, which is described below. The hemicelluloses are hydrogen bonded to the cellulose fibrils in a manner which varies with the degree of substitution of the hemicellulose backbone. Pectins (homo- and hetero- galacturonans and arabinans) form an additional meshwork around the other molecules, possibly as an amorphous matrix. Pectins comprise 34%, 10%, and 22% of the primary cell walls of dicots, monocots or softwoods (Douglas fir), respectively (Thomas et al. 1987). Much less is known about the structure of secondary plant walls due to their highly lignified nature, but xylans are abundant in woody tissue and comprise the major hemicellulose in wood from angiosperms, as well as a substantial portion (7-12% of dry weight) of the wood from gymnosperms (Timmell 1962, Whistler and Richards 1970).

Xylans comprise a class of hemicellulose widely distributed among both terrestrial and aquatic plants. They are complex heteropolymers typically consisting of a β-1,4-linked xylopyranose backbone variously substituted with L-arabinofuranose, 4-*O*-methyl-D-glucuronic acid, and *O*-acetyl side groups. The arabinose substituents are typically linked to C-3 of the xylose residues while the acetic acid and methyl-glucuronic acid residues are usually linked to C-2. The nature and degree of substitution varies with the stage of plant growth as well as within different tissues of the same plant (Wong et

al. 1988, Wilkie 1983). Typical chemical procedures used to isolate xylans from plant material employ alkaline hydrolysis which may alter acidic constituents or hydrolyze ester linkages, leading to a loss of characteristic *in situ* features of the xylan, such as the acetyl and feruloyl linkages described above (Wilkie 1983).

The use of enzymes from plant-polymer degrading fungi and bacteria has been extremely useful in elucidating the structures of plant cell wall polysaccharides. The xylans of maize primary cell walls have been particularly well studied by both enzymatic and chemical methods and have been shown to exhibit several of the properties of the hypothetical xylan (Kato and Nevins 1984 a-d, 1985, Nishitani and Nevins 1988, 1989, 1990, 1991). Kato and Nevins (1984a) found that the predominant noncellulose polysaccharide of a water insoluble fraction of *Zea mays* shoot cell walls is an arabinoxylan. Treatment of the water insoluble fraction with an endo-(1,4)-β-xylanase purified from a commercial preparation of *Bacillus subtilis* α-amylase resulted in glucuronoarabinoxylan (GAX) fragments having a β-1,4-D-xylopyranose backbone with 60-70% substitution at C-2 or C-3 with arabinose, glucuronic acid or other substituents (1984d). The GAX further contained ferulic acid, later shown to be bound to the GAX as *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1-3)-*O*-β-D-xylopyranosyl-(1-4)-D-xylopyranose (1985). The *Bacillus subtilis* enzyme released only 20% of the total GAX from the cell wall preparations (1984d). A second *Bacillus subtilus* enzyme degraded glucuronic acid substituted xylans of both bean (*Vigna*) and *Zea mays* cell walls to characteristic α-1,2-glucuronic acid substituted xylosyl oligomers (Nishitani and

Nevins 1991). This constitutes the first evidence of a repeating unit structure in any xylan.

Non-enzymatic analyses have also been useful in elucidating the structure of xylans in the maize cell wall. Carpita (1983) extracted hemicellulose from *Zea mays* coleoptile cell walls by using alkali gradients and showed that hemicellulosic polymers comprised 43% of these PCW's. Three separate fractions [GAX I, GAX II and MG-GAX (a mixture of 60% mixed-linked glucans and 40% GAX)] comprised 20, 30 and 50% respectively of the total extracted hemicellulose. GAX I (extracted wth 0.01-0.045 N KOH) was highly substituted; 6 of every 7 xylose residues carried a terminal arabinose (t-ara) or an α-1,2-linked glucuronic acid. The t-ara substituents were most common although some galactose residues comprised a very small proportion of substituents. GAX II (0.45-0.8 N KOH) was substituted on 2 of every 3 xylose residues, with similar substituents to GAX I. GAX I may be a precursor of other GAX in the primary cell wall (Carpita and Whittern 1986). Carpita (1986) also examined ferulic acid in maize cell walls and found aromatic material (including aromatic amino acids) comprising only 0.3-1.2% of 2-4 day coleoptiles. Most of the radioactivity from incorporated ^{14}C-phenylalanine or ^{14}C-tyrosine was incorporated into the hemicellulose fraction. Based on differential extraction Carpita (1986) proposed etherified as well as esterified aromatics as substituents of hemicelluloses in *Zea mays* (see also Scalbert et al. 1985). He suggested, based on ^{14}C-proline incorporation, that hydroxyproline-rich proteins comprise only a small percentage of maize cell walls. Finally, he demonstrated that ^{14}C

labelled glucose or xylose are metabolized to hexoses before being incorporated into the cell wall polymers, but over 80% of ^{14}C-arabinose is incorporated directly into non-cellulosic polymers (Carpita et al. 1982). No analysis to date has been made of the distribution of acetyl groups in maize hemicelluloses; however, Bacon et al. (1975) showed *O*-acetyl groups to be common in grasses.

Xylan degrading enzymes.

From the foregoing discussion of the nature of plant cell walls it is obvious that one important characteristic of a xylan-degrading microorganism would be possession of the requisite depolymerizing enzymes. The suite of enzymes commonly thought to be necessary for the complete degradation of a xylan include endoxylanase (E.C. 3.2.1.8) to degrade the xylan backbone, xylobiase (E.C. 3.2.1.37) to cleave terminal xylose residues from xylooligomers produced by endoxylanase activity, and arabinofuranosidase (E.C. 3.2.1.55) to remove arabinosyl side groups. Reviews on the nature and sources of xylanases, xylosidases, and arabinofuranosidases have appeared previously (Biely 1985, Dekker and Richards 1975, Kaji 1984, Reilly 1981, Wong et al. 1988). As our concept of the structure of xylan *in situ* has been refined, it has become apparent that additional enzymes would be needed to remove methyl-glucuronic, as well as acetyl and feruloyl substituents. The importance of acetyl or acetyl-xylan esterases (Biely et al. 1986, Wood and McCrae 1986, Hespell and O'Bryan-Shah 1988), α-4-*O*-Me-D-glucuronidase (Puls et al. 1987, Johnson et al. 1989) and ferulic acid esterase (MacKenzie and Bilous 1988, Borneman et al. 1990) has only recently been recognized. Acetyl

esterases and glucuronidases have been shown to be synergistic with xylanases in the degradation of isolated xylans, but little information on the nature and mode of action of these enzymes exists (Biely et al. 1986, Puls et al. 1987). The lack of a suitable substrate for in vitro tests of enzyme activity has hampered the study of both the glucuronidases and the ferulic acid esterases.

Theoretically, the enzymes described above should be sufficient to fully degrade a hypothetical xylan, and should constitute the basic components of a xylan-degrading enzyme system. However, the picture is complicated by the evidence that some xylanases exhibit a preference for, or may be specialized to degrade, xylans with specific substituents (Dekker and Richards 1975, Nishitani and Nevins 1991, Reilly 1981, Wong et al. 1988). In many (most) cases, a single microorganism possesses multiple endoxylanases which differ in substrate specificity, mode of action, or properties such as isoelectric point and molecular weight (Wong et al. 1988). It is now clear that many bacteria possess multiple xylanase genes (Flint et al. 1989, 1990, Gilbert et al. 1988, Hamamoto et al. 1987, Hazlewood et al. 1988, Honda et al. 1985, Kluepfel et al. 1990, MacKenzie et al. 1989, Sakka et al. 1989, 1990, Schwarz et al. 1990, Vats-Mehta et al. 1990, Yang et al. 1989). Wong et al. (1988) observed that many fungi and bacteria produce two forms of endoxylanase: a high molecular weight/acidic form and a low molecular weight/basic form. These authors also reviewed evidence from their own work with *Trichoderma harzianum*, as well as from the work of Takenishi and Tsujisaka (1975) on *Aspergillus*, that multiple endoxylanases from a single

microorganism can cooperate in the degradation of xylan. However, in both examples, the existence and degree of cooperativity of the different endoxylanases was dependent on the nature of the test substrate (Wong et al. 1988). Finally, many endoxylanases exhibit substrate cross-specificity (Wong et al. 1988) degrading, for example, carboxymethylcellulose or lichenan. Conversely, many endoglucanases have some activity on xylan. It is clear that xylan-degrading enzyme systems can be extremely complex; however, as pointed out by Wong et al. (1988) biotechnological applications of isolated enzymes, enzyme combinations or whole organisms will require an understanding of this complexity.

Described bacterial xylan-degrading enzyme systems

Xylan-degrading enzymes have been purified, or cloned (or both) from several genera of bacteria (Table 1). In no case have all the relevant enzymes been studied in a single bacterial genus. Often, research on xylan-degrading enzymes has followed naturally from previous purification or cloning of the cellulose-degrading enzymes of a particular microorganism (e.g., *Clostridium thermocellum*). Other studies have employed combinations of denaturing and non-denaturing gel electrophoresis, isoelectric focusing, and activity stains (e.g. Bachmann and McCarthy 1991) to give an overview of the number and nature of the enzymes which comprise the xylan-degrading enzyme system of a previously unstudied microorganism. This review extends the information on bacterial xylan-degrading enzyme systems provided by Wong et al. (1988), and specifically focuses on those bacterial genera for which there is substantial information on one or more of the

enzymes involved in xylan degradation. In the sections below, aspects of the xylan-degrading enzyme systems of these focal genera are discussed in greater detail.

Table 1. Bacterial xylan-degrading enzyme systems.

Organism	Enzyme	Activity	MW	pI	Reference
Aeromonas					
caviae W-61	xyn (P)	Xn->X2,>X2;transxyl	22	9.2	Viet et al. 1991
sp. no. 212	xyn L (P,C)	Xn-> X2, >X2	145	ND	Kudo et al. 1985
	xyn M (P)	+	37	ND	1985, Ohkoshi et
	xyn S (P)	+	23	ND	al. 1985
Bacillus					
sp. C-125	xyn A (P,C)	Xn->X2,>X2;transXyl	43	ND	Honda et al. 1985a,b; Hamamoto
	xyn N (P)	Xn->X2,>X2;transXyl	16	ND	et al. 1987
circulans WL-12	xyn A (P)	Xn-> X1 - X4	85	4.5	Esteban et al.
	xyn B (P)	Xn-> X2 - X4	15	9.1	1982
	xyl (P)	Xyl	85	4.7	
circulans NRC 9024	xyn 1 (C)	+	22	9.0	Yang et al. 1989
	xyn 2 (C)	+	20.5	9.0	
	xyn 3 (C)	+	59	9.0	
pumilus IPO	xyn (P,C)	+	22.5	ND	Panbangred et al.
	xyl I (P,C)	Xyl	130	ND	1983a,b, 1984;
	xyl II (C)	(PNPX,ONPG,PNPG)	130	ND	Moriyama et al. 1987
pumilus 12	xyl (P)		ND	4.4	Kersters-Hilderson et al. 1969

Table 1. (cont'd.)

Organism	Enzyme	Activity	MW	pI	Reference
Bacillus					
subtilus PAP115	xyn (P)	Xn->X, X2, X3	32	ND	Bernier et al.
	xyn (C)	+	22	8.9	1983a,b; Paice et al. 1986
subtilus	xyn (P)	+	9.5	ND	Kato and Nevins 1984
	xyn (P)	GlcAXn->GlcAX6, GlcAX1	44	ND	Nishitani and Nevins 1991
subtilus F-11	araf (P)	araf	65	5.3	Weinstein and Albersheim 1979
Bacteroides					
ovatus	xyn (C)	+	ND	ND	Whitehead and
	xyl/araf (C)	xyl/araf	38	ND	Hespell 1990a
ruminicola	xyn (C)	Xn-> X3	60	ND	Whitehead and Hespell 1990
Butyrvibrio					
fibrisolvens 49	xyn A (C)	+	42.9	8.6	Mannarelli et al. 1990
fibrisolvens H17c	xyn B (C)	(Xn, PNPC)	73	ND	Lin and Thomson 1991
fibrisolvens GS113	xyl B (C)	xyl/araf	60	ND	Utt et al. 1991

Table 1. (cont'd.)

Organism	Enzyme	Activity	MW	pI	Reference
Clostridium					
acetobutylicum ATCC 824	xyn A (P)	Xn-> X2 - X6	65	4.45	Lee et al. 1987,
	xyn B (P,C)	Xn-> X2, X3	29	8.50	Lee and Forsberg 1987
P262	xyl (P)	PNPX, X2-X6->X1	224	5.85	Zappe et al. 1990
stercorarium	xyn A (P)	Xn-> X2, X3	44	4.53	Berenger et al.
	xyn B (P)	Xn-> X2, X3	72	4.43	1985
	xyn C (P)	Xn-> X2, X3	62	4.39	
stercorarium NCIB 11745	xyn A (C)	+	42,62,70	ND	Schwarz et al.
	bxyl A (C)	xyl	ND	ND	1990
	bxyl B (C)	xyl/araf	85	ND	
	arf A (C)	xyl/araf	49	ND	
	arf B (C)	araf	30	ND	
	cel W (C)	(Xn, CMC, PNPC)	42	ND	
	cel X (C)	(Xn, CMC, PNPC)	120, 80	ND	
thermocellum	xyn Z (P,C)	(Xn, MUC, PNPC)	90	ND	Grepinet et al. 1988a,b
	cel E (C)	(CMC, Xn)	90	ND	Hall et al. 1988
	xyn 1 (C)	+	ND	several	MacKenzie et al.
	xyn 2 (C)	+	25	several	1989
	xyn 3 (C)	+	ND	several	

Table 1. (cont'd.)

Organism	Enzyme	Activity	MW	pI	Reference
Clostridium thermocellum	Cellulosomal	+	60	4.5	Morag et al. 1990
	S2	(Xn, CMC)	170	ND	
	S7	(Xn, MUC)	84	ND	
	S9/10	(Xn, MUC)	67	ND	
	S13	(Xn, CMC)	54	ND	
	S3	(Xn, MUC)	150	ND	
	S5 (=xynZ?)	(Xn, MUC)	98	ND	
	S11	+	60	ND	
	Non-Cellulosomal				
	1	Xn only	65	ND	
	2	Xn only	47	ND	
	3	Xn only	45	ND	
	4	Xn only	40	ND	
	5	Xn only	30	ND	
	Cell-associated				
	xyl		200	ND	
Fibrobacter succinogenes S85	xyn (C)	+	ND	ND	Sipat et al. 1987
	xyn 1 (P)	AXn->A,X3,X4;no araf	53.7	>1,basic	Matte and
	xyn 2 (P)	AXn->X,X2,X3,X4 (CMC)	66	>1,basic	Forsberg 1992

Table 1. (cont'd.)

Organism	Enzyme	Activity	MW	pI	Reference
Pseudomonas fluorescens					
subsp. *cellulosa*	xyn A	+	60	4.5	Hall et al. 1989
	xyn B	AXn->X, X2	61	ND	Kellett et al.
	xyn C	AXn->A;no araf	59	ND	1990, Gilbert et al. 1988
Ruminococcus					
albus 8	xyn 1	+	720	3.8	Greve et al.
	xyn 2	+	260	4.3	1984b
	araf	araf	~305	~4.0	Greve et al. 1984a
flavefaciens FD-1	xyn A (C)	Xn->X,X2;PNPX,PNPC	30	ND	Flint et al. 1991
	xyn B (C)	Xn-> X2,>X2	20-30	ND	
Streptomyces					
lividans 66	xyn A (P,C)	Xn oligo->X,X2	43	5.2	Morosoli et al.
	xyn B (P,C)	Xn-> X5 - X8	31	8.4	1986, Mondou et al. 1986, Kluepfel et al. 1990, Vats-Mehta et al. 1990
roseiscleroticus	xyn (P)	AXn->A, X2, X3	22.6	9.5	Grabski and Jeffries 1991
thermoviolaceus OPC-50	xyn I (P)	Xn-> X, X2	54	4.2	Tsujibo et al.
	xyn II (P)	Xn-> X, X2	33	8.0	1992

Table 1. (cont'd.)

Organism	Enzyme	Activity	MW	pI	Reference
Thermomonospora					
fusca BD21	xyn	+	32	7.9	Bachmann and
	xyn	+	32	8.2	McCarthy 1991
	xyn	+	32	8.6	
	xyn	+	24	>1, acidic	
	araf (P)		92	ND	
	ACE (P)	also AC-Xn	80	ND	
	xyl (P)		168	4.37	Bachmann and McCarthy 1989
fusca YX	xyn (C)	+	30	ND	Ghangas et al. 1985

Enzyme designations: xyn, endoxylanase; xyl, -xylosidase; araf, -L-arabinofuranosidase; cel, endoglucanase; ACE, acetyl esterase; (P), purified; (C), cloned.

Activity: Xn, xylan; Axn, arabinoxylan; GXn, glucuronoxylan; AC-Xn, acetyl xylan; X2, xylobiose; X3, xylotriose; transxyl,transxylosidase; ( ), additional activities noted: PNPX, p-nitrophenyl-xylosidase; PNPG, p-nitrophenyl-glucosidase; PNPC, p-nitrophenyl-cellobiosidase; CMC, carboxymethylcellulase; MUC, 4-methylumbelliferyl-cellobiosidase.

