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CHARACTERIZATION OF AN α-L-ARABINOFURANOSIDASE AND OTHER COMPONENTS OF THE XYLANASE SYSTEM OF *Cytophaga xylanolytica*

By

Michael Jay Renner

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Submitted to
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ABSTRACT

CHARACTERIZATION OF AN α-L-ARABINOFURANOSIDASE AND OTHER COMPONENTS OF THE XYLANASE SYSTEM OF *Cytophaga xylanolytica*

By

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All land plants, and most aquatic plants and algae, contain some form of xylan, a plant cell wall hemicellulose second only to cellulose as the most abundant polysaccharide on earth. Although xylans are abundant and widely distributed, studies of their degradation have been rather limited and almost entirely restricted to cellulose-degrading aerobic bacteria or fungi and rumen anaerobes, microorganisms that usually secrete their polysaccharide-degrading enzymes. Research described herein examined the largely cell-associated xylan-degrading enzyme system of *Cytophaga xylanolytica*, a ubiquitous anaerobic gliding bacterium found in anoxic freshwater and marine sediments.

An α-L-arabinofuranosidase (ArfI) was purified 85-fold from cells of *Cytophaga xylanolytica* strain XM3. The native enzyme had a pI of 6.1 and an apparent molecular mass of 160-210 kDa, and it appeared to be a trimer or tetramer consisting of 56 kDa subunits. The enzyme exhibited a K_m of 0.504 m*M* and a V_{max} of 319 μmol · min^{-1} · mg $protein^{-1}$, and it had optimum activity at pH 5.8 and 45°C. ArfI was relatively stable over a pH range of 4 to 10 and at temperatures up to 45°C. The enzyme released arabinose from a variety of arabinose-linked artificial and natural substrates and was specific for the α-linked furanose form. ArfI interacted synergistically with three partially purified endoxylanase fractions from *C. xylanolytica* in hydrolyzing rye arabinoxylan. However,

cell fractionation studies revealed that ArfI was largely, if not entirely, cytoplasmic, so its activity *in vivo* is probably most relevant to hydrolysis of arabinose-containing xylooligosaccharides small enough to pass through the cytoplasmic membrane. Antibodies prepared against purified ArfI also cross-reacted with arabinofuranosidases from other freshwater and marine strains of *C. xylanolytica*.

DEDICATION

This thesis is dedicated to my good friend and colleague Dr. Richard M. Behmlander, whose untimely death at such a young age shocked us all. I will remember you as one of the finest friends and scientists I ever knew.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

INTRODUCTION AND BACKGROUND

Plant cell walls consist mainly of cellulose, hemicelluloses, and lignin. Cellulose, an unbranched polysaccharide chain consisting of β-1,4-linked glucose units, is the main structural polymer in plant cell walls. This homopolysaccharide can form insoluble microfibril bundles through hydrogen bonding of numerous single chains having degrees of polymerization averaging 14,000 glucose units in length (McNeil, *et al.* 1984). Hemicellulose is a generic term, first introduced by Schulze in 1891, to describe the alkali extractable carbohydrate fraction remaining after hot and cold water extraction of plant material (Schulze 1891). Hemicelluloses are typically branched heteropolysaccharides that are classified according to their resident backbone sugar moieties. For example, xylans are composed of a β-1,4-linked xylose backbone that can be up to 200 units in length and contain side groups of arabinose, galactose, glucuronic acid, and acetate. Similarly, there are xyloglucans (β-1,4-linked glucose backbone with some β-1,6-linked xylose side chains), mannans (β-1,4-linked mannose backbone), arabinans (α-1,5-linked arabinose backbone with α-1,2- and α-1,3-linked arabinose side groups), and arabinogalactans (α-1,3-linked galactose backbone with α-1,6-linked galactose and α-1,3-linked arabinose side groups) (Aspinall 1980).