MW: molecular weight (kD); ND, not determined

pI: isoelectric point

Aeromonas. The genus *Aeromonas* includes Gram negative, facultatively anaerobic bacteria related to the vibrios. The species in Table 1 were isolated in one case on the basis of alkalophilic growth as well as xylan hydrolysis (sp. no. 212), and in the other case simply on the ability to degrade xylan. All the studied enzymes were produced extracellularly but there is no indication that cell-associated enzymes, if present, were investigated. The xylanase of *A. caviae* was induced by xylan and not by xylo-oligosaccharides or cellulose. This enzyme possessed transxylosidase activity, which has been noted in several other xylanases (Table 1, Reilly 1981, Biely 1985, Wong et al. 1988). The xylanase L gene, cloned from sp. no. 212, hybridized with only one genomic fragment from sp. no. 212, suggesting that xylanases S and M from this species were encoded by separate genes.

Bacillus. Several species in the Gram positive, endospore-forming genus *Bacillus* have been investigated with respect to xylan-degradation. All the investigated xylanases have been produced extracellularly, and as was the case for *Aeromonas*, there is no indication of whether these bacteria produce cell-associated xylanases. The xylosidase of *B. circulans* WL-12 was produced extracellularly while those of *B. pumilus* 12 and IPO were located intracellularly. This is the only genus in which two xylosidase genes have been identified (*B. pumilus* IPO, Table 1). In *B. pumilus* IPO, the xylanase gene and xylosidase I gene were encoded on the same genomic restriction fragment (Panbangred et al. 1983a,b). A *B. subtilus* PAP115 22 kD xylanase exhibited 50% amino acid homology with the *B. pumilus* IPO xylanase, but no identity with a 32 kD xylanase

previously cloned from the same organism (Paice et al. 1986). While several *Bacillus* species are recognized to produce a number of polysaccharide-degrading enzymes other than xylanases (Esteban et al. 1982, Nishitani and Nevins 1991), there have been no comprehensive studies, in one species, of the circumstances under which these multiple enzyme activities are expressed.

Rumen bacteria. The rumen bacterial genera *Bacteroides*, *Butyrivibrio*, *Fibrobacter* and *Ruminococcus* have been studied in some detail to determine the conditions under which their polysaccharide-degrading enzymes are expressed (Williams and Withers 1982a,b, 1985; Martin and Akin 1988, Hespell and O'Bryan-Shah 1988, Sewell et al. 1988, Pettipher and Latham 1979, Greve et al. 1984c). *Butyrvibrio* is considered to be the major xylan/hemicellulose degrading bacterium in the rumen (Hespell et al. 1987). Although *Bacteroides*, *Fibrobacter* and *Ruminococcus* produce xylan-degrading enzymes (Table 1), these genera grow poorly on xylan (Hespell et al. 1987) and are generally thought to utilize primarily cellulose in the rumen. Williams and Withers (1982a,b) comprehensively examined the effect of carbohydrate growth substrate on glycosidase (14 enzymes), depolymerase (6 enzymes) and *Lolium multiflorum* cell wall degrading activities of *Ruminococcus*, *Fibrobacter*, *Bacteroides*, and *Butyrivibrio* isolates after growth on several monomeric or polymeric carbohydrates. Interestingly, although not pointed out by the authors, each of these genera best degraded *L. multiflorum* cell walls after growth on the carbohydrate substrate (different for each genus) which best induced cellulase activity (Williams and Withers 1982a). Only in *Fibrobacter succinogenes* S85

were the highest levels of xylanase activity found after growth on xylan. In the other genera the highest levels of xylanase activty were noted after growth on xylose (*Ruminococcus albus* RUM5), arabinan (*Ruminococcus flavefaciens* 123), maltose (*Bacteroides ruminicola* strains), or glucose (*Butyrivibrio fibrisolvens* H10b). All of these isolates also produced xylanase (although at lower levels) after growth on xylan. *Bacteroides ovatus*, included in Table 1, is not a rumen isolate, but occupies the human colon.

Both *Bacteroides* and *Butyrivibrio* have yielded enzymes which on good evidence appear to be bifunctional xylosidase/ arabinofuranosidases (Table 1). In *Bacteroides ovatus* the gene for this bifunctional enzyme appears on a 3.8 kb genomic fragment which also contains a xylanase gene (Whitehead and Hespell 1990). However, the xylanase gene was regulated independently of the bifunctional gene, and there was some evidence for a second arabinofuranosidase gene. The bifunctional xylosidase/ arabinofuranosidase of *Butyrivibrio fibrisolvens* GS113 did not exhibit amino acid homology with any known xylan-degrading enzymes or glycosidases. The *xyn*A gene product from *Butyrivibrio fibrisolvens* 49 exhibited 37% amino acid homology with the *Bacillus* sp. C-125 xynA protein (Mannarelli et al. 1990), but hybridized to chromosomal sequences in only two of five closely related *Butyrivibrio* strains. The *B. fibrisolvens* H17c *xyn*B gene product exhibited 32%, 38%, and 40% amino acid homology with the cellobiohydrolase/endocellulase of *Clostridium saccharolyticum*, the xylanases of *Bacillus* sp. C-125, and the xylanase of *B. fibrisolvens* 49, respectively (Lin and Thomson 1991).

Fibrobacter and *Ruminococcus* each produce more than one xylanase protein (Table 1). There was clear evidence for at least two genes (possibly more) in *R. flavefaciens* FD-1, as well as indication of multiple copies of both the *xyn*A and *xyn*B genes in the *R. flavefaciens* FD-1 genome (Flint et al. 1991). There was also evidence of increased mRNA production from both *xyn*A and *xyn*B in *R. flavefaciens* FD-1 grown on xylan as compared to cellobiose (Flint et al. 1991). The endoxylanases purified from the non-sedimentable culture fluid of *Fibrobacter succinogenes* S85 exhibited single bands on SDS-PAGE gels but multiple bands (4 each) after isoelectric focusing (Matte and Forsberg 1992). This phenomenon was also observed by Bachmann and McCarthy (1991) in their study of the xylanases of *Thermomonospora fusca*, as well as by MacKenzie et al. 1989, in work on the xylanases of *Clostridium thermocellum*. The causes and significance of this heterogeneity remain unknown. Endoxylanase 1 from *F. succinogenes* S85 exhibited the interesting property of removing arabinose residues from arabinoxylan without accompanying activity on aryl-α-L-arabinofuranosides, arabinan, or arabinogalactan (Matte and Forsberg 1992). Similar activity has been shown for *Streptomyces roseiscleroticus* (Table 1) as well as for a xylanase from the fungus *Trichoderma koningii* (Wong et al. 1988).

Clostridium. This genus includes Gram positive, anaerobic, endospore formers widely recognized for diverse substrate utilization. Many of the species studied with regard to xylan-degradation were originally isolated for their ability to degrade cellulose, and in fact the original *C. acetobutylicum* ATCC 84 strain, as well as *C. thermocellum*

grow very poorly on xylan. During growth on xylan as sole substrate, xylose oligomers and xylose accumulated in the *C. thermocellum* culture fluid, indicating a preference by this species for high molecular weight xylan fragments (Weigel et al. 1985). Other bacterial genera which grew rapidly on xylan utilized xylose first, and then xylo-oligomers.

C. thermocellum excretes a high molecular weight complex comprised of 26 subunits separable by SDS-PAGE (Bayer et al. 1983, Kohring et al. 1990), which binds tightly to cellulose, and has been designated the "cellulosome." Xylanolytic activity in the cellulosome has only recently been investigated. A similar, secreted high molecular weight cellulolytic and xylanolytic complex has also been examined in the mesophilic *Clostridium* sp. C7 (Cavedon et al. 1990). Non-cellulosome associated cellulases and xylanases have been found in the culture fluid of both species (Morag et al. 1990).

A total of 18 genes cloned from *C. thermocellum* were found to encode 15 endoglucanases, 2 xylanases, and one β-glucosidase (Hazlewood et al. 1988). Of the endoglucanases, 6 were observed to have xylanase activity as well. Kohring et al. (1990) found that 15 of 26 bands appearing after SDS-PAGE exhibited endoglucanase activity, and 13 had xylanase activity. Both activities were found in 8 of the 26 bands. Many (14) of these proteins were glycosylated, and debranching activities were also detected. Grepinet et al.(1988a,b) determined the nucleotide sequence of the *xynZ* gene, and purified and studied the encoded protein (Table 1). MacKenzie et al. (1989) obtained an additional three cloned fragments encoding xylanase

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activity, none of which appeared to be identical to the *xyn*Z gene. The *xyn*Z gene encoded a polypeptide with a centrally located 60 amino acid sequence, containing duplicated 24-amino acid segments, which was similar to conserved domains found at the carboxy-terminus of *C. thermocellum* endoglucanases (Grepinet et al. 1988b). The conserved domain was not required for catalysis. The *xyn*B gene product from *C. acetobutylicum* P262 exhibited 65% homology with the *Bacillus pumilus* IPO xylanase, and 40% homology with the *B. subtilus* PAP115 xylanase (Zappe et al. 1990).

Actinomycetes. The actinomycetes have been found to be excellent producers of extracellular xylanases, as well as of other depolymerases (Ball and McCarthy 1989, Johnson et al. 1988, Zimmermann et al. 1988). Advances in the molecular biology and genetics of these bacteria have allowed cloning between species, avoiding problems encountered with expression and excretion when foreign genes are cloned into *Escherichia coli* (e.g., Vats-Mehta et al. 1990). Two xylanase-encoding genes have been cloned from *Streptomyces lividans* 66 (Mondou et al. 1986, Vats-Mehta et al. 1990) into a xylanase/endo-xylanase double mutant of *S. lividans*. The genes appear to encode two proteins previously purified from *S. lividans* 66, which are not immunologically related (Kluepfel et al. 1990, Morosoli et al. 1986). There appears to be little xylanase associated with *S. lividans* cells, but the production of extracellular enzyme was increased by the addition of Tween 80 or olive oil to cultures during growth (Bertrand et al. 1989). The gene for one of four xylanases found in the culture medium of *Thermomonospora fusca* was cloned into *S. lividans* TK24

(Ghangas et al. 1989). The xylanase encoded by this gene bound to and hydrolyzed insoluble xylan. The partial amino acid sequence for xylanase I from *S. thermoviolaceus* OPC-520 showed 47% homology with the *Cellulomonas fimi* exoglucanase, while the xylanase II protein exhibited 46% homology with the *Bacillus pumilus* IPO xylanase (Tsujibo et al. 1992).

Thermomonospora fusca BD21 produced extracellular and intracellular xylanase, acetyl esterase, and arabinofuranosidase proteins; however, the β-xylosidase was located intracellularly (Bachmann and McCarthy 1989, 1991). In this species, acetyl esterase was responsible for all observed acetyl-xylan esterase activity. Addition of purified β-xylosidase to *T. fusca* endoxylanase increased the hydrolysis of xylan, and similar addition of purified α-L-arabinofuranosidase resulted in increased saccharification of wheat straw (Bachmann and McCarthy 1991). MacKenzie et al. (1987) examined the induction of extracellular cellulase and xylanase enzymes from *S. flavogriseus* and *S. olivochromogenes* after growth on cellulose, oat spelt xylan or wheat bran. Xylan-containing substrates induced cellulase, xylanase, and debranching enzyme activities (acetyl-xylan esterase, α-L-arabinofuranosidase, and α-L-O-Me-glucuronidase) in both species, as well as ferulic acid esterase in *S. olivochromogenes*.

Pseudomonas. Two genomic fragments encoding three xylan-degrading enzymes have been cloned from *Pseudomonas fluorescens* subsp. *cellulosa* (Gilbert et al. 1988). The *xyn*A gene was encoded on the same genomic fragment as an endoglucanase gene with which it exhibited substantial

homology due to the possession by both of highly conserved domains (see below; Hall et al. 1989). The gene for *xyn*B appeared on a different fragment along with the *xyn*C gene. The product of the *xyn*C gene was shown to release only arabinose from arabinoxylan (i.e., there appeared to be no endoxylanase activity) and this protein exhibited no activity on aryl-α-L-arabinofuranosides (Gilbert et al. 1988). Both *xyn*B and C possessed conserved sequences which conferred on the protein products the ability to bind to cellulose (but not xylan), and which were not required for catalysis (Kellett et al. 1990). These results elicit intriguing questions with regard to the origin of polymer-degrading enzymes of similar function, a topic recently reviewed for the β-1,4-glycanases by Gilkes et al. (1991). These authors suggest that cellulases and xylanases of both procaryotic and eucaryotic origin can be grouped into families based on conserved amino acid sequences in their putative catalytic domains. Many of these enzymes exhibit additional sequences, again frequently conserved across species, genus and procaryotic-eucaryotic boundaries, which are similar to sequences known to confer the ability of some enzymes to bind to a substrate (usually cellulose), or which encode for protein segments rich in proline or threonine residues which may be glycosylation sites (Gilkes et al. 1991). They propose that fusion or shuffling of these different domains may account for the somewhat bewildering array of enzymes which hydrolyze the β-1,4-glycosidic bond, with varying degrees of specificity, in an equally diverse array of polysaccharides.

Factors affecting the complexity of xylan-degrading enzyme systems

It is obvious that we have learned a great deal in recent years about the number and nature of xylan-degrading enzymes from several genera of bacteria. Use of denaturing and non-denaturing gel electrophoresis, isolelectric focusing and activity stains, has revealed information about the xylan-degrading enzyme systems of genera for which genetic systems are not well-established. Concurrently, molecular techniques have been developed for several well-studied genera and protein isolation methods have become more routine. The combination of these diverse methods has begun to reveal a previously unsuspected complexity in bacterial xylan-degrading enzyme systems. It is now clear that most bacteria possess multiple xylanase genes encoding proteins which exhibit further heterogeneity in physical characteristics, substrate specificity, or mode of action. In turn, many xylanase genes may be part of a larger gene complex encoding polysaccharide degrading activities in a multitude of procaryotic and eucaryotic organisms.

Although this diversity will enhance the probablity of finding very specific enzymes for biotechnological applications, it will encumber the search for pattern and definition. It seems clear that xylan-degrading enzyme systems will not be as neatly packaged as, for example, bacterial cellulase systems, where three types of enzymes (endoglucanase, exoglucanase, and β-glucosidase) essentially perform the same functions in all bacteria, and are sufficient to degrade cellulose wherever it may be found. The heterogeneity of bacterial

xylan-degrading enzyme systems is probably most directly a function of the heterogeneity of xylan, and thereafter a function of the heterogeneous environment of the plant cell-wall. While enzymatic tests for xylanase activity employ isolated and soluble substrates, bacteria degrading xylan in nature will encounter xylan inextricably linked to a multitude of other polysaccharides. Most bacteria probably do not need to fully degrade xylan in order to survive. Instead, multiple enzyme activities which partially degrade xylan, as well as other plant cell wall polysaccharides, or which clip away residues from a variety of parent molecules, may be more advantageous than a "complete" xylan-degrading enzyme system. In this respect, it seems logical that many xylanases have some activity on other polysaccharide substrates, and vice versa. It also seems logical that, as for the rumen bacteria discussed above, xylan-degrading enzymes would be induced by mono- or polysaccharides other than xylan.

It is also important to recognize that our understanding of the complexity of bacterial xylan-degrading enzyme systems is restricted by the types of questions we have asked. In some cases the primary research objective has been to obtain enzymes with unique substrate specificities or tolerances, and no effort has been made to examine other components of the xylan-degrading enzyme system, or to determine the circumstances under which the producing organism utilizes xylan in nature. In some cases, no effort has been made to determine whether a given xylanase has activity on other substrates, or whether the conditions under which the enzyme was studied represent those under

which it would typically, or optimally, be expressed. As more xylan-degrading enzymes are isolated we will have a better concept of the criteria to be met by specific classes of xylanases.

Finally, much of our knowledge comes from research with bacteria which do not grow on xylan. Should we expect the components of the xylan-degrading system of *Clostridium thermocellum* (which, as noted above, does not grow well on xylan) to be similar to those of, for example, *Butyrivibrio*? The evolutionary constraints on these two systems will be quite different. The *C. thermocellum* system does not need to produce xylose, or even apparently, xylo-oligomers for the cells (Wiegel et al. 1985) and may exist only to free cellulose for further cellulase attack. It may be that some competition for plant cell wall polymers is based on the ability of a cell to "carve out" of the cell wall, and utilize, the unique products of its own polymer-degrading enzyme system.

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Chapter One

Cytophaga xylanolytica sp. nov., a xylan-degrading, anaerobic gliding bacterium

Abstract. Gliding bacteria attached in masses to, and dominated the fermentation of, xylan powder in methanogenic and sulfidogenic enrichments from various freshwater sediments. Isolates of such bacteria were all Gram negative, slender rods (0.4 x 4-24 μm) that formed no endospores, microcysts or fruiting bodies. Representative strain XM3 was a mesophilic, aeroduric anaerobe that grew by fermentation of mono-, di-, and polysaccharides (but not cellulose) in a mineral medium containing up to 3% NaCl. However, CO_2/HCO_3^- was required in media for consistent initiation of growth. Fermentation products included acetate, propionate, succinate, CO_2, and H_2. Xylan-grown cells had xylanase and various glycosidase activities that were mainly or almost entirely cell-associated, respectively. Strain XM3 was weakly catalase positive, but oxidase negative; it possessed sulphonolipids and carotenoid, but not flexirubin, pigments; and its total cellular fatty acids were dominated by $C_{15:0}$ *anteiso* (75%), *n* (13%) and *iso* (2%) isomers. Strain XM3 had 45.5 mol% G + C in its DNA, and partial sequencing of its 16S rRNA placed XM3 within the *Bacteroides-Flavobacterium* phylogenetic group. Similar strains have recently been isolated from marine sediments. Strain XM3 is herewith proposed as the type strain of the new species, *Cytophaga xylanolytica*. Results, which are discussed in terms of our current concept of the genus *Cytophaga*, suggest that the importance of *C. xylanolytica* in anaerobic biopolymer decomposition has not been fully appreciated.

Xylans are a class of cell wall hemicelluloses widely distributed among both terrestrial and aquatic plants. They are heteropolysaccharides consisting of a β-1,4-linked xylopyranose backbone, often with α-1,3-arabinofuranoside and α-1,2-methylglucuronic acid side groups. In addition, C_2 and C_3 of some of the xylopyranose residues may be acetylated (Biely 1985). Next to cellulose, xylans are probably the most abundant polysaccharides in nature; and it has been estimated that nearly 10^{10} metric tons are turned over annually (Wilkie 1983). Unfortunately, however, our understanding of the microbiology and biochemistry of xylan decomposition is fairly limited (Biely 1985). This is particularly true for anaerobic decomposition of xylans by free-living microbial communities. Accordingly, the present study was initiated.

This paper reports on the isolation and characterization of freshwater and marine cytophagas capable of decomposing xylan and other polysaccharides under strictly anaerobic conditions. Freshwater isolates are typified by strain XM3 which is proposed as the type strain of the new species, *Cytophaga xylanolytica*. The properties of *C. xylanolytica* reported herein extend our current concept of the genus *Cytophaga* and suggest that the importance of such organisms in anaerobic decomposition of biopolymers has not been fully appreciated.

Materials and Methods

Enrichment and isolation of bacteria Approximately 1 ml samples of aquatic sediment slurry were inoculated into 50 ml of Na_2S-reduced, CO_2/HCO_3^--buffered, defined mineral media containing, as sole fermentable substrate, 53 mg of xylan (larchwood or oat spelt; pre-extracted with 70% ethanol; Sigma Chem. Co.) in powdered (i.e. non-heat-sterilized) form. Media contained 0 to 20 m*M* SO_4^{-2} (for methanogenic or sulfidogenic enrichments, respectively) and a salt concentration compatible with the source of inoculum (freshwater or marine; see below) and were held in rubber-stoppered serum bottles under N_2/CO_2 (80/20, v/v). Incubation was at 30°C. From robust enrichments, pure cultures of xylanolytic bacteria were isolated by using 1% agar shake tubes of homologous medium containing 0.1-0.2% heat-sterilized (solubilized) xylan. Isolated strains were maintained in liquid medium or on 1.5% agar bottle plates (Hermann et al. 1986).

Media and growth studies Defined "freshwater" mineral medium #1 contained (per liter): KH_2PO_4, 0.2 g; NH_4Cl, 0.25 g; KCl, 0.5 g; $CaCl_2.2H_2O$, 0.15 g; NaCl, 1.0 g; $MgCl_2.6H_2O$, 0.62 g; $NaHCO_3$ solution (84 g/l; Widdel and Pfennig 1984), 30 ml; SL-10 trace element solution (Widdel et al. 1983), 1 ml; W/Se solution (0.1 m*M* each of Na_2SeO_3 and Na_2WO_4 in 20 m*M* NaOH), 1 ml; vitamin B_{12} solution (50 mg/l), 1 ml; mixed vitamin solution (below), 1 ml; and 1 *M* Na_2S (prepared and stored under N_2), 1 ml. The mixed vitamin solution contained (per liter): 4-aminobenzoic acid, 40 mg; D-(+)-biotin, 10 mg; nicotinic acid, 100 mg; Ca-D(+)-pantothenate, 50 mg; pyridoxamine

dihydrochloride, 100 mg; and thiamine dihydrochloride, 100 mg. All solutions were heat-sterilized, except for the mixed vitamin solution which was filter-sterilized. Energy sources (eg. xylan, xylose, etc.) were sterilized separately and included at a final concentration of 0.1-0.2% or 5-10 m*M*. Freshwater mineral medium #2 was identical to #1, except that $MgCl_2.6H_2O$ was replaced by $MgSO_4.7H_2O$ (0.74 g/l). Defined "marine" medium was identical to freshwater medium #2, except that the following salts were increased to (g/l): NaCl, 25; $CaCl_2.2H_2O$, 1; and KCl, 1; and $MgSO_4.7H_2O$, 5. "Brackish" water medium was prepared by mixing equal volumes of marine medium and freshwater medium #2.

Broth and agar media were usually prepared and incubated under N_2/CO_2 (80/20, v/v), which resulted in a final pH of 7.2 - 7.4. Adjustment of the pH, if necessary, was made by adding sterile 1*M* Na_2CO_3 or HCl, or by varying the $CO_2/NaHCO_3$ ratio (Costilow 1981). The requirement by cells for CO_2/HCO_3^- was examined by omitting $NaHCO_3$ from the medium, but including 10 m*M* 3-(N-morpholino)propanesulfonic acid (MOPS buffer; adjusted to pH 7.3 and heat-sterilized separately) and incubating cells under a gas phase of 100% N_2. To test aerobic growth, cells were incubated under air in sulfide-free, bicarbonate-free freshwater medium #2 which was buffered with Tris/TRIZMA base (pH 7.3; 20 m*M* final concentration).

Turbidimetric measurement of cell growth, cell dry mass determinations, and assessment of utilization of organic and inorganic substrates were done as previously described (Breznak et al. 1988).

Metabolic studies Material balances for the fermentation of uniformly-labeled ^{14}C-glucose and ^{14}C-xylose were done with cells growing in freshwater medium #2 containing these substrates. Products were analyzed as previously described (Potrikus and Breznak 1977), but analyses also included separation and quantification of soluble products by high performance liquid chromatography (HPLC), trapping of $^{14}CO_2$, and determinations of specific ^{14}C radioactivity all as described by Breznak and Switzer (1986).

Tests for aerobic respiration of xylose were done by using a Warburg type respirometer and conventional manometric techniques (Umbreit et al. 1957). Reaction mixtures (3.0 ml) were held in double sidearm respirometer vessels and contained: 20 m*M* Tris buffer and 10 m*M* K-phosphate buffer, both at pH 7.3; NaCl, 0.17 *M*; xylose, 20 m*M*; dithiothreitol (DTT; for anoxic incubations only), 3 m*M*; and cells [grown anaerobically on xylose; harvested by centrifugation and resuspended in Tris-phosphate-NaCl (above)], 2.54 mg dry cell mass equiv. Reactions were initiated by introducing xylose from one of the sidearms, and they were terminated by adding 0.2 ml of 5*N* H_2SO_4 from the other sidearm. Incubation was at 30°C under air or 100% N_2, and any gas exchange was corrected for that occuring in xylose-free (endogenous metabolism) reaction mixtures.

Enzyme assays Late exponential to early stationary phase xylan-grown cells were harvested at 5°C by centrifugation at 10,000 x *g* for 10 min to obtain extracellular (supernatant fluid) and cell-associated (pellet) enzyme fractions. The extracellular fraction was either used

without further treatment or was dialyzed (12-14 kD mol. wt. cutoff) for 3 h at 4°C against 3 changes of ca. 50 volumes each of 0.01 *M* N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] buffer (pH 6.0) containing 1 m*M* $CaCl_2$ (= HEPES/$CaCl_2$). Dialyzed preparations were then concentrated by ultrafiltration through an Amicon PM10 membrane under 20 psi N_2. Pellets were resuspended in HEPES/$CaCl_2$ (pH 6.8), and cells were disrupted at 4°C by using a Branson sonicator operating for 1.5 min at 60% duty cycle and an output setting of 5.

Xylanase and carboxymethylcellulase (CMCase) were determined by measuring the release of reducing sugar from xylan or CMC, respectively. Reaction mixtures contained 2.5 ml of 0.1% (w/v) heat-solubilized xylan (oat spelt, lot no. 116F-0240; Sigma Chemical Co.) or CMC (Na salt; Sigma Chem. Co.), 1.5 ml of 0.1 *M* HEPES/$CaCl_2$ (pH 6.8), and 1 ml enzyme solution (added to start the reaction) and were incubated at 30°C. Reducing sugar liberated was quantified by colorimetric assay (Nelson 1944) with xylose as standard. Glycosidase and acetylesterase activities were determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl substrates (Greve et al. 1984). Reaction mixtures contained 1 ml of substrate (10 m*M* in 0.1 *M* HEPES, pH 6.8) and 0.2-1.0 ml enzyme solution. Incubation was at 30°C.

Chemical assays Glucose was determined by HPLC (Breznak and Switzer 1986) and by assay with glucose oxidase (Sigma Chem. Co.). Xylose was determined by HPLC (above) and by the orcinol assay (Herbert et al. 1971). Xylan was determined by using the orcinol or phenol-H_2SO_4 assay (Herbert et al. 1971). Pyruvate was quantified and qualitatively

identified as the 2,4-dinitrophenylhydrazone derivative (Freidmann and Haugen 1943), as well as by HPLC of the underivatized acid (above). Protein was determined by using the Bradford reagents (Bio-Rad Chem. Co.). Sulfide was assayed as described by Cord-Ruwisch (1985), and production of methane and volatile fatty acids (VFA's) in enrichment cultures was determined by gas chromatography (Schink and Pfennig, 1982).

Other procedures For sequencing of 16S rRNA, total nucleic acids were extracted from cells with hot phenol and were precipitated from the aqueous phase with ethanol (Lane et al. 1985). Nucleotide sequences were determined by using the dideoxynucleotide method with: reverse transcriptase; 16S rRNA as template; and three universal oligonucleotide primers (Sanger et al. 1977; Lane et al. 1985) provided by Dr. D. Stahl (Univ. Illinois, Urbana, USA).

Total cellular fatty acids were analyzed by using the MIDI system (Microbial ID, Inc., Newark, DE, USA) according to procedures described in the technical manual which accompanied the MIDI equipment. Briefly, this involved: 1) saponification of cellular lipids with methanolic NaOH; 2) esterification of fatty acids with acidic methanol; and 3) extraction of fatty acid methyl esters (FAMEs) into methyl *tert*-butyl ether/hexane, then into NaCl-saturated 0.3 *N* NaOH, prior to analysis by gas chromatography on a 25 m fused silica capillary column.

Spot tests for carotenoid pigments were performed as described by Skerman (1967). Carotenoids were also extracted from cell pellets of strain XM3 with acetone/ methanol (7/3, v/v) for determination of absorption spectra. Tests for flexirubin pigments were done on cell pellets as described by Reichenbach et al. (1974). Sulphonolipid analysis was kindly performed by Drs. W. Godchaux and E. R. Leadbetter (Univ. of Connecticut, Storrs, USA) as described by these same workers (1983).

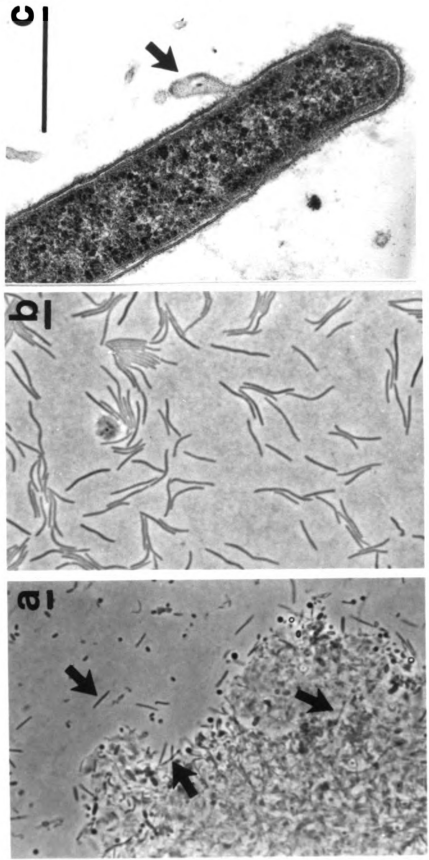
DNA base composition of strain XM3 was kindly determined by Dr. J. Flossdorf (GBF, Braunschweig, FRG) by using a buoyant density method (Flossdorf 1983). Previously described methods were also used for electron microscopy (Breznak and Pankratz 1977) and for determination of nitrate reduction (Smibert and Krieg 1981). Phase contrast photomicrographs were made by using the procedure of Pfennig and Wagener (1986).

Results

Enrichment and isolation of bacteria Various freshwater sediments collected in the vicinity of Konstanz, FRG, all gave rise to methanogenic and sulfidogenic enrichments when inoculated into mineral medium containing xylan powder. During the first 2 weeks of incubation, most enrichments became turbid, and the otherwise readily-dispersible xylan powder became gummy and dispersible only with vigorous swirling. Phase contrast microscopy of xylan particles at this time revealed large numbers of slender, rod-shaped bacteria attached to, and gliding over, the particle surface (Fig. 1a). In methanogenic enrichments, 4 m*M* acetate and 3 m*M* propionate appeared as the dominant (but transient) VFA's in culture fluid, and after 8 weeks approximately 94% of the original xylan carbon could be accounted for as CH_4 and CO_2. In sulfidogenic enrichments, acetate (4-5 m*M*) was the single dominant VFA produced, but xylan decomposition did not proceed to completion, presumably because sulfide accumulated to inhibitory levels (4-5 m*M*).

From such enrichments, 20 strains of xylanolytic gliding bacteria were isolated, representing the numerically dominant colonies which developed in xylan shake tubes. All strains were virtually identical in size and in cell and colony morphology, and they all resembled the gliding bacteria seen on the surface of xylan particles in enrichments, so one of these (strain XM3) was selected for detailed study as reported below. Subsequently, similar gliding bacteria were enriched and isolated from freshwater muds (including a swine waste

Figure 1.a. Phase contrast micrograph of a mass of *Cytophaga xylanolytica*-like cells attached to the surface of a xylan particle in a methanogenic, freshwater enrichment culture. Individual cells can be discerned on some portions of the particle, along its edges, and after having glided out on the agar-covered slide (arrows). **b.**Phase contrast micrograph of *C. xylanolytica* XM3 cells in pure culture. **c.** Transmission electron micrograph of a thin section of xylan-grown *C.xylanolytica* XM3. Note the vesicular evagination of the outer membrane (arrow). Marker bars = 5 μm (a & b); 0.4 μm (c).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lagoon sediment) collected in East Lansing, Michigan (Table 1) and from marine sediments collected in Woods Hole, Massachusetts USA (Table 2). On first transfer into broth media, XM3 and many other strains grew as blotches attached to the inside wall of culture tubes or as macroscopically visible clumps in the liquid. However, such a phenotype was usually lost after several transfers, with most strains eventually growing as uniformly suspended cells.

Isolates grew as swarms, not discrete colonies, on the surface of bottle plates. Moreover, when the Michigan and Woods Hole enrichments were streaked directly onto xylan bottle plates, thin, spreading swarms of gliding bacteria developed among, and extending out from, the more typical colonies of non-gliding bacteria. Selective transfer of the leading edge of such swarms to fresh plates resulted in pure populations of gliding bacteria, from which isolated colonies could ultimately be obtained by using agar shake tubes. In shake tubes solidified with $\geq$ 1% agar, isolated colonies of XM3 were about 2 mm in diameter, dense, lenticular or round in shape, and usually pigmented (see below). In less solid agar medium, XM3 colonies were spherical in shape, less dense, and increased in diameter with time (up to 1 cm). Gliding bacteria were never isolated from enrichments by streaking conventional Petri plates of xylan medium which lacked $NaHCO_3$ and Na_2S [but which contained 10 mM MOPS buffer (7.3), 1 mM DTT, and 3 mM SO_4^{-2}], and which were incubated in an anaerobe chamber under N_2/H_2 (92/8, v/v). This was true even if the gas phase was enriched with 20% (v/v) CO_2. Furthermore, if enrichments were established by using heat-

Table 1. Properties of freshwater isolates of *Cytophaga xylanolytica*.[a]

	XM3	SL1[b]	MA3	EW1
Cell size (μm)	0.4 x 5-10	0.4 x 4-24	0.4 x 4-24	0.4 x 3-15
Substrates fermented[c]				
xylan	+	+	+	+
starch	+	+	-	+
pectin	+	+	-	-
inulin	-	-	+	-
laminarin	+	+	+	+
lichenin	+	+	+	+
pullulan	+	+	-	-
cellobiose	+	+	+	+
lactose	+	+	+	+
maltose	+	+	-	-
sucrose	-	-	+	-
D-glucose	+	+	+	+
D-fructose	-	-	+	-
D-galactose	+	+	+	+
D-mannose	+	+	+	+
D-xylose	+	+	+	+
L-arabinose	+	+	+	+
D-glucuronic acid	+	+	-	+
D-galacturonic acid	+	+	-	+

[a]All strains were orange pigmented (subsurface colonies), catalase +, and oxidase -, and all formed acetate, propionate and succinate as major acidic products of xylan fermentation.

[b]Isolated from a swine waste lagoon sediment.

[c]None of the strains fermented: cellulose (Sigmacell 20 microcrystalline cellulose; or ball-milled Whatman #1 filter paper), CMC, arabinogalactan, mannan, chitin, N-acetylglucosamine, L-sorbose, D-ribose, or L-rhamnose. Strain XM3 did not ferment: chitosan, sorbitol, glycerol, malate, citrate, formate, benzoate, gallate, syringate, caffeate, phenylacetate, xanthine, uracil, methanol, ethanol, phenylalanine, Brij 58, or Tween 20.

Table 2. Properties of marine strains of xylanolytic *Cytophaga*.