Cellulose microfibers are thought to be encased by hemicellulose polymers in the maturing secondary plant cell walls, possibly through hydrogen bonds and van der Wahl

forces (Atkins 1992, Wong, *et al.* 1988). In turn, this complex is then thought to combine with lignin (an amorphous, complex polyphenolic polymer) and other minor components such as pectins (polygalacturonic acid backbone), and proteins (hydroxyproline-rich, arabinose substituted extensins; expansins; and other glycosylated proteins and enzymes), through covalent cross-linkages (to arabinose side groups) and hydrogen bonds (McNeil, *et al.* 1984, Monro, *et al.* 1976, Shcherban, *et al.* 1995). The resulting insoluble matrix is thought to provide plants with their structural rigidity and cell wall impermeability (Whistler and Richards 1970).

Xylan, the most common hemicellulose, is second only to cellulose as the most abundant polysaccharide on earth (Biely 1985), and it has been estimated that 10^{10} metric tons are recycled annually, with the degradative arm occurring largely through the action of microbes (Wilkie 1983). Xylans are found in the growing primary and mature secondary cell walls of all terrestrial, and nearly all marine plants and algae (Wilkie 1983, Wong, *et al.* 1988). The amount of xylan found in plants can vary from 35% to as little as 7% of the dry weight of birchwood and gymnosperms, respectively (Aspinall 1959, Timell 1967). Xylans consist of a β-1,4-linked xylopyranose backbone, to which are often attached side groups of α-1,2- and/or α-1,3-linked arabinose, α-1,2-linked 4-*O*-methyl-glucuronic or glucuronic acid, and ester- or ether-linked ferulic, *p*-coumaric and/or acetic acid side groups depending on the plant source (Aspinall 1959, Timell 1967). Xylans can vary in structure from plant to plant, from tissues within the same plant, and even within the same tissue at different growth stages (Wilkie 1983, Wong, *et al.* 1988). They can be found in various forms from simple, unbranched linear xylans (*e.g.*, Esparto grass and tobacco stalks) to low and high branching arabinoxylan with

arabinose side groups (*e.g.*, monocots and grains) to glucuronoxylan with 4-*O*-methyl-glucuronic and glucuronic acid side groups (*e.g.*, soybeans, hardwoods, and legumes); to the most complex glucuronoarabinoxylans containing arabinose, glucuronic acid, and galactose side groups (*e.g.*, softwoods and dicots) (Joseleau, *et al.* 1992). These various xylans can have further complexity by the addition of short chain oligosaccharides of various composition (Zinbo and Timell 1965).

In 1889, Hoppe-Seylor set out to determine the microbes present in anoxic river mud enrichments that could degrade hemicellulose (Hoppe-Seylor 1889). The evolved gases he observed could have been due in part to the action of cytophagas, since they are widely distributed in nature and are well known, but poorly studied, for their biopolymer-degrading capabilities (Reichenbach 1989). The usually saprophytic organisms in the genus *Cytophaga* are classified as aerobic, Gram-negative, unicellular gliding rods, that form no endospores, microcysts, or fruiting bodies (Reichenbach 1992). They are the most commonly found gliding bacteria and typically inhabit organically rich marine, freshwater, and terrestrial environments (*e.g.*, soils (Reichenbach and Dworkin 1981), decaying plant material (Liao and Wells 1986), and sewage treatment plants (Gude 1980)). The cytophagas were originally classified, in part, on the basis of their ability to degrade cellulose (Christensen 1977, Christensen 1974, Christensen and Cook 1972, Colwell 1973, Goodfellow 1967, Mitchell, *et al.* 1969, Reichardt 1974, Reichenbach and Dworkin 1981, Reichenbach and Kleinig 1972, Soriano 1973, Weeks 1969), but the original classification scheme has been modified to include non-cellulose degraders (Jooste, *et al.* 1985, Lysenko, *et al.* 1995, Reichenbach 1989, Reichenbach 1992, Schindler and Metz 1989). A large group of non-cellulose degraders include the