	OP2E	OP2F	EPA	EPB	PR2L	EPFW
Cell size (μm)						
width	0.4-0.6	0.4-0.5	0.4-0.6	0.4-0.6	0.6-0.7	0.4-0.5
length	2-8	3-12	4-9	4-34	2-7	4-28
Color[a]	orange	white	gold	gold	salmon	white
Carotenoid	+	-	+	+	-	-
Flexirubin	+	-	+	+	-	-
Catalase	+	+	+	+	+	+
Oxidase	-	+	-	-	+	ND[b]
Growth in (medium)						
brackish water	+	+	+	+	+	+
freshwater #2	-	-	+	+	-	+
Acidic fermentation products[c]	A,P	A,P	A,P	A,P,S,B	ND	ND
Agarolytic	+	+	-	-	+	-

[a]Subsurface colonies or agar surface swarms.

[b]ND, not determined

[c]During growth on xylan. A, acetate; P, propionate; S, succinate; B, butyrate.

sterilized (solubilized) xylan instead of xylan powder, *Cytophaga*-like bacteria (although microscopically visible) were far less abundant.

Morphology Cells of strain XM3 were Gram negative rods, possessing both an inner (cytoplasmic) and outer membrane and measuring 0.4 x 5-10 μm (Fig. 1b,c). In thin sections, the outer membrane appeared relatively smooth, but possessed evaginations at various points to form cushion-like knobs or elongated vesicles. The latter were often 0.2-0.4 μm long (Fig. 1c) and resembled those observed on certain cytophagas (Follett and Webley 1965; Oyaizu *et al.* 1982). Cells occured singly or in pairs, less frequently as chains or asterisk-shaped aggregates, and they lacked flagella. However, cells of XM3 and other isolates in liquid media could bend or flex slightly, translocate parallel to their long axis when attached to surfaces, transport minute particles of debris over the cell surface, and pivot on one cellular pole as described for typical cytophagas (Reichenbach 1989c). No endospores, microcysts, or fruiting bodies were formed, although ghost-like spherical bodies (both free and attached to cells) were formed in old cultures. The size range of all strains is shown in Tables 1 & 2. Based on their general phenotypic properties (described more fully below), isolates were concluded to be members of the genus *Cytophaga* (Reichenbach 1989c) and will be referred to by this epithet for the remainder of this paper.

Nutrition and growth characteristics *Cytophaga* XM3 (as well as other freshwater isolates) grew in defined mineral media with various mono-,

di-, and polysaccharides as sole fermentable energy source (Table 1). Three of the 6 marine isolates were also agarolytic (Table 2). XM3 grew within a pH (initial) range of 6.1-8.7 (optimum 7.2-8.2) and temperature range of 19-37°C (optimum 30-32°C). No growth occurred at 4°C or 45°C. When grown at 30°C with 10 m*M* glucose as energy source, cells had a doubling time of 2.6 h and attained an $OD_{600\ nm}$ of 1.0-1.2 (equiv. 0.40 mg dry cell mass/ml). *Cytophaga* XM3 could use NH_4^+, glutamine (but not glutamate, NO_3^-, or N_2), or peptone as sole N source, and did not require vitamins for growth. However, cells did not respire anaerobically with NO_3^- or SO_4^{-2} as terminal electron acceptor. Surprisingly, strain XM3 and other freshwater isolates grew equally well in media containing up to 3% NaCl. Conversely, all marine isolates grew in brackish water medium; 2 strains also grew in freshwater medium (Table 2).

Luxurious growth of all freshwater isolates required the presence of $CO_2/NaHCO_3$ in the medium. Cells grew poorly or not at all in $CO_2/NaHCO_3$-free, MOPS-buffered media under 100% N_2. Likewise Na_2S, used as the reducing agent in anoxic media, was the best S source. Strain XM3 grew poorly and inconsistently in Na_2S-free medium containing 1 m*M* SO_4^{-2}, cysteine, or glutathione (with or without 1 m*M* DTT included as reducing agent). No growth at all was observed in similar media containing methionine or thiosulfate. None of the other freshwater isolates obtained so far will use SO_4^{-2} as S source (with or without DTT as reducing agent). Interestingly, however, increasing the NaCl concentration in freshwater media to 10-20 g/l permitted maintenance of strain XM3 on marginal S sources such as SO_4^{-2} and

glutathione.

Cytophaga XM3 grew in the presence of air in non-reduced, Tris/TRIZMA-buffered medium with glucose as energy source, but only if the following conditions were met: [NaCl] $\geq$ 1%; [CO_2 + HCO_3^-] $\geq$ [energy source]; and the surface/volume ratio of the culture broth was small (i.e. held in a vertical culture tube). Under these conditions, SO_4^{-2} was provided as S source. However, cells of XM3 (and other freshwater isolates) grew poorly or not at all if such cultures were shaken, or if streaked on the surface of homologous agar medium exposed to air. Moreover, cell suspensions of strain XM3 did not exhibit xylose-dependent O_2 uptake in manometric experiments. Based on these characteristics, XM3 was concluded to be an aeroduric anaerobe. The recently-isolated marine strains have not been examined in sufficient detail to state their precise relationship to oxygen.

Fermentation products During anaerobic growth in CO_2/$NaHCO_3$-buffered medium, *Cytophaga* XM3 fermented ^{14}C-UL-glucose and ^{14}C-UL-xylose to acetate, propionate, succinate, and CO_2 as major products (Table 3). The same, or similar, major organic acid profiles were displayed by XM3 (and other isolates examined) when grown on xylan, with the exception that marine strain EPB also produced butyrate (Table 2). H_2 was also produced by XM3, as was (occasionally) pyruvate (Table 3). However, although 98% of the substrate ^{14}C was recovered in both experiments, only about 70% was associated with specifically identified products + cells: the remainder was present in HPLC fractions (F1-F4) that elicited little or no response from the

Table 3. Products of xylose and glucose fermentation by growing cells of *Cytophaga xylanolytica* XM3.

Substrate[a]	Products							
	Propionate	Acetate	Succinate	CO_2	H_2	Pyruvate	Unk[b]	Cells
UL-^{14}C-Xylose								
Recovery of ^{14}C (%)	11.2	9.2	9.5	11.5	-	12.2	27.5	17.5
mmol per 100 mmol xylose fermented[c]	32.9	36.4	21.0	57.5	7.2[d]	19.0	-	-
dpm per mmol (x 10^7)	2.2	1.7	3.0	1.3	-	2.0	-	-
mmol exogenous CO_2 fixed								
per mmol product	1.3	0.7	1.7	-	-	1.5	ND[e]	ND
per 100 mmol xylose	42.7	26.9	36.5	-	-	27.7	ND	ND
UL-^{14}C-Glucose								
Recovery of ^{14}C (%)	14.6	10.0	5.7	14.4	-	0	32.5	21.0
mmol per 100 mmol glucose fermented[c]	53.1	41.6	9.8	86.2	3.2[d]	0	-	-
dpm per mmol (x 10^7)	1.3	1.1	2.8	0.8	-	0	-	-
mmol exogenous CO_2 fixed								
per mmol product	1.4	0.6	0.5	-	-	0	ND	ND
per 100 mmol glucose	71.7	23.9	4.7	-	-	0	ND	ND

Table 3 Cont'd.

[a]Specific activities were (10^7 dpm/mmol): UL-^{14}C-glucose, 4.78; UL-^{14}C-xylose, 6.54.

[b]Unknown soluble material (see text). Fermentation products tested for, but not detected in significant amounts, included: glycerol, sorbitol, dulcitol, mannitol, inositol, propanol, butanol, ethanol, 2,3-butanediol, acetoin, diacetyl, lactate, formate, butyrate, *iso*butyrate, valerate, *iso*valerate, crotonate, fumarate, citrate, oxalate, oxaloacetate, α-ketoglutarate, α-ketogluconate, fructose, mannose, galactose, ribulose, and glucuronic acid.

[c]Based on direct chemical analyses, except for CO_2 which was based on radioactivity determination.

[d]Determined in separate experiments.

[e]ND, not determined.

refractive index detector (Table 3; Fig. 2). The material in such fractions remains to be identified, but it was not residual glucose or xylose, nor was it any of 28 other common fermentation products including tricarboxylic acid cycle intermediates and sugar alcohols (Table 3, footnote d). Based on its retention time during HPLC, it may be a heterogeneous mixture of di- or oligosaccharides and/or derivatives thereof.

Comparison of the specific radioactivity of identified products with that of the initial substrate indicated that CO_2/HCO_3^- fixation (or exchange) was involved in sugar fermentation by strain XM3. From 0.5 to 1.7 mmol exogenous CO_2/HCO_3^- was incorporated per mmol organic acid formed (Table 3). Overall, however, about 1 mmol CO_2/HCO_3^- was incorporated into soluble products per mmol glucose or xylose dissimilated.

Additional phenotypic and genotypic characteristics Cytophaga XM3 produced a cell-associated pigment which resulted in orange subsurface colonies in xylan shake tubes, orange to salmon colored swarms on xylan plates, and light pink cell pellets from glucose broth cultures. Spot tests suggested that the pigment was carotenoid, and acetone/methanol extracts of glucose-grown cells displayed an absorption spectrum typical of that of carotenoids (Zechmeister 1962) with major peaks at 458 (shoulder), 484 and 518 nm, and minor peaks at 357, 373, and 673 nm (Fig. 3). Qualitatively and quantitatively similar pigmentation was observed whether cells were grown in the dark or with incandescent illumination. No evidence was obtained for the

Figure 2. Histogram depicting the distribution of ^{14}C radioactivity in HPLC fractions of spent culture fluid from a *C. xylanolytica* fermentation of 20 m*M* UL-^{14}C-xylose and 10 m*M* UL-^{14}C-glucose. Py, S, A, and P are fractions containing pyruvate, succinate, acetate, and propionate, respectively, as indicated by the peaks in detector response. F1-F4 are arbitrary fractions.

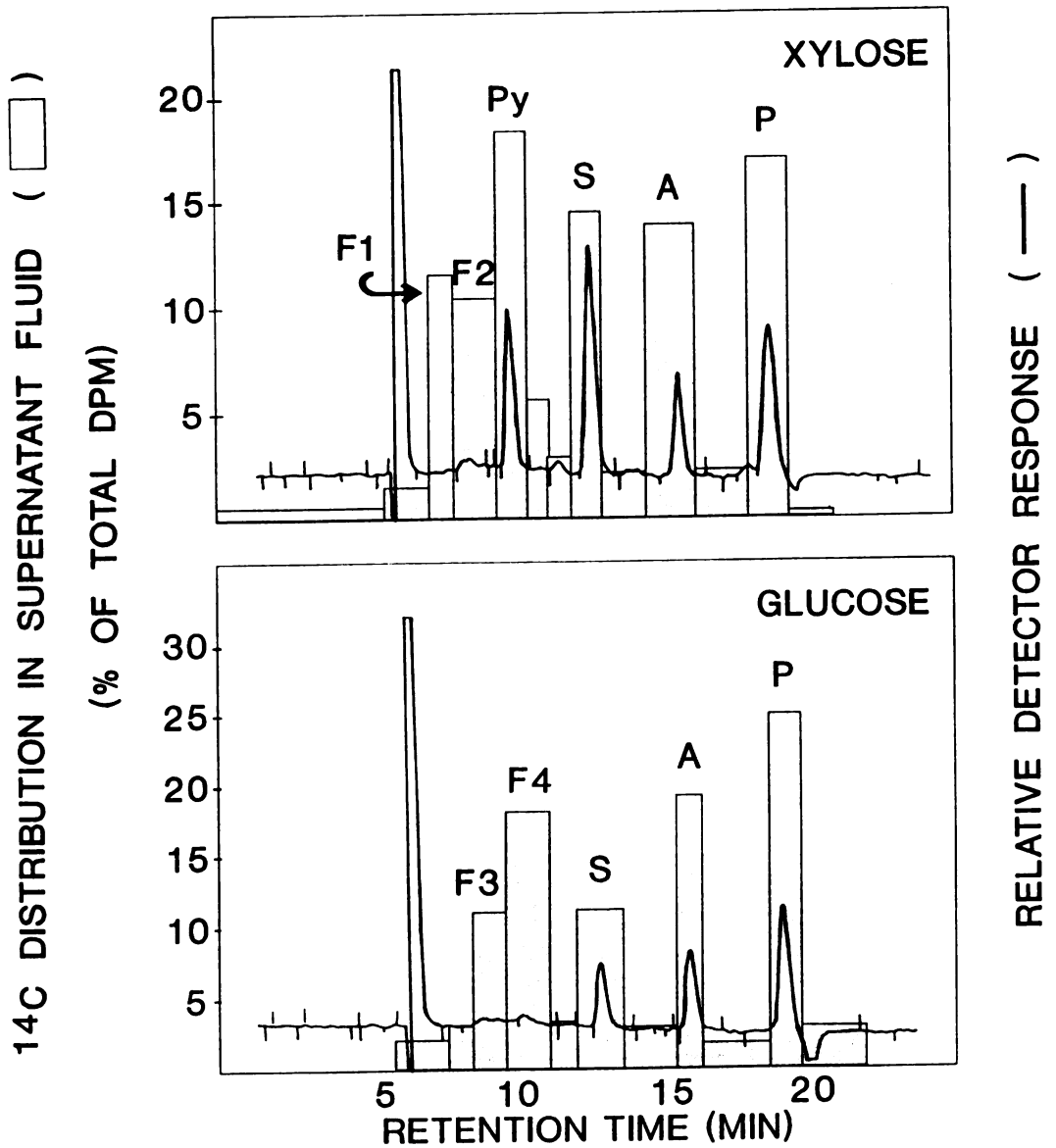
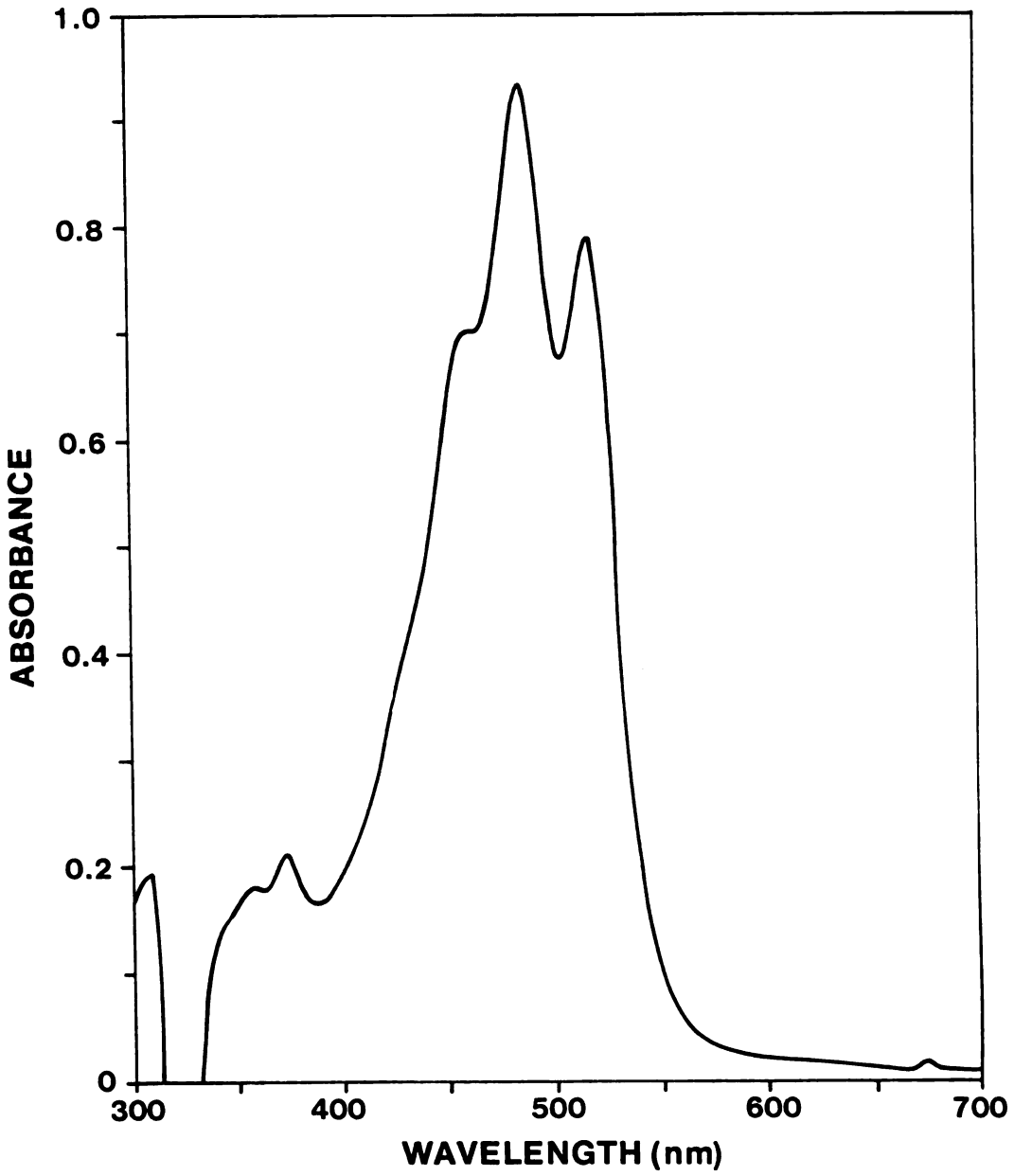

XYLOSE
Py
P
S
A
F1
F2
20
15
10
5
GLUCOSE
P
A
F4
S
F3
30
25
20
15
10
5
5
10
15
20
RETENTION TIME (MIN)
14C DISTRIBUTION IN SUPERNATANT FLUID ( )
(% OF TOTAL DPM)
RELATIVE DETECTOR RESPONSE ( )

Figure 3. Absorption spectrum of an acetone/methanol (7/3, v/v) extract of glucose-grown cells of *C. xylanolytica* XM3.