Cytophaga-like bacteria (CLB), which are typically pathogens of fish and shellfish (Amin, *et al.* 1988, Anderson and Conroy 1969, Bernardet and Kerouault 1989, Bragg 1991, Brown, *et al.* 1996, Cipriano, *et al.* 1995, Dungan, *et al.* 1989, Gennari and Tomaselli 1988, Hansen, *et al.* 1992, Jack, *et al.* 1992, Kent, *et al.* 1989, Kueh and Chan 1985, Lee and Pfeifer 1977, Lee and Preifer 1975, Murchelano and Bishop 1969, Nomura 1997, Pacha 1968, Potin, *et al.* 1991, Shotts 1991, Sinskey, *et al.* 1967, Speare, *et al.* 1995, Speare and Mirsalimi 1992, Vasconcelos and Lee 1972). More recent studies involving 16S rRNA sequences have strengthened the classification of the *Cytophaga* branch, but have also confirmed the diversity within the genus (Manz, *et al.* 1996, Nakagawa and Yamasato 1993).

The only known strictly anaerobic *Cytophaga* described to date is *Cytophaga xylanolytica* (Haack and Breznak 1993). Other unusual characteristics of this bacterium include its inability to degrade cellulose and its ability to grow under either low or high salt conditions (*e.g.*, freshwater and marine media); two traits that are atypical for bacteria in this genus. *C. xylanolytica* was originally isolated in Germany from methanogenic and sulfidogenic enrichment cultures. These enrichments contained powdered xylan as sole fermentable substrate and were inoculated with freshwater sediments. Similar organism(s) were also isolated, using the above methods, from freshwater sediment samples from several locations on the campus of Michigan State University and from samples of freshwater and marine sediments found in Woods Hole, Massachusetts (Haack and Breznak 1993). Luxuriant growth of *C. xylanolytica* was seen on xylan, and it was observed that gliding cells were attached in masses to the xylan particles. When examined further, it was found that greater than 90% of xylan-degrading

enzymes of *C. xylanolytica* were cell-associated (Haack and Breznak 1992). Although polysaccharide-degrading enzymes have been described for a number of fungi and bacteria [*e.g.*, (Anderson 1995, Bachmann and McCarthy 1991, Castanares, *et al.* 1995, Costa-Ferreira, *et al.* 1994, Flipphi, *et al.* 1994, Gasparic, *et al.* 1995, Gasparic, *et al.* 1995, Harada 1983, Kimura, *et al.* 1995, Lee, *et al.* 1993, Luonteri, *et al.* 1995, Ransom and Walton 1997, Salyers 1993, Schwarz, *et al.* 1990, van der Veen, *et al.* 1991, Wood and McCrae 1996)]. the information available on xylan-degrading microorganisms has been more limited [*e.g.*, (Bachmann and McCarthy 1991, Belancic, *et al.* 1995, Castanares, *et al.* 1995, Clermont, *et al.* 1970, Costa-Ferreira, *et al.* 1994, Fernandez, *et al.* 1995, Gasparic, *et al.* 1995, Gasparic, *et al.* 1995, Haack and Breznak 1992, Lee, *et al.* 1993, MacKenzie, *et al.* 1989, Saraswat and Bisaria 1997, Schwarz, *et al.* 1990, Sunna and Antranikian 1997, Whitehead and Hespell 1990)].

Studies of polysaccharidases from cytophagas have been mostly limited to agarases, cellulases, amylases, carrageenases, and pectinases (Bacon, *et al.* 1970, Bacon, *et al.* 1970, Chang and Thayer 1977, Charpentier 1965, Clermont, *et al.* 1970, Duckworth and Turvey 1969, Duckworth and Turvey 1969, Duckworth and Turvey 1968, Duckworth and Turvey 1969, Jeang, *et al.* 1995, Li, *et al.* 1996, McKay 1991, Osmundsvag and Goksoyr 1975, Potin, *et al.* 1991, Sarwar, *et al.* 1987, Sarwar, *et al.* 1985, Sundarraj and Bhat 1972, Sundarraj and Bhat 1971, Turvey and Christison 1967, van der Meulen and Harder 1976, van der Meulen and Harder 1975, van der Meulen and Harder 1976, van der Meulen, *et al.* 1974). Two cellulases, one secreted and one cell-associated, were found to be produced by *Sporocytophaga myxococcoides* (Osmundsvag and Goksoyr 1975). A cellulolytic soil *Cytophaga*, sp. LX-7, secreted a cellobiose-oxidizing enzyme (Li, *et al.*