1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
300
400
500
600
700
WAVELENGTH (nm)

presence of flexirubins or bacteriochlorophyll in XM3, nor was phototrophic growth observed in freshwater medium #1 with with acetate as organic substrate. Spot tests also revealed the presence of carotenoids in other freshwater isolates (Table 1). By contrast, the pigmented marine isolates contained both carotenoids and flexirubins; nonpigmented strains contained neither (when assayed by spot test only; Table 2).

Cells of XM3 incorporated $^{35}SO_4^{-2}$ into the cell lipid fraction during growth. Thin layer chromatography of that fraction revealed radioactivity migrating with an R_f of 0.74, suggesting the presence of a sulphonolipid somewhat less polar than N-acylcapnine (R_f = 0.65). When hydrolyzed to remove any N-acyl moiety, the resulting labeled material chromatographed with an R_f = 0.30, compared to an R_f of 0.39 for an authentic capnine standard.

The total cellular fatty acid content of freshwater isolates was dominated by $C_{15:0}$ isomers, with *anteiso* > *n* > *iso*. Among the hydroxy fatty acids, 3-OH-*n*-$C_{15:0}$ was a major and consistently present species (Table 4).

Of 1542 nucleotides typically present in 16S rRNA, 927 of those present in XM3 were sequenced, spanning *Escherichia coli* position numbers (Woese 1987) 218-510, 554-906, and 1093-1380. These were aligned against the *E. coli*, *Bacteroides fragilis*, and *Flavobacterium* [= *Cytophaga*; Christensen (1980)] *heparinum* complete sequences as reported by Weisburg et al. (1985). At position numbers 290 (G), 310

(C), 501 (G), 569 (U), 881 (A), 570 (U), 680 (G), 710 (C), 724 (U), 866 (A), and 1340 (U), strain XM3 bore the sequence signature of the *Bacteroides-Flavobacterium* subgroup (Weisburg et al. 1985). At position numbers 450 (G), 483 (C), and 484 (G), it resembled that of other eubacteria. Seven additional nucleotide positions useful in distinguishing the bacteroides and flavobacteria were not determined in the present study. Of 12 nucleotide positions found by Woese (1987) to be useful in distinguishing bacteroides from flavobacteria: 4 would place XM3 with the bacteroides; 4 would place it with the flavobacteria; and 1 is more typical of other eubacteria. Three others remain to be determined. The partial sequence for strain XM3, based on three internal nonoverlapping fragments, has been deposited with GenBank (accession numbers M80585, M80586, and M80587), or it can be obtained by writing the authors directly.

Cells of *Cytophaga* XM3 were catalase and oxidase negative when first isolated, but became weakly catalase positive upon prolonged laboratory subculture. Other isolates were also weakly catalase positive, and all but 2 marine strains were oxidase negative (Table 2). The DNA base composition of strain XM3 was 45.5 mol% G + C.

Xylan-degrading enzymes Oat spelt xylan-grown *Cytophaga* XM3 possessed xylanase activity which was largely cell-associated (91%) and which exhibited pH and temperature optima of 6.8 and 45°C, respectively (Table 5). The extracellular activity did not appear to be an artifact of cell lysis, inasmuch as 7 glycosidase activities assayed in similar experiments were almost exclusively cell-associated (Table 6).

Table 4. Cellular fatty acids in freshwater isolates of *Cytophaga xylanolytica*[a].

Fatty acids[b]	% of Total XM3	SL1	MA3	EW
Straight chain				
n 14:0	ND[c]	ND	3.7	ND
n 15:0	19.9 ± 3.4	12.4	27.4	42.7
n 16:0	4.1 ± 1.8	1.4	1.1	ND
Branched				
i 14:0	ND	ND	3.7	ND
i 15:0	3.7 ± 0.8	3.7	10.2	4.0
a 15:0	57.2 ± 8.7	71.0	49.3	45.1
a 17:0	1.8 ± 0.3	2.6	ND	ND
3-Hydroxy				
n 15:0	5.5 ± 2.0	1.7	4.3	8.1
a 17:0	3.3 ± 0.6	5.2	ND	ND
Total	94.1 ± 1.6	97.9	99.7	100

[a]Values for strain XM3 are the mean ± SEM of 3 independent determinations. Values for other strains are the results from a single analysis.

[b]C atoms:double bonds. *n*, normal; *i*, *iso*-branched; *a*, *anteiso*-branched.

[c]ND, not detected.

Table 5. Xylanase activity of *Cytophaga xylanolytica* XM3

	Cell-associated activity	Extracellular activity
pH optimum	6.8	6.8
50% activity	5.5 and 8.0	5.5 and 8.0
Temperature (°C)		
optimum	45	45
50% activity	30 and 49	26 and 56
Specific activity[a]	1.35	4.14
% of total culture activity	90.3	9.7

[a] μmol reducing sugar liberated per min per mg protein. Substrate was 0.5% (final conc.) oat spelt xylan (Lot #116F-0240; Sigma Chem. Co.).

Table 6. Cell-associated glycosidase activities of *Cytophaga xylanolytica* XM3[a].

	Specific Activity[b]
α-D-glucopyranosidase	0.344
β-D-glucopyranosidase	0.671
α-D-galactopyranosidase	0.703
β-D-galactopyranosidase	0.384
β-D-xylopyranosidase	5.156
β-D-fucopyranosidase	0.184
α-L-arabinofuranosidase	4.628

[a] α-D- and β-D-mannopyranosidase and acetylesterase activities were not detected. Except for a trace of α-L-arabinofuranosidase, no activity of any of the enzymes tested was found in extracellular culture fluid.

[b] μmol *p*-nitrophenol released per min per mg protein.

Xylanase-containing cell extracts (9.7-11.2 μg protein/ml) typically hydrolyzed about 50% of added oat spelt xylan (500 μg/ml) after 1 h at 40°C and pH 6.8. A maximum of 71.9% hydrolysis was observed after 48 h. Addition to reaction mixtures of fresh xylan resulted in an abrupt increase in the rate of release of reducing sugar, but addition of fresh enzyme did not. This observation suggested that some glycosidic linkages present in the xylan substrate were resistant to attack by XM3 xylanase enzyme(s). Strain XM3 possessed no α-D- or β-D-mannopyranosidase or acetylesterase activity (Table 6); and CMCase specific activity was typically 100- to 1000-fold lower than that of xylanase (data not shown). The latter observation was consistent with the inability of XM3 to grow on various celluloses or CMC (Table 1).

Discussion

Taxonomy of xylanolytic isolates The generic epithet "*Cytophaga*" currently embraces a heterogeneous assembly of chemoorganotrophic, free-living, rod-shaped gliding bacteria that have the abiltity to degrade a variety of biopolymers, but which form neither resting cells nor fruiting bodies (Reichenbach 1989c). It is a genus that is likely to undergo extensive revision once the natural relationships amongst its members become more precisely defined (Reichenbach 1989c, *personal communication*). However, until such revision dictates otherwise it seems reasonable to assign the isolates described herein (both freshwater and marine strains) to *Cytophaga*, inasmuch as most of their phenotypic properties are consistent with the genus description. In particular we note their gliding motility, uniform cellular morphology, and free-living habitat which, taken together, are inconsistent with the decriptions of other genera within the *Cytophagaceae* (Reichenbach 1989b) or *Cytophagales* (Reichenbach 1989a). Our decision is supported by the apparent presence in strain XM3 of sulfonolipids, a class of lipids widely distributed among members of the *Cytophaga* supergroup (Godchaux and Leadbetter 1983). Furthermore, the major soluble fermentation products of freshwater and marine isolates (i.e. acetate and propionate $\pm$ succinate; Tables 1-3) are the same as those formed by facultatively anaerobic *Cytophaga* species during fermentative growth (Reichenbach 1989c).

Nucleotide sequence analysis of the 16S rRNA of strain XM3 was

not especially helpful in generic assignment, because such data are almost nonexistant for cytophagas. Nevertheless, results of such analysis placed strain XM3 within the *Bacteroides* - *Flavobacterium* subgroup of the eubacteria. This is the same subgroup to which *Cytophaga johnsonae* and *Flavobacterium heparinum* [= *Cytophaga heparina*, Christensen (1980)] have been assigned (Weisburg *et al.* 1985).

The freshwater strains described herein possess several properties which make them distinct from existing *Cytophaga* species, however. Most notable among these is their relationship to oxygen. Whereas all known *Cytophaga* species are strict or (rarely) facultative aerobes, the present freshwater isolates are strict anaerobes which, although aeroduric, will not grow under conventional oxic conditions nor will use oxygen as terminal electron acceptor for metabolism (strain XM3). Fatty acid analyses (Table 4) revealed additional contrasts. Cellular fatty acid profiles of known *Cytophaga* species are dominated by $C_{15:0}$ branched isomers of the *iso*-type, whereas in the present isolates these were *anteiso*. Where *anteiso*-fatty acids have been found to occur in *Cytophaga* as major constituents, as in *C. hutchinsonii*, they have been $C_{17:0}$ (Reichenbach 1989c, and references therein). By contrast, little or no *anteiso* $C_{17:0}$ fatty acids were detected in the present isolates. Likewise hydroxy fatty acids, which occur in relatively large amounts in cytophagas (15-55% of total), were present in low amount in the current isolates (4-9%) and were identified as 3-OH-*n*-$C_{15:0}$ and 3-OH-*a*-$C_{17:0}$, two forms poorly represented among known *Cytophaga* species (Reichenbach 1989c). In

addition, the salt tolerance of the present freshwater isolates (up to 3% NaCl) was much greater than that of other freshwater or terrestrial cytophagas, which rarely grow above 1 % NaCl (Reichenbach 1989c). Finally, the mol% G+C in the DNA (45.5; strain XM3) is nearly the highest of any *Cytophaga*, with the possible exception of *C.* (*Flavobacterium*) *heparinum* (40-46) and *C. arvensicola* (43-47) which are both strict aerobes (Reichenbach 1989c).

We believe that the above-mentioned differences are significant enough to warrant creation of a new species, *Cytophaga xylanolytica*, to accommodate the xylan-degrading freshwater isolates described herein, with strain XM3 as the type strain. A description of *C. xylanolytica* is presented below. Regarding the recently-isolated marine cytophagas, it seems wisest to postpone taxonomy of these strains until they have been studied in greater detail.

Ecological significance The repeated, ready enrichment and isolation of *C. xylanolytica* and xylanolytic marine cytophagas from various sites in the United States and southern Germany suggest that they are important to the decomposition of xylan in anoxic freshwater and marine sediments. As described above, insoluble (i.e. non-heat-sterilized) xylan particles in primary enrichments quickly became colonized by an attached mass of *C. xylanolytica*-like cells (Fig. 1a) which glided over, and grew to occupy, virtually every square μm of surface area on the particles and which dominated their hydrolysis and initial fermentation. Not surprisingly, strain XM3 was found to possess most of the enzyme activities necessary for effective xylan

degradation (Biely 1985). Inasmuch as over 90% of the xylanase activity of *C. xylanolytica* XM3 is cell-associated (Table 5), we suspect that both attachment to, and gliding over, the surface of xylan particles were important in bringing the bulk of the cells' xylanase activity into contact with its substrate and in allowing such enzymes to continually gain access to new, perhaps more labile, microsites for hydrolysis. That attachment and motility are competitive traits for xylan degradation by *C. xylanolytica* is supported by the observation that such cytophagas appeared to be far less abundant in enrichments established with heat-sterilized (hence solubilized) xylan, wherein other, non-gliding, bacteria came to the fore.

Presumably, *in vitro* enrichment cultures with powdered xylan mimic natural habitats, wherein xylan-containing plant materials are normally presented to degradative microbial communities in insoluble, particulate form. Such enrichments allow for selection of xylan-degrading bacteria in which phenotypic properties other than xylan hydrolysis *per se* are equally (and perhaps more) relevant to their competitive success. However, xylan is never found in pure form in nature. It is normally complexed with, and secondary in abundance to, cellulose in plant cell walls (Biely 1985, Wilkie 1983). Therefore, effective decomposition of xylan by *C. xylanolytica* in nature probably occurs within microbial consortia containing cellulolytic bacteria, because *C. xylanolytica* strains could not grow on cellulose and possessed only low levels of CMCase activity (strain XM3).

Their attachment to, and conspicuous enrichment with, particulate xylan as opposed to heat-solubilized xylan, as well as their anaerobicity, probably both contribute to the obscurity of *C. xylanolytica* until now. Moreover, their inability to form dense, discrete, "typical" bacterial colonies on the surface of agar media, instead of film-like swarms, would decrease the chances that they would be noticed by the unsuspecting eye, a problem compounded by the tendency of cells to attach together to particles and be quickly diluted out if source material were diluted prior to direct plating (Reichenbach 1988). In retrospect, we might well have overlooked them ourselves if original anoxic enrichments were not prepared with powdered xylan and followed by isolation with agar shake tubes. These considerations suggest that the importance of *C. xylanolytica* to xylan degradation in anoxic habitats may not be fully appreciated.

Description of Cytophaga xylanolytica sp. nov. Cytophaga xylanolytica sp. nov. [xy.lan.o.ly'ti.ca. *xylan* xylan, a xylose-containing heteropolysaccharide in plant cell walls; Gr. v. *lyein* to loosen, dissolve; M.L. fem. adj. *lytica*; M.L. fem. adj. *xylanolytica* xylan-dissolving].

Slender, flexible, Gram negative rods with slightly tapering ends, 0.4 x 3-24 μm. Move by gliding motility. No endospores, cysts, or fruiting bodies formed.

Cells orange (subsurface colonies in xylan agar medium) or orange to salmon (surface swarms on xylan agar) due to the presence of carotenoid pigment(s). Flexirubins absent. Subsurface colony morphology (lenticular or spherical) and size (2 mm or spreading up to

1 cm) in xylan agar both depend on agar concentration, the latter characteristics being prevalent in media solidified with <1% agar.

Best growth occurs in Na_2S-reduced, HCO_3^-/CO_2-buffered, anoxic mineral media with mono-, di-, or polysaccharides as fermentable energy sources. Vitamins not required (strain XM3). Sulfide used as sole S source. Poor and inconsistent growth with sulfate, cysteine, or glutathione as sole S source. Methionine and thiosulfate not used as S sources. NH_4^+ (but not NO_3^- or N_2), glutamine (but not glutamate), and peptone used as sole N sources. HCO_3^-/CO_2 required or greatly stimulatory for growth. All strains fermented xylan, laminarin, lichenin, cellobiose, lactose, glucose, galactose, mannose, xylose, and arabinose as energy sources for growth. Individual strains fermented starch, pectin, inulin, pullulan, maltose, sucrose, fructose, glucuronic acid, and galacturonic acid. None fermented cellulose, carboxymethylcellulose, arabinogalactan, mannan, chitin, N-acetylglucosamine, sorbose, ribose or rhamnose. Growth occurs within a pH range of 6.1-8.7 (optimum 7.2-8.2), a temperature range of 19-37°C (optimum 30-32°C), and in media containing up to 3% NaCl.

Aeroduric anaerobe; metabolism fermentative. Weakly catalase positive (may be negative when first isolated); oxidase negative. Acetate, propionate, and succinate are major products of xylan, xylose, and glucose fermentation. Small amounts of CO_2 and H_2 also detected in detailed analyses of fermentations by strain XM3. NO_3^- and SO_4^{-2} not used as terminal electron acceptors during anaerobic growth. No growth under conventional aerobic conditions, with or without CO_2 enrichment of the atmosphere. O_2 not used as terminal electron acceptor (only strain XM3 tested).

Cellular fatty acids dominated by $C_{15:0}$ isomers, with *anteiso* > *n* > *iso* forms. Hydroxy fatty acids include 3-OH-*n*-$C_{15:0}$ and 3-OH-*a*-$C_{17:0}$ types. Preliminary analyses suggest the presence in cells of a sulfonolipid(s) of a type slightly less polar than N-acylcapnine (strain XM3).

Partial nucleotide sequencing of the 16S rRNA of the type strain places it in the *Bacteroides - Flavobacterium* subgroup of the eubacteria. The mol% G+C in the DNA of the type strain is 45.5 (Bd).

Source: Freshwater sediments and swine waste lagoon sediment.

Type strain: Strain XM3; deposited with the Deutsche Sammlung von Mikroorganismen, Braunschweig (DSM No. 6779).

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Chapter Two

The xylan-degrading enzyme system of *Cytophaga xylanolytica*

Abstract. Xylanase activity of *Cytophaga xylanolytica* strain XM3 was mainly (90-95%) cell-associated, the remainder was secreted into the culture fluid. The pH and temperature optima (6.8 and 45 ^{o}C, respectively) were similar for both the cell-associated and extracellular activities. The extracellular activity was not sedimentable by ultracentrifugation, however approximately half of the cell-associated activity was associated with cell wall fragments. Treatment of whole cells with 0.2% Triton X-100 released 74% of the xylanase activity without lysing the cells, suggesting that xylanase was associated with the outer membrane and/or with the periplasmic space. This was supported by transmission electron microscopy of Triton-treated cells. Isoelectric focusing of Triton extracts of cells, followed by activity stains, suggested that xylose- and xylan-grown cells had common endoxylanase (pI 4.3), arabinofuranosidase (pI 5.85), and xylosidase (pI 4.6) (iso)enzymes. However, xylan-grown cells produced additional xylanase activity bands (pI 4.5 - 5.1 and > 5.5) as well as several lichenanases. An arabinofuranosidase with the same pI as the cell associated protein was found in supernatants of both xylan- and xylose-grown cultures, but neither supernatant contained a β-xylosidase. Both supernatants also exhibited endoxylanase activity bands not found in the Triton extracts. Xylanase and lichenanase were fully induced only when cells were grown on the homologous polymer; however, cells retained low, constitutive levels of xylanase and lichenanase when grown on monosaccharide components of the respective polymer, and even when grown on other polymers. Glycosidase activities followed similar patterns.

Next to cellulose, hemicelluloses are the most abundant polysaccharides in nature (Wilkie 1983), yet our understanding of the decomposition of these heteropolysaccharides by microbes is limited (Biely 1985, Wong et al. 1987). Xylans are a major class of cell-wall hemicellulose widely distributed among both terrestrial and aquatic plants. They are complex polymers consisting of a β-1,4-linked xylopyranoside backbone variously substituted with α-1,3-arabinofuranoside, α-1,2-methylglucuronic acid, and acetyl side groups, the nature and number of which may vary from plant to plant or within different tissues of the same plant (Wong et al. 1987, Wilkie 1983). Typically, xylan-degrading fungi and bacteria possess endoxylanase (E.C. 3.2.1.8) and xylobiase (E.C. 3.2.1.37) activities which degrade the xylan backbone (Biely 1985). In many cases, a given microorganism possesses multiple endoxylanases which differ in substrate specificity or mode of action (Wong et al. 1987). Xylan-degrading microbes may also possess debranching activities, such as arabinofuranosidase (E.C. 3.2.1.55), α-4-O-Me-D-glucuronidase (Puls et al. 1987, Johnson et al. 1989), acetyl- or acetyl-xylan esterase (Biely et al. 1986, Wood and McCrae 1986, Hespell and O'Bryan-Shah 1988), or ferulic acid esterase (MacKenzie and Bilous 1988, Borneman et al 1990). However, these accessory enzymes have been much less studied (Biely 1985).

When microbes encounter xylan in nature,it will exist in insoluble, relatively refractory complexes with other plant polymers. Among the microbes typically associated with the degradation of plant materials, those studied most extensively with respect to their

ability to produce xylan-degrading enzymes include white-rot fungi such as *Trichoderma*, the yeast *Cryptococcus*, the rumen bacteria *Bacteroides*, *Ruminococcus*, *Butyrivibrio*, and *Fibrobacter*, the Gram positive bacterial genera *Clostridium* and *Bacillus*, and the two actinomycetes *Streptomyces* and *Thermomonospora*. Most of the above microorganisms secrete xylanases into the culture fluid. Consequently, it has been these secreted enzymes which have been best studied. Additionally, many of these microorganisms were originally isolated and studied for their cellulose-degrading activities, and in some cases (*Clostridium thermocellum* and *acetobutylicum*, *Fibrobacter succinogenes*, *Cellulomonas fimi*) will not grow on xylan.

Bacteria of the genus *Cytophaga* are widely recognized for their ability to degrade a diverse array of biopolymers, including cellulose (Reichenbach 1988). Ironically, little work has been done on the mechanisms of biopolymer decomposition by this ubiquitous group of bacteria. Cytophagas are typically aerobic, Gram negative, non-fruiting rods which exhibit gliding motility (Reichenbach 1989). We are studying a new, anaerobic, non-cellulolytic *Cytophaga* (*C. xylanolytica* sp. nov.; manuscript accepted) originally isolated from freshwater sediments on the basis of its ability to adhere tightly to, and dominate the hydrolysis of, particulate xylan. The majority of the xylanase activity of *C. xylanolytica* was found to be cell-associated. We have hypothesized that several traits of this and other species of *Cytophaga* are central to their biopolymer degrading ability, including their attachment to and ability to glide over, insoluble substrates and the location and pattern of regulation of

their polymer-degrading enzymes. This study describes some of the basic characteristics of the xylan-degrading enzyme system of *C. xylanolytica* XM3, from which we hope to gain a better understanding of how bacteria which retain almost all of their polymer-hydrolyzing enzymes compete for polymeric substrates in nature.

Methods and materials

Microorganism and culture conditions

Cytophaga xylanolytica strain XM3 was used in all studies. Its enrichment, isolation, and detailed description were previously published (Haack and Breznak 1992). Cells were routinely cultured at 30 °C in a defined mineral medium with 0.1-0.2% oat spelt xylan (Lot # 116F-0240, Sigma Chem. Co., extracted with 70% ethanol before use) or 10 mM xylose as carbon source.

Enzyme assays

Depolymerizing activities were determined by measuring the release of reducing sugars from polymeric substrates. Reaction mixtures (1 ml) typically contained: 500 ug oat spelt xylan (previously extracted with 70% ethanol) or lichenan (both Sigma Chem. Co.; solubilized by heating a 0.1% aqueous solution at 80 °C for 10 min) or 2.5 mg carboxymethylcellulose (CMC, Na salt; Sigma Chem. Co.); N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) buffer (pH 6.8), 50 umol; $CaCl_2$ 500 nmol; and enzyme solution (added to start the reaction). The HEPES and $CaCl_2$ were usually prepared as a single stock solution containing, respectively, 0.1 M and 1 mM of each component (=HEPES/CaCl). Unless stated otherwise, incubation was at 40 °C for 20 min for xylanase or lichenanase assays, and up to 24 hr for CMCase assays. Reducing sugar liberated was quantified by colorimetric assay [Somogyi (1952) adaptation of Nelson 1944] with xylose or glucose as standard. Glycosidase and acetylesterase activities were determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl

substrates (Greve et al. 1984). Reaction mixtures contained 1 ml substrate (10 mM in 0.1 M HEPES/$CaCl_2$) and 0.2-1.0 ml enzyme solution. Assays were conducted at 40 ^{o}C and were terminated when color development was measureable or for a maximum of 20 min. Determination of pH optima was performed by using series of 0.1 M Good buffers (Ferguson and Good 1980) for pH values between 6.0 and 10, and 0.1 M acetate buffer for pH values between 4.0 and 5.0. Determination of pH stability was done by suspending enzyme preparations in 0.05 M buffers of various pH for 3 h at 30 ^{o}C, then diluting the preparation 1/5 with 0.1 M HEPES/$CaCl_2$ before assay. Protein was measured by the modified Lowry method (Peterson 1977) after solubilization with sodium dodecylsulfate and precipitation with trichloroacetic acid according to Sigma Chem. Co. protein assay protocol P5656.

Localization of xylanase activity

Crude preparations of cell-associated xylanase were obtained after separating cells (late exponential to early stationary phase) from culture fluid by centrifugation (10000 x *g*, 20 min, 10^{o}C), resuspending them in 1/10 their original volume with 0.01M HEPES/$CaCl_2$ or with medium containing no added substrate, and then disrupting the cells at 4 ^{o}C by using a Branson Sonifier (1.5-2 min, 60% duty cycle, output 4-5). The culture supernatant fluid (crude extracellular fraction) was filtered (Millipore GSWP or Anotop; 0.22 um pore size) to remove unpelleted cells, and then was either used without further treatment or was dialyzed (12-14 kD mol. wt. cutoff) for 3 h at 4^{o}C against 3 changes of ca. 50 volumes each of 0.01 M HEPES/$CaCl_2$. Dialyzed preparations were subsequently concentrated by

ultrafiltration through an Amicon PM10 membrane under 20 psi N_2. For ultracentrifugation (100,000 x *g*, 2 h, 5°C), the crude extracellular fraction was used without concentration, and the crude cell-associated fraction was resuspended to the original culture volume in medium with no added substrate.

Fractionation of extracellular xylanase activity

The crude extracellular fraction (150 - 500 ml) was concentrated by $(NH_4)_2SO_4$ precipitation (80% saturation) at 4°C for 4 h. The precipitate was resuspended to 1/100 the original volume with 0.01% Triton X-100 (w/v; RPI) and dialyzed against 3 changes of 2 l each of 0.01% Triton X-100 at 4°C. The dialyzed sample was then centrifuged (10,000 x *g*, 20 min, 10 °C) to remove particulate material (especially noted in xylan cultures) and the resulting supernatant, which contained the majority of protein and xylanase activity, was concentrated with a Centricon 10 or 30 centrifugal microconcentrator (Amicon) to a typical working concentration of 1-10 mg protein/ml (ca. 2000-fold volume reduction from original culture for xylose-grown cells and ca. 600-fold for xylan-grown cells). For xylose-grown cultures, this procedure resulted in a tan to brown solution which could be used directly in isoelectric focusing (IEF) experiments.

In xylan-grown cultures, the supernatant contained a yellow soluble material (presumably carbohydrate) which, if the above procedure was followed, resulted in an extremely dark brown solution containing substances which interferred with IEF. The yellow material could be removed from the crude extracellular fraction by anion-exchange chromatography on DEAE-Sephadex A50 (Sigma Chem. Co.)

equilibrated with 0.01 M potassium phosphate buffer (pH 7). Substantial protein and xylanase activity appeared in the void volume as well as in fractions eluted with 0.1 M potassium phosphate buffer (pH 7) containing > 0.9 M NaCl. Neither NaCl in concentrations up to 1.5 M nor a reduction of the pH to 4.0 would remove the yellow material from the DEAE-Sephadex A50 column. Therefore, a batch procedure was employed as follows: DEAE-Sephadex A50 previously equilibrated with 0.01 M potassium phosphate buffer (pH 7) was added to the crude extracellular fraction at a ratio of 1:6 (v/v). The mixture was stirred gently at 4°C for 30 min, then filtered through a 0.45 um filter (Millipore GSWP) under vacuum, being careful not to allow the gel to become dry. The filtrate was designated Fraction 1 (F1). The gel was washed from the filter with 10 ml of 0.1M potassium phosphate buffer (pH 7) containing 1.2 M NaCl, stirred at 4 °C for 60 min, then filtered again, this time allowing the gel to become dry; this filtrate was designated Fraction 2 (F2). These fractions were each concentrated (Amicon, PM10) to 1-2 mg protein/ml.

Fractionation of cell-associated xylanase activity

Since xylan was believed to be too large a molecule to enter the cells, it was assumed that the cell-associated activity must be located in or on the cell wall; therefore, various treatments were employed in an effort to extract the cell-associated activity without disrupting the cells. Unsonicated cell pellets were suspended to 1/10 the original culture volume with each of the following solutions: medium containing no added substrate; distilled water; 50 mM EDTA; 0.2 or 1 M NaCl; 50 mM potassium phosphate buffer (pH 6.8); 0.1M

HEPES/$CaCl_2$ (pH 6.8); or 0.01% or 0.1% Triton X-100 (w/v; dissolved in either distilled water or 50 mM potassium phosphate buffer pH 6.8). The suspensions were mixed gently for 30 min at 4 ^{o}C. In a separate experiment, cells were resuspended in 1/50 culture volume (1.5 ml) of phospholipase C (Celesk and London 1987; Sigma Chem. Co., 62.5 U) in 0.15 M triethanolamine (pH 7.6) containing 0.03 M $CaCl_2$. Cells were kept at 37 ^{o}C for 3 h with periodic gentle mixing before being brought to 1/10 culture volume with 0.01 M HEPES/$CaCl_2$. The extracts (supernatants) and cells (sonicated as previously described) obtained from each of the above treatments after centrifugation at 10,000 x *g* (20 min, 10 ^{o}C), were assayed for distribution of protein and xylanase activity.

In an additional experiment, cells were subjected to osmotic shock essentially as described in Huang and Forsberg 1987. Cells previously washed with 0.1 M HEPES/$CaCl_2$ were sequentially suspended in sucrose/EDTA, then in ice cold distilled water. After centrifugation at 30,000 x *g* (20 min, 10 ^{o}C), the resulting supernatant was collected as the periplasmic fraction. The cell pellet, resuspended in 0.01 M HEPES/$CaCl_2$, was sonicated and centrifuged again to obtain membrane (pellet) and cytoplasmic (supernatant) fractions. All supernatants, as well as designated fractions, were assayed for distribution of protein and xylanase activity.

Extraction with 0.1% Triton X-100 (whether dissolved in water or phosphate buffer) removed the most protein and activity from the

cells. For all subsequent experiments, 0.2% (chosen to optimize the detergent to extractable protein ratio) Triton X-100 (w/v in distilled water) was used to extract xylanase activity from the cells. The 0.2% Triton extract from xylan-grown cells was further subjected to fast protein liquid chromatography (FPLC) Triton extract (4.1 ml containing ca. 3.4 mg protein) was applied to a MONO-Q column equilibrated with 50 mM HEPES pH 7.1. Fractions were eluted with a linear gradient of 0-2 M NaCl. The flow rate was 1 ml/min, and 1 ml fractions eluted from the column were each analyzed for protein (A_{280} and Lowry) and xylanase activity. Fractions exhibiting xylanase activity were combined (4-7 ml), concentrated to ca. 0.5 ml (Amicon, PM10). Triton X-100 was then added to a final concentration of 0.2% and the solution was further concentrated (Centricon 10) to 1-4 mg protein/ml.

Isoelectric focusing and activity stains

Analytical thin layer IEF was performed by using the Bio-Rad Bio-Phoresis Horizontal Electrophoresis Cell and application protocols supplied by the manufacturer. Typical procedures employed 1% agarose gels (0.8 mm) with Bio/Lyte ampholytes and both Bio-Rad and Pharmacia (low pH range) IEF standards. Gels were stained for protein with Coomassie R-250 (Bio-Rad). After IEF the separation gel was overlayered with a 0.4 mm thick activity gel of 1% agarose (prepared in 0.1M HEPES/$CaCl_2$) cast on agarose support film (Bio-Rad) as described in Lee and Forsberg (1987) and Biely and Markovic (1988). Activity gels contained either 0.5-1% Remazol Brilliant Blue-dyed xylan (RBB-xylan, Biely 1985), 1% oat spelt xylan (Lot # 116F-0240),

1% lichenan, or 0.1-0.2% 4-methylumbelliferyl-glycosides (MU-glycosides). All substrates were from Sigma Chem. Co. Activity gels were prewarmed to 40 ^{o}C and the sandwich was kept at 40 ^{o}C for 1-2 h (RBB-xylan, oat spelt xylan or lichenan) or 10 min-1 h (MU-glycosides). MU-glycoside hydrolysis was visualized by exposure of the gels to UV light (Biely and Markovic 1988). RBB-xylan hydrolysis was observed as a clear zone after 30 min- 1 h rinse with 0.05 M acetate buffer (pH 5.4) in 95% ethanol (1:2, v/v, Biely et al. 1985). Oat spelt xylan and lichenan hydrolysis were observed as clear zones after staining the activity gel with Congo Red (1 mg/ml for lichenan; 10 mg/ml for xylan) for 15 min with subsequent rinsing in 1 M NaCl (Teather and Wood 1982). Since freshwater strains of *Cytophaga* have been reported to hydrolyze agar (Agbo and Moss 1979), a control activity gel containing no added substrate was tested on one occasion.

Other procedures

Previously described methods were used for electron microscopy (Breznak and Pankratz 1977) and high performance liquid chromatography (HPLC; Breznak and Switzer 1986). To test for a requirement by the cells for contact with xylan for growth, a method similar to that of Kauri and Kushner (1985) was used in which xylose- or xylan-grown cells were inoculated onto the surface of 0.05 um filters (Millipore) held on the surface of agar medium plates containing either 0.4% xylan or no added substrate.

Results

Characteristics of crude enzyme fractions

General characteristics of the crude extracellular and cell-associated fractions were previously reported (Haack and Breznak 1992). Both exhibited similar pH and temperature optima (6.8 and 45 °C, respectively). Both fractions exhibited 50% or greater activity from pH 5.5 to 8.0, and from 30 to 50 °C. The crude cell-associated fraction maintained stable levels of activity for 3 h at 30 °C in the pH range 5.5-8.3. The crude cell-associated fraction typically hydrolyzed 40-50% of added (0.5%) oat spelt xylan in 1 h at 30 °C and pH 6.8; a maximum hydrolysis of 70% was obtained in one assay. The decrease in activity after 1 h was not due to enzyme instability, because addition of fresh xylan at 3 h caused an immediate increase in activity, but addition of fresh enzyme did not. In addition to xylanase activity, the crude cell-associated fraction exhibited α- and β-D-glucopyranosidase, α- and β-D-galactopyranosidase, β-D-fucopyranosidase, β-D-xylopyranosidase, and α-L-arabinofuranosidase activities, but no α- or β-D-mannopyranosidase or acetylesterase activity (Haack and Breznak 1992). Except for a trace of arabinofuranosidase, no glycosidase or acetylesterase activity was detected in the crude extracellular fraction. There was no evidence of synergism when the crude extracellular and crude cell-associated fractions were recombined.

Localization of enzyme activity

Between 90 and 95% of the total whole-culture activity of xylan-grown cultures was typically associated with the cells (Table 1; pellet),

TABLE 1. Distribution of xylanase in *Cytophaga xylanolytica* XM3 grown on 0.2% oat spelt xylan (Sigma, Lot # 116F-0240) or 10 mM xylose.