1996) whereas another soil species secreted an amylase (Jeang, *et al.* 1995). A mostly cell-associated amylase was described for *Cytophaga johnsonae* (McKay 1991). *C. johnsonii* (sic) produced a cell-associated dextranase (Jansen 1975). The most thorough study of a *Cytophaga* species (Chang and Thayer 1977) described a completely cell-associated cellulose-degrading system with cellulases found in the periplasm, cytoplasm, and also membrane-associated. However, the only known studies of xylanases of cytophagas are those of Haack and Breznak with *C. xylanolytica* (Haack and Breznak 1993, Haack and Breznak 1992) and Clermont *et al.* with *Sporocytophaga myxococcoides* (Clermont, *et al.* 1970).

Microorganisms degrade plant material by a variety and/or combination of strategies. The most well known strategy described is that of enzyme secretion. Examples include the polysaccharidases of most fungi and actinomycetes (McCarthy 1987, McCarthy and Ball 1987, Ramachandra, *et al.* 1987, Wood 1989). Although this strategy allows organisms to degrade polysaccharides from a distance, it also enables other organisms to compete for the breakdown products. A second strategy is to keep the enzymes on or near the outer surface so that the breakdown products released are more readily accessible for uptake by the degrading organism. The most thoroughly studied example of this strategy is the cellulose-degrading "cellulosome" of Gram-positive *Clostridium* spp. This large cell surface-associated complex is composed of numerous polysaccharidases and structural proteins (Beguin 1996, Beguin and Aubert 1994, Lamed, *et al.* 1987, Lamed, *et al.* 1983, Lamed, *et al.* 1983, Leschine 1995) and is thought to be anchored to the crystalline S-layer located just external to the peptidoglycan. The cellulosome also appears to help anchor the bacterium to cellulose,

allowing prolonged, intimate contact and therefore enhancing its competition for this substrate. The third, and the most recently described, strategy for polymer degradation is exemplified by Gram-negative bacteria of the genus *Bacteroides* (Reeves, *et al.* 1997, Salyers, *et al.* 1996). *B. thetaiotaomicron* appears to bind starch or maltooligosaccharides (up to 7 glucose units in length) to its outer surface where they are then projected into the polysaccharidase-containing periplasmic space. This strategy is unusual since it was previously thought that only small, *e.g.*, mono- and disaccharides could penetrate outer membranes. In addition to the three main examples above, there are also organisms that use combinations of the above strategies (Salyers, *et al.* 1996). Although some of these enzyme systems have only recently been described, others have been studied for over thirty years (*e.g.*, the cellulase system of *C. thermocellum*), yet no one system has been completely defined.

Owing to the complexity of xylans, it is generally assumed that a microorganism would require a suite of enzymes working in a synergistic/cooperative manner to completely degrade these heteropolysaccharides to their monomeric constituents. The degradation of a typical xylan is believed to be accomplished through the action of endoxylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) which cleave randomly within the xylan backbone, usually at unsubstituted β-1,4-xylosyl linkages; β-xylosidases (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37) which release xylose through exo-acting activity on xylooligosaccharides and xylobiose; and the debranching enzymes, which remove side chain substituents. Debranching enzymes include α-L-arabinofuranosidases (α-L-arabinofuranoside arabinofuranohydrolase; EC 3.2.1.55) which hydrolyze non-reducing terminal α-1,2- and α-1,3-linked arabinofuranose side groups, glucuronidase

(EC 3.2.1.31) that releases α-1,2-linked glucuronic and (4-*O*-methyl) glucuronic acid residues, and xylan acetylesterase or other esterases (EC 3.2.1.6) that remove acetic, ferulic, or *p*-coumaric acid *O*-1,2- or *O*-1,3-linked groups (Biely 1985, Wong, *et al.* 1988).