	XYLAN-GROWN		XYLOSE-GROWN	
	SP. ACT.[a]	% TOTAL CULTURE ACTIVITY	SP. ACT.	% TOTAL CULTURE ACTIVITY
WHOLE CULTURE	0.543	100.0	0.057	100.0
SUPERNATANT[b]	0.101	7.6	0.000	0.0
ULTRACENTRIFUGED[c]				
Supernatant	0.149	7.1	ND[d]	ND
Pellet	2.000	0.4	ND	ND
SEPHADEX FRACTIONS				
F1	0.693	2.3	ND	ND
F2	0.343	1.4	ND	ND
PELLET[b]	0.753	92.4	0.064	100.0
SONICATED/ULTRACENTRIFUGED[c]				
Supernatant	0.474	53.9	ND	ND
Pellet	0.552	55.2	ND	ND
TRITON EXTRACT	1.660	73.9	0.156	141.7
FPLC Fractions				
FA	0.555	1.3	ND	ND
FB	0.265	4.7	ND	ND
FC	0.276	2.1	ND	ND
FD	0.291	0.4	ND	ND
FX	0.000	0.0	ND	ND
FA+FB+FC+FD+FX	0.484	14.0	ND	ND

[a] umol reducing sugar released x min^{-1} x mg $protein^{-1}$
[b] 10,000 x *g*, 20 min;sonicated
[c] 100,000 X *g*, 2 hr
[d] ND, not determined

and could be sedimented by low speed centrifugation. For xylose-grown cultures, concentration of the supernatant revealed extracellular activity which could not be detected in unconcentrated preparations. When both crude fractions of the xylan-grown cultures were ultracentrifuged, the majority of the original supernatant extracellular activity remained in the soluble fraction (supernatant; Table 1); however, approximately half of the original culture pellet activity remained with the insoluble (pellet) fraction after ultracentrifugation (Table 1).

Exposure to 0.2% Triton X-100 for 1 h at 4 ^{o}C released almost all of the xylanase from cells, accounting for 73.9% of the culture activity for xylan-grown cells and 142% for xylose-grown cells (Table 1). Similar treatments with 0.2 M or 1 M NaCl, 50 mM potassium phosphate buffer (pH 6.8), 50 mM EDTA, 0.1M HEPES/$CaCl_2$ (pH 6.8), distilled water, growth medium with no added substrate, and enzymatic attack with phospholipase C were not effective in removing substantial amounts of protein or activity from the cells. The specific activity of xylanase in the cell-free Triton extracts of both xylan- and xylose-grown cells was approximately 3-fold higher than for the corresponding cell-associated activity (Table 1). The Triton extracts of both xylan- and xylose-grown cells were remarkably stable, retaining nearly full activity for > 6 mo. when stored at 4 ^{o}C.

Triton treatment did not appear to cause marked disruption of cells, which retained their rod-shaped morphology and phase-dark character when observed by phase contrast microscopy. However,

electron microscopy revealed that the Triton treatment appeared to draw the outer membrane closer to the cell wall, thereby collapsing somewhat the rather thick periplasmic space typical of untreated cells (Fig. 1). Moreover, vesicles found as outer membrane blebs or free in the intracellular space around untreated cells were not as apparent in the Triton-treated preparations (Fig. 1). Similarly, after osmotic shock, 38.6% of the cell-associated activity was cytoplasmic, 47.8% was found in the membrane fraction, and 13.5% was periplasmic. In this same experiment, virtually all β-D-galactosidase activity was associated with the cytoplasmic fraction, while 16.3%, 71.0%, and 12.8% of the β-D-xylosidase activity was membrane associated, cytoplasmic, or periplasmic, respectively. Together, the results from Triton treatment as well as from osmotic shock suggested a cell wall (both membrane and periplasmic space) location for the majority of the xylanase activity of *C. xylanolytica* XM3,

Fractionation of xylanase activity

Treatment of the culture supernatant of xylan-grown cells with DEAE-Sephadex A50 removed (presumed) residual carbohydrate and yielded two fractions with combined protein and activity representing approximately 50% of that found in the crude supernatant fraction (Table 1). This treatment was not required for the supernatants of xylose-grown cells.

FPLC, used to fractionate xylanase activity present in the Triton extracts of xylan-grown cells, yielded four fractions with xylanase activity (FA-FD) and one non-active fraction (FX) (Fig.2). These

FIGURE 1. Transmission electron micrographs of thin sections of xylan-grown cells of *Cytophaga xylanolytica* strain XM3. 1A, 1B; normal, untreated cells. 2A, 2B; cells treated with 0.2% Triton X-100 for 1 hr at 4 ^{o}C. Bars: 1A, 2A, 0.5 um; 1B, 2B, 0.2 um.

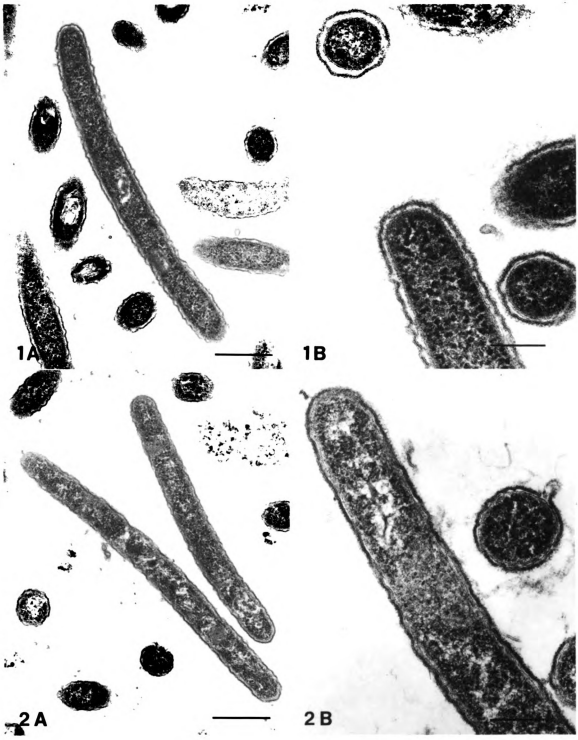
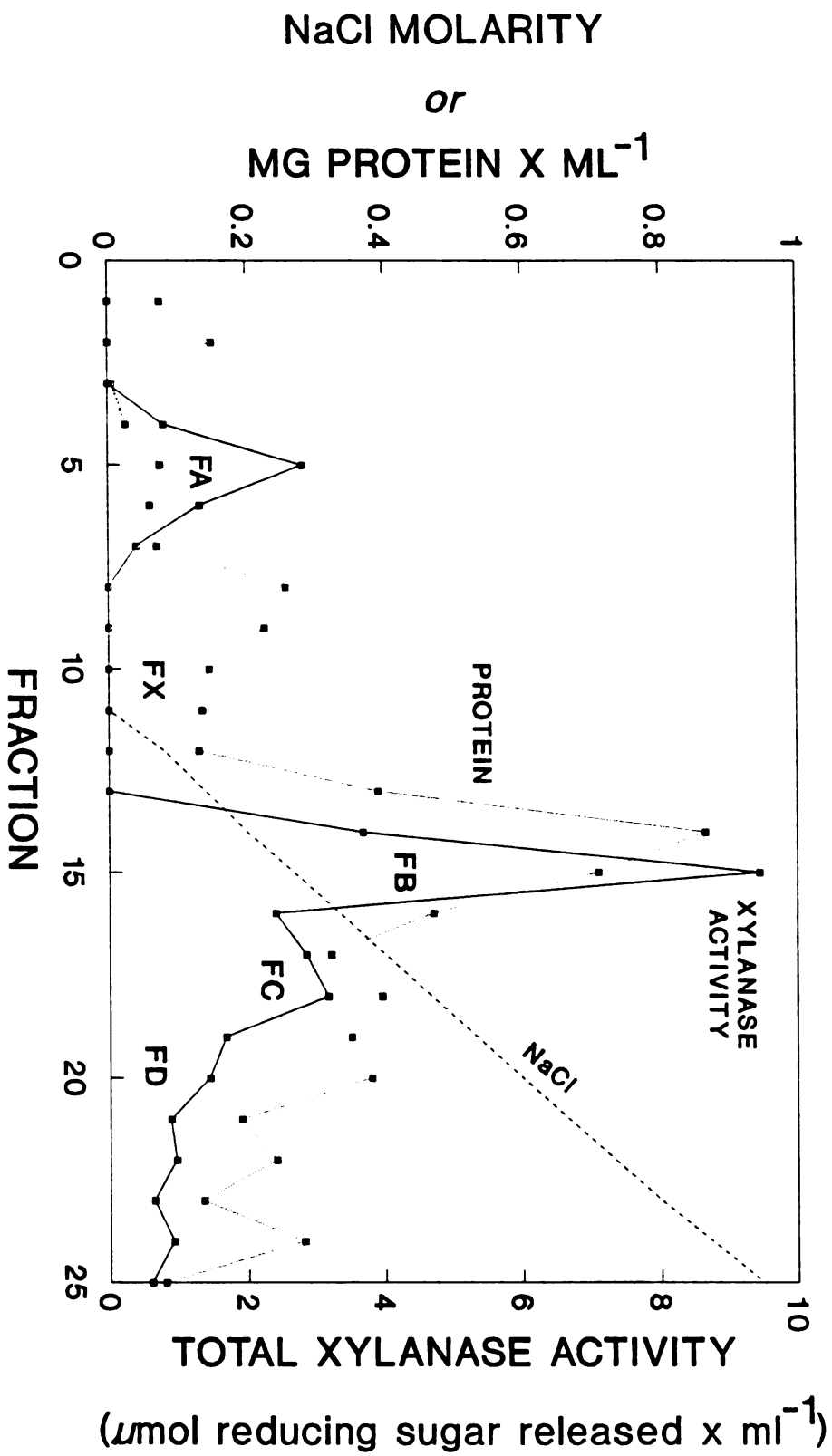

1A
1B
2A
2B

FIGURE 2. FPLC fractionation of protein and xylanase activity from the Triton extract of cells of *Cytophaga xylanolytica* XM3. MONO-Q column; linear NaCl concentration gradient in 0.05 M HEPES pH 7.1 **FA**: Fractions 1-7; **FX**: Fractions 8-13; **FB**: Fractions 14-17; **FC**: Fractions 18-21; **FD**: Fractions 22-26.


NaCl MOLARITY
or
MG PROTEIN X ML-1
0
0.2
0.4
0.6
0.8
1
FRACTION
0
5
10
15
20
25
FA
FX
FB
FC
FD
PROTEIN
XYLANASE ACTIVITY
NaCl
0
2
4
6
8
10
TOTAL XYLANASE ACTIVITY
(μmol reducing sugar released x ml-1)

fractions together accounted for 100% of the protein but only 19% of the total activity applied to the column. The activity of individual fractions, when summed, accounted for only 8.5% of the total culture activity (Table 1). However, when these five fractions were recombined (maintaining the same relative proportions of total protein per fraction) the total activity was increased to ca. 14% of the total culture activty (Table 1), suggesting a synergistic interaction of enzyme activity between the fractions. Although FPLC fractionation apparently resulted in significant loss of enzyme activity, it allowed increased resolution of the pI's of endoxylanases in the 4.5-5.1 range (see below).

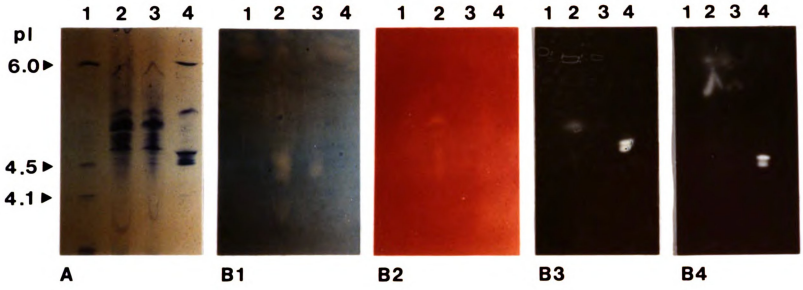
Isoelectric focusing

Analytical isoelectric focusing indicated that the Triton extracts of both xylan- and xylose-grown cells contained several proteins, all with pI < 6.5 (Fig. 3). Activity stains suggested that xylan- and xylose-grown cells possess common xylanase, arabinofuranosidase, and B-xylosidase (iso)enzymes (Fig. 3, Table 2); however, xylan-grown cells exhibited additional xylanase activity bands representing proteins with pI's ranging from 4.5 - 5.9, as well as several bands with lichenanase activity, none of which were easily separated by isoelectric focusing of the complete Triton extract (Fig. 3). Xylan-grown cells exhibited extracellular xylanase activity at five distinct pI's, whereas xylose-grown cells exhibited only three (Table 2). An arabinofuranosidase activity band with the same pI as the cellular protein was found in the supernatant of both xylan- and xylose-grown cells, but neither supernatant displayed a xylosidase activity band

TABLE 2. Isoelectric point (pI) of carbohydrases of *C. xylanolytica* XM3.

	XYLANASE	XYLOSIDASE	ARABINOSIDASE	LICHENANASE
XYLAN-GROWN				
TRITON EXTRACT OF CELL PELLET	4.3	4.6	5.85	4.8
	4.5			4.9
	4.7			5.0
	4.8			5.4
	5.0			
	5.4			
	5.9			
CULTURE SUPERNATANT	4.0		5.85	
	4.2			
	4.5			
	4.7			
	5.1			
XYLOSE-GROWN				
TRITON EXTRACT OF CELL PELLET	4.3	4.6	5.85	
CULTURE SUPERNATANT	4.0		5.85	
	4.6			
	4.7			

FIGURE 3. Analytical isoelectric focusing of Triton extracts of xylan- or xylose-grown cells of *Cytophaga xylanolytica* strain XM3. **A**: Coomassie protein stain. **B**: activity stain; B1, xylan hydrolysis (Remazol Brilliant Blue dyed xylan); B2, lichenan hydrolysis (Congo Red); B3, Methylumbelliferylxyloside hydrolysis (Photographed under UV illumination); B4, Methylumbelliferylarabinofuranoside hydrolysis. **LANES**: 1 and 4, pI Standards (positions of selected pI markers are indicated); 2, Triton extract of xylan-grown cells; 3, Triton extract of xylose-grown cells.


1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4
pI
6.0
4.5
4.1
A
B1
B2
B3
B4

(Table 2). This pattern suggested that the extracellular arabinofuranosidase activity was not the result of cell lysis. FPLC fraction FB, which contained the greatest total xylanase activity (Fig. 2), exhibited xylanase activity bands at pI 4.5, 4.7, 4.8, 5.0, and 5.4, as well as the arabinofuranosidase and the xylosidase activity bands (Table 2, Fig. 4). Three of the xylanase activity bands in FPLC fraction FB appeared to also have lichenanase activity; however, this fraction also contained an additional lichenanase activity band at pI 4.9 (Table 2). Some xylanase activity bands present in fraction FB also had analogues in fractions FC (pI 4.5) and FA (4.7 and 4.8); however, fractions FA and FC both contained a xylanase activity band at pI 4.3 which was not seen in fraction FB. Fraction FA also displayed a band at pI 5.9 (Table 2, Fig. 4). FPLC fraction FD exhibited no discrete activity band on IEF. No activity bands corresponding to β-glucosidase or cellobiohydrolase were detected in FPLC fractions FA, FB, or FC after 1 h or 24 h incubation.

Induction of enzyme activity

In *C. xylanolytica* XM3, the enzymes for degrading xylan or lichenan were fully induced only when cells were grown on the appropriate polymer (Table 3); however, cells retained low constitutive levels of xylanase and lichenanase when grown on monosaccharide or disaccharide constituents of the appropriate polymer, and even when grown on other polymers (Table 3). Glycosidase activities followed similar trends (Table 3). *C. xylanolytica* could not grow on cellulose (ball-milled filter paper, or Sigmacell 20) or carboxymethylcellulose (Haack and Breznak 1992). Thus, it was not surprising that CMCase specific

FIGURE 4. Analytical isoelectric focusing of FPLC fractions of Triton extracts of xylan-grown cells of *C. xylanolytica* strain XM3. A: Coomassie protein stain. B: activity stain; clear zones after staining with Congo Red indicate xylan hydrolysis. Lanes: STDS, pI standards (positions of selected pI markers are indicated); FA, FB, FC, FPLC fractions (see Fig. 2).

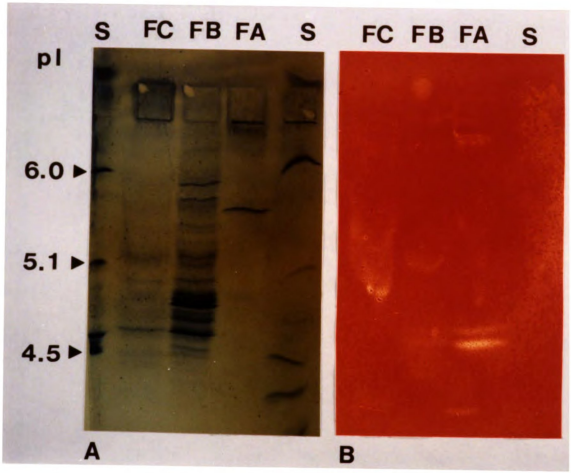

S
FC
FB
FA
S
FC
FB
FA
S
pI
6.0
5.1
4.5
A
B

TABLE 3. Depolymerase and glycosidase specific activities[a] of *Cytophaga xylanolytica* XM3 when grown on various polymer or monomer substrates.