Xylanases have been used in studies of plant polysaccharide structure (Nishitani and Nevins 1989), as animal feed supplements (Bedford 1995, Lewis, *et al.* 1996, Selinger, *et al.* 1996), in bread making (McCleary 1994, Rouau 1993), in paper and pulp production (Jimenez, *et al.* 1997, Paice, *et al.* 1995), for fruit juice clarification (Brillouet, *et al.* 1996, Gueguen, *et al.* 1995), and in nutraceuticals and chemical feedstock production (Prade 1996, Wong, *et al.* 1988). Extensive reviews have been written on the sources and activities associated with xylanases (Bajpai 1997, Biely 1985, Dekker 1985, Dekker and Richards 1976, Prade 1996, Reilly 1981, Sunna and Antranikian 1997, Thomson 1993, Wong, *et al.* 1988), but most authors recognize the lack of information available on the debranching enzymes, even though all acknowledge the important role debranching enzymes play in xylan degradation.

The only review on arabinosidases was written in 1984 (Kaji 1984). At that time, less than a dozen arabinosidase enzymes had been purified and none of the genes encoding them had been cloned. The arabinosidases were described and classified (in *Enzyme Nomenclature*) into two main groups: (i) those enzymes that hydrolyze from the nonreducing, terminal end of the substrate molecule; and (ii) those enzymes that act randomly at internal sites. The enzymes of the first group are the α-L-arabinofuranosidases (α-L-arabinofuranoside arabinofuranohydrolase; EC 3.2.1.55) that

have activity against low molecular weight substrates [*e.g.*, the synthetic substrates *para*-nitrophenyl- and 4-methylumbelliferyl-α-L-arabinofuranosides (*p*NP-AF and MU-AF, respectively), and arabinooligosaccharides], containing α-1,2- and α-1,3-linked (to carbons C_2 and C_3 of the backbone residue, respectively) arabinose side groups. The second group of arabinosidases are the α-1,5-endoarabinases (EC 3.2.1.99) that effect random hydrolysis of the internal α-1,5-linked backbone of arabinans.

Kaji further classified the α-L-arabinofuranosidases (ARAFs) into two groups: (i) the *Aspergillus niger* type that releases arabinosyl residues from arabinans, arabinoxylan, and arabinogalactan, but is also active towards the simple synthetic substrates *p*NP-AF and MU-AF and has low α-1,5-endoarabinase activity on arabinans; or (ii) the *Streptomyces purpurascens* type that acts on low molecular weight synthetic substrates and arabinooligosaccharides, but does not act on arabinans, arabinoxylans, or arabinogalactans.

The above classification was based on information only available for a relatively few purified ARAFs, and mostly those secreted by, and purified from, actinomycetes and fungi. Since then, numerous other ARAFs have been purified and/or cloned from mesophilic and thermophilic bacteria and fungi, as well as several plants (Table 1). Characteristics of these newer ARAFs have clouded the classical groupings of α-L-arabinofuranosidases. In particular, a previously purified ARAF (AXH; described as a β-1,4-D-arabinoxylan arabinofuranohydrolase) from *Aspergillus awamori* showed arabinose-releasing activity with arabinoxylooligosaccharides, but had no activity towards the synthetic substrate *p*NP-AF, nor did it act on arabinans or arabinogalactans

(Kormelink, *et al.* 1991). The enzyme did possess weak activity towards arabinoxylans, but only after prolonged incubations. The authors also described a second, unpurified ARAF from *A. awamori*, which had no activity on *p*NP-AF but did have significant activity on arabinoxylans. A recently purified ARAF from *Bifidobacterium adolescentis*, also showed no activity on *p*NP-AF, nor did it act on sugar beet arabinan, soy arabinogalactan, arabinooligosaccharides, and arabinogalactooligosaccharides (Van Laere, *et al.* 1997). However, the enzyme did possess activity on wheat flour arabinoxylan, but only on terminal C_3-linked arabinosyl groups from double-substituted, and not single-substituted, xylose residues derived from arabinoxylans. These same authors describe preliminary findings of enzymes that only release C_2-linked arabinosyl groups from single-substituted xylose residues and similar activities in *Trichoderma reesei* (to be published by K. Rakasi).