	ENZYME[b]						
	XYLANASE	LICHENANASE	CM-CELLULASE	ARABINOSIDASE	XYLOSIDASE	GLUCOSIDASE	CELLOBIOSIDASE
GROWN ON							
10 mM XYLOSE	0.041	ND[c]	0.0002	0.369	0.009	0.021	0.0006
5 mM XYLOBIOSE	0.074	ND	0.0009	0.871	0.005	0.025	0.0008
5 mM XYLOSE AND 5 mM ARABINOSE	0.031	ND	0.0004	2.290	0.015	0.015	ND
0.1% XYLAN	0.423	0.026	0.0006	1.038	0.318	0.030	0.0007
0.1% XYLAN AND 5 mM XYLOSE	0.308	0.015	0.0002	1.795	0.066	0.031	ND
10 mM GLUCOSE	0.060	0.022	0.0007	1.145	0.029	0.023	0.0005
5 mM CELLOBIOSE	0.028	0.078	0.0004	0.383	0.019	0.287	0.0144
0.1% LICHENAN	0.068	0.345	0.0011	1.274	0.072	1.572	0.0301
0.05% XYLAN AND 0.05% LICHENAN	0.293	0.131	0.0008	0.718	0.018	0.220	0.1026

Table 3 (cont'd.)

[a] umol reducing sugar or *p*-nitrophenol released from a given polymeric or *p*-nitrophenyl-glycoside substrate x min^{-1} x mg^{-1}

[b] Lichenanase, 1,3-1,4-β-D-glucan 4 glucanohydrolase (EC 3.2.1.73); CM-cellulase, carboxymethyl-cellulase; Arabinosidase, α-L-arabinofuranosidase (EC 3.2.1.55); Xylosidase, β-D-xylopyranosidase (EC 3.2.1.37); Glucosidase, β-D-glucopyranosidase (EC 3.2.1.21); Cellobiosidase, cellobiohydrolase or exoglucanase (EC 3.2.1.91)

[c] ND, not detected.

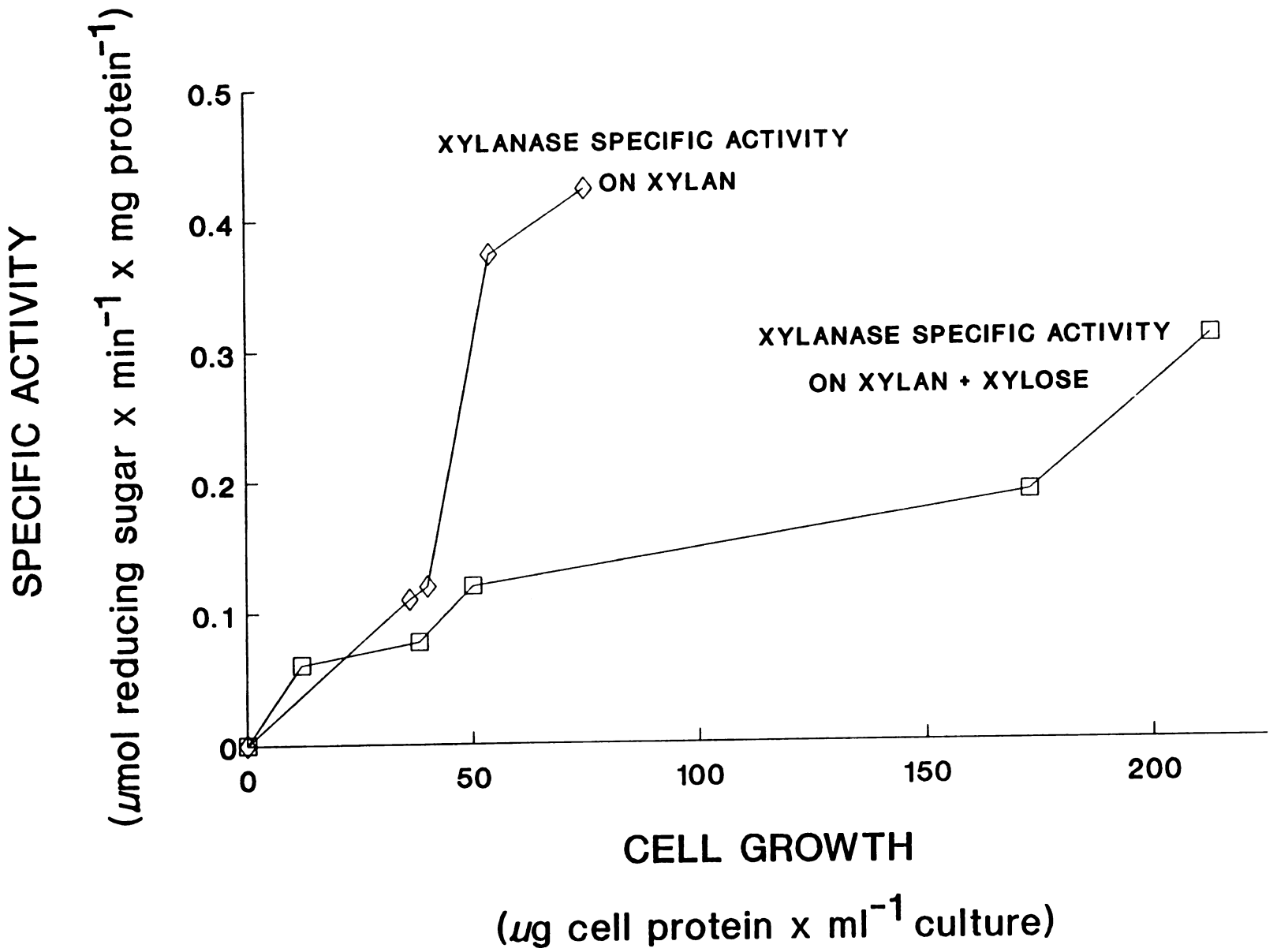
activity was 100- to 1000-fold lower than that of xylanase activties.

Starvation for 48 hr after growth on xylose also did not result in induction of xylanase activity (data not shown). When grown in a medium in which xylose or lichenan was included with oat spelt xylan, late exponential phase cultures exhibited an appreciable level of xylanase (and, in the latter case, also lichenanase) activity, but which was still less than that observed when grown on xylan alone (Table 3). The induction of xylanase activity during cell growth on 5 mM xylose plus 0.1% xylan was investigated at several points in the growth curve (Fig. 5). Xylose steadily disappeared from the culture medium and was completely utilized by the time culture protein values had reached 100 ug/ml data not shown). Xylanase specific activity increased steadily during growth, and was approximately 5-fold greater than uninduced levels even at early time points in the growth curve when as much as 3 mM xylose remained in the medium; the greatest increases in specific activty occurred after xylose was depleted (ca. 150 ug/ml cell protein; Fig. 5). Cells grown on xylan alone exhibit fully induced xylanase activity at much lower cell densities (Fig. 5). Such cells do not appear to release xylose into the medium during growth on xylan (detection limit 0.1 mM).

Other studies

In experiments with *C. xylanolytica* XM3 in which cells were grown with combinations of sugars (5 mM xylose + 5 mM arabinose, or 5 mM cellobiose + 5 mM glucose), both substrates disappeared from the medium concurrently, as detected by HPLC (data not shown).

FIGURE 5. Effect of xylose on induction of xylanase activity by xylan in *Cytophaga xylanolytica* XM3. Xylose-grown cells were inoculated into medium containing only 0.1% oat spelt xylan (Sigma; Lot # 116F-0240) or 0.1% xylan + 4.2 mM xylose.


SPECIFIC ACTIVITY
(μmol reducing sugar x min^{-1} x mg protein^{-1})
0.5
0.4
0.3
0.2
0.1
0
XYLANASE SPECIFIC ACTIVITY
ON XYLAN
XYLANASE SPECIFIC ACTIVITY
ON XYLAN + XYLOSE
0
50
100
150
200
CELL GROWTH
(μg cell protein x ml^{-1} culture)

In a test similar to that of Kauri and Kushner (1985), it was found that *C. xylanolytica* (previously grown on either xylose or xylan) could grow on 0.05 um membrane filters which were placed on the surface of agar plates containing 0.4% xylan as sole carbon source. No growth occurred on filters placed on 1.5% agar alone, and clearing of the underlying xylan could be seen when the filter was removed, although all growth was confined to the filter surface.

Conclusions

The majority of the xylan-degrading enzyme system of *C. xylanolytica* XM3 is cell-associated. The effectiveness of Triton treatment in removing cell-associated activity, taken together with the intact appearance of the cells after Triton treatment, and the association of substantial activity with insoluble material after ultracentrifugation of sonicated cells strongly suggest that the xylanase activity of *C. xylanolytica* is largely associated with the outer membrane of the cell wall. However, the release of some xylanase by osmotic shock suggests that some of the xylanase activty may reside in the periplasmic space.

An outer membrane or cell-surface location was originally proposed for *Cytophaga* polymer-hydrolyzing enzymes by Stanier in 1942, and this has been confirmed for several enzymes or enzyme complexes of cytophagas including agarase (Duckworth and Turvey 1969), protease(s) (Christison and Martin 1971), carboxymethycellulase (Chang and Thayer 1977), amylase (McKay 1991), and a dextranase (Janson 1975). In the closely-related *Sporocytophaga myxococcoides,* from 10% to 100% of the cellulase activity was reported to be located in the cell wall, and both groups found the activity to be extractable with Triton X-100 (Charpentier and Robic 1974, Osmundsvag and Goksoyr 1975). Additionally, in *Bacteroides* a genus related to *Cytophaga* on the basis of 16S rRNA homology (Reichenbach 1989), an outer membrane mannanase (*B. ovatus*; Gherardini and Salyers 1987), an inner membrane associated polygalacturonic acid lyase (*B. thetaiotaomicron*; McCarthy et al. 1985), and a protease located on the surface of the outer membrane (*B. gingivalis*; Grenier and McBride 1989) could all be released from

isolated membranes by Triton X-100.

In no case is the exact nature of the interaction of these enzymes with the cell wall understood, and in almost all the *Cytophaga* enzyme studies cited above, some activity could also be found in the culture supernatant. Both Duckworth and Turvey (1969) and Christinson and Martin (1971) proposed an interaction of cell-associated enzymes with extracellular polysaccharide (slime) which is produced by many *Cytophaga* species (Reichenbach 1989). Bacon et al. (1970) found yeast-cell-wall-degrading enzymes of *C. johnsonae* to be primarily extracellular, although some activities were cell-associated, and Agbo and Moss (1979) noted both extracellular and cell-associated enzyme complexes after growth of several *Cytophaga* isolates on agar. Vance et al. (1980) reported the cellulase of *S. myxococcoides* to be extracellular. However, in that study the cells were grown in a stirred fermentor with a surfactant added to control foaming, and the cellulase could have been stripped from the cells by this procedure.

Inasmuch as most of the xylanase of *C. xylanolytica* is cell-associated, it would seem that the most efficient dissociation of xylan by this bacterium would occur when cells are in direct contact with insoluble xylan---a situation entirely consistent with the lifestyle of ths gliding bacterium. However, *C. xylanolytica* does exhibit extracellular xylanase activity, and such activity does not appear to be an artifact of cell lysis, as the supernatants and Triton extracts of either xylan- or xylose-grown cells exhibited different patterns of xylanase and β-xylosidase activity bands after

electrophoresis. Since *C. xylanolytica* could grow on microporous membrane filters placed on the surface of 0.4% oat spelt xylan plates, and in so doing hydrolyze the xylan beneath the filter, as Kauri and Kushner found for cellulolytic *Cytophaga* spp., *C. xylanolytica* apparently does not absolutely require direct contact with a polymeric growth substrate in order to utilize it. This further suggests that the extracellular activity of this bacterium may specifically function to attack polymeric substrates at some distance from the cell. The fact that even xylose-grown cells, though not fully induced for xylanase activity, still displayed extracellular xylanase activity bands on IEF gels, may indicate that this extracellular activity serves an important function in initiating degradation of polymeric substrates to produce fragments which in turn serve to induce full xylanase activity.

C. xylanolytica appears to have a complex enzyme system which is induced by xylan and is relatively specific for xylan degradation. Clearly, growth on xylan increases the xylanolytic activity of the cells and results in the appearance of a multitude of new xylanase activity bands after isoelectric focusing. Multiple xylanase activity bands have been observed by others, but the significance of multiple IEF activity bands is not yet clear. Multiple xylanase genes have been clearly identified in several bacterial species (*Bacillus*: Yang et al. 1989, Hamamoto et al. 1987, Honda et al. 1985, Sakka et al. 1989 and 1990; *Clostridium*: Schwarz et al. 1990, MacKenzie et al. 1989, Hazlewood et al. 1988; *Streptomyces*: Kluepfel et al. 1990, Vats-Mehta et al. 1990; *Ruminococcus*: Flint et al. 1989 and 1990;

Pseudomonas: Gilbert et al. 1988). In addition, two xylosidase genes have been identified in *Bacillus* (Panbangred 1984). However, a 2.8 kb genomic fragment from *Clostridium thermocellum* cloned into *E. coli* encoded a single molecular weight band (25,000 kD) on SDS-PAGE gels but multiple (at least 4) bands on IEF gels (MacKenzie et al. 1989). Studies with proteins isolated from the host species have produced similar results. For example, in *Thermomonospora fusca*, Bachmann and McCarthy (1991) demonstrated that a single 32-kD endoxylanase produced three activity bands at pI 7.9, 8.2 and 8.6. This 32-kD protein, itself active on denaturing PAGE gels developed as zymograms, was proposed to be a subunit of a yet larger protein which was active on native PAGE gels. A second proposed subunit of 24 kD was presumed to account for three additional IEF activity bands at lower pI's. Three bands of acetyl-esterase activity revealed by IEF, were correlated with an 80-kDa intracellular protein secreted to yield 40-kD active subunits. Similarly, purified endoxylanase 1 from *Fibrobacter succinogenes* (Matte and Forsberg 1992) produced a single band of 53.7 kDa by SDS-PAGE analysis, but four activity bands at pI 7.3, 8.1, 8.3 and 8.9 after IEF. This heterogeneity may be due to a variety of postranslational events, including subunit activity as noted above, glycosylation (Kluepfel et al. 1990), or proteolytic processing (Biely 1985). In preparations containing several proteins, multiple activity bands may also be due to the possession of multiple activities by a single protein, such as the apparent lichenanase activity of three of the endoxylanase activity bands in our study and also observed in several of the purified or cloned enzymes noted above. Finally, heterogeneous activities of purified proteins or

cloned gene products toward the commonly used test substrates have been described (Kluepfel et al. 1990, Gilkes et al. 1984, Sakka et al. 1989). For example, MacKenzie et al. (1989) observed that one gene product from *Cl. thermocellum* formed clear halos in RBB-xylan plates, clearings on Congo Red-stained xylanase activity gels, and substantial quantities of reducing sugar in typical xylanase assay procedures. However, two additional clones also formed clear halos on RBB-xylan, and on Congo Red-stained aspen or larchwood xylan activity gels, but not on oat spelt xylan activity gels, and both converted less than 1% of xylan substrates to reducing sugar. Determination of the exact nature and significance of the multiple IEF activity bands in enzyme preparations from *C. xylanolytica* XM3 must await further analysis.

C. xylanolytica XM3 grows on few compounds other than sugars or sugar polymers, but can grow on a diverse array of both (Haack and Breznak 1992), and it appears to be able to utilize more than one sugar substrate concurrently. We believe that cytophagas grow primarily on polymers in nature and we assume that the natural habitat of *C. xylanolytica* and other cytophagas is a complex matrix of biopolymers, varying in degree of substitution and linkage, over and through which these bacteria glide or swarm. Potentially, an individual cell will encounter a multitude of sugar residues, in a variety of linkages, within relatively short spatial or temporal intervals. In this environment, the ability to completely degrade a given polymer may not be as important to survival as the ability to utilize many of the sugar residues, regardless of linkage or parent molecule association. Hofle (1982) demonstrated that *C. johnsonae*

possessed two different glucose uptake systems, both of which were unspecific in that mannose and D-glucosamine or N-acetyl-D-glucosamine also appeared to be transported by these uptake systems. He proposed that this would be advantageous in environments where monomer components of the degradation of cellulose, starch, mannan and chitin would be available concurrently. Agbo and Moss (1979) showed that agar-degrading enzyme complexes in freshwater *Cytophaga* species were induced by agar, but also (and occasionally at higher levels), by galactans and other plant cell wall polysaccharides. These complexes also degraded a variety of polysaccharides. Similarly, whether grown on xylose, glucose, cellobiose or lichenan, *C. xylanolytica* retained the ability to respond to xylan through constitutive expression of xylanase, xylobiase and arabinofuranosidase enzymes. Xylan-grown cells produced additional endoxylanases, and retained lichenanase activity, while cells grown on xylan plus lichenan expressed induced levels of both depolymerase activities. Although *C. xylanolytica* does not release xylose or arabinose into the medium during growth on xylan, the presence of xylose in the medium with xylan (potentially encountered in real environments through the degradative activities of other microbes) did not repress xylanase activity.

Apparently, *C. xylanolytica* and other cytophagas are well adapted, through the location and regulation of their polymer degrading activities, for growth in a complex insoluble matrix of multiple potential growth substrates. However, *C. xylanolytica* cannot grow on or degrade cellulose, nor can it effectively degrade more complex plant cell wall derived substrates containing xylan, yet it

possesses a complex enzyme system which is inducible by xylan. This suggests that in the anoxic environments where *C. xylanolytica* exists, xylan residues are sufficiently exposed by the activities of other polymer-degrading microorganisms to warrant the retention of this enzyme system as one of several depolymerizing systems ecologically advantageous to these cells. It is anticipated that continued study of these ubiquitous biopolymer degrading cytophagas will greatly increase our understanding of the biochemistry, and microbial ecology of polymer degradation in natural environments.

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