As is evident from the above discussion, sorely-needed new information about ARAFs has been slowly accumulating since the last review by Kaji in 1984 (Kaji 1984). In general, almost all ARAFs have activity on synthetic substrates (typically only the furanoside configuration) and numerous plant polysaccharides (although with a narrow substrate specificity). However, as a group they display a variety of pIs, molecular weights and subunit compositions, pH and temperature optima, K_m and V_{max} constants, and cellular location(s). The following review consolidates the information currently available on ARAFs (Table 1), and the organisms producing them are discussed in more detail.

Table 1. Origin and Properties of Enzymes with α-L-arabinofuranosidase Activity.

Organism	Enzyme/Location/ Degree of Purity[a]	pI[b]	Optimum pH	Optimum Temp (°C)	M_r[c] (kDa)	K_m (m*M*)/Other Hydrolytic Activities[d]	Reference
Bacteria							
Bacillus							
sp. no. 430	I/Sec/P	ND[e]	6.5	40	ND	ND/ -	[Yasuda, 1983 #492]
polymyxa CECT 153	AF166/Sec/P	4.7	6.5	50	65 and 33/ n166	ND/ -	[Morales, 1995 #493]
polymyxa CECT 153	AF64* /Sec/P	8.7	6.5	55	64	ND/ -	[Morales, 1995 #255]
	AF53* /Sec/P	9.0	6.5	55	53	ND/ -	
subtilis F-11	II/Sec/P	5.3	6.5	ND	65	ND/ -	[Weinstein, 1979 #494]
subtilis 3-6	α-L-AFase/Sec/P	ND	7.0	60	61	ND/ -	[Kaneko, 1994 #268]
stearothermophilus L1	- /Sec/P	ND	7.0	70	52 and 57/ n110	0.22/ -	[Bezalel, 1993 #467]
stearothermophilus T-6	AF/Sec and CA_p/P	6.5	5.5-6.0	70	64/ n256	0.42/ -	[Gilead, 1995 #264]
Bacteroides							
ovatus V975	-* /CA_p/pP	ND	ND	ND	38	ND/XYL	[Whitehead, 1990 #296]
xylanolyticus X5-1	AF/Sec and CA_p/P	ND	5.5-6.0	50	61/ n364	0.5	[Schyns, 1994 #495]

Table 1. (cont'd).

Organism	Enzyme/Location/ Degree of Purity[a]	pI[b]	Optimum pH	Optimum Temp (°C)	M_r[c] (kDa)	K_m (m*M*)/Other Hydrolytic Activities[d]	Reference
Bifidobacterium							
adolescentis	AH/CA_e/P	ND	6.0	30	n100	ND/AXH	[Van Laere, 1997 #238]
Butyrivibrio							
fibrisolvens	*xylB** / - /Cr	ND	ND	ND	60	ND/XYL	[Utt, 1991 #290]
fibrisolvens GS113	AF/CA_e/P	6.0	6.0-6.5	45	31/ n240	0.66/ -	[Hespell, 1992 #349]
Clostridium							
acetobutylicum	AF/Sec/P	8.2	5.0-5.5	ND	94/ n90	4/ -	[Lee, 1987 #333]
stercorarium	*arfA** / - / -	ND	ND	ND	49	ND/ -	[Schwarz, 1990 #295]
	*arfB** /Sec/P	ND	5.0	70	52/ n195	ND/ -	[Schwarz, 1995 #257]
stercorarium F-9	*xylA** / - /P	ND	7.0	65	53/ n220	17.6/XYL	[Sakka, 1993 #282]
Cytophaga							
xylanolytica XM3	*arfI* * ArfI /CA_e/P	6.1	5.85	45	56/ n160-210	0.504/ -	This study
	*arfII** ArfII / - / -	ND	ND	ND	59[f]	ND/ -	

Table 1. (cont'd).

Organism	Enzyme/Location/ Degree of Purity[a]	pI[b]	Optimum pH	Optimum Temp (°C)	M_r[c] (kDa)	K_m (mM)/Other Hydrolytic Activities[d]	Reference
Fibrobacter							
succinogenes S85	1/Sec/P	8.9	7.0	39	54	ND/ deb.ENDOX	[Matte, 1992 #353]
Prevotella							
ruminicola B_14	L3* / - / -	ND	ND	ND	ND	ND/ -	[Gasparic, 1995 #395]
Pseudomonas							
Fluorescens sp. *cellulosa*	*xynC** XylC / - / -	ND	ND	ND	59[f]	ND/ -	[Kellett, 1990 #294]
Ruminococcus							
albus 8	α-AF/Sec/P	3.8	6.9	ND	75/ n305-310	1.6/ -	[Greve, 1984 #313]
Streptomyces							
sp. no. 17-1	- /Sec/P	4.4	6.0	ND	92	3.6/ -	[Kaji, 1981 #474]
diastaticus ET/BW200	C1/Sec/P	8.8	4.0-7.0	ND	38	10/ -	[Tajana, 1992 #285]
	C2/Sec/P	8.3	4.0-7.0	ND	60	12.5/ -	
diastatochromogenes	AFase/Sec/P	ND	6.1	55	ND	ND/ -	[Higashi, 1983 #496]
	AFase/CA_s/P	ND	5.9	55	ND	ND/ -	

Table 1. (cont'd).

Organism	Enzyme/Location/ Degree of Purity[a]	pI[b]	Optimum pH	Optimum Temp (°C)	M_r[c] (kDa)	K_m (mM)/Other Hydrolytic Activities[d]	Reference
Streptomyces							
lividans 66 strain 1326	*abfA** AbfA /CA$_s$/P	4.6	6.0	60	69/ n380	0.6/ -	[Manin, 1994 #267]
lividans 66 strain 1326	*abfB** AbfB /Sec/P	7.4	6.0	55	43	ND/ -	[Vincent, 1997 #368]
massasporeus IFO 3841	- /Sec/P	ND	5.0	ND	n54	1.67/ -	[Kaji, 1982 #497]
purpurascens IFO 3389	- /Sec/P	3.9	6.5	ND	62/ n495	0.08/ -	[Komae, 1982 #498]
roseiscleroticus	- /Sec/P	9.5	6.5-7.0	60	23/ n5.5	ND/deb.ENDOX	[Grabski, 1991 #499]
Thermoanaerobacter							
ethanolicus JW 200	- /CA$_s$/P	4.6	6.0	75-80	85/ n165	4.6/XYL	[Shao, 1992 #500]
Thermomonospora							
fusca BD21	- /Sec/P	ND	6.0	50	46/ n92	0.1/ -	[Bachmann, 1991 #346]
Fungi							
Aspergillus							
awamori IMI 142717	- /Sec/P	3.6/3.2	4.6	50	64	1.39/ -	[Wood, 1996 #251]

Table 1. (cont'd).

Organism	Enzyme/Location/ Degree of Purity[a]	pI[b]	Optimum pH	Optimum Temp (°C)	M_r[c] (kDa)	K_m (mM)/Other Hydrolytic Activities[d]	Reference
Aspergillus							
awamori CMI 142717	- /Sec/P	ND	5.0	50	32	ND/AXH	[Kormelink, 1991 #501]
nidulans	α-AFase/Sec/P	4.3	4.5-5.5	55	36	2.7/ -	[Fernandez-Espinar, 1994 #271]
nidulans WG096	B/Sec/P	3.3	4.0	65	65	0.68/ -	[Ramon, 1993 #276]
niger	- /Sec/P	6.0-6.6	4.1	50	128	0.6/ -	[Rombouts, 1988 #512]
	- /Sec/P	5.5-6.0	3.7	60	60	0.48/ -	
niger KI	- /Sec/P	3.6	3.8	60	n53	4.9/ -	[Kaji, 1967 #502]
niger MRC11624	*abfB** Abf2 /Sec/ -	ND	ND	ND	70/ 53[f]	ND/ -	[Crous, 1996 #248]
niger N400	A/Sec/P	3.3	3.4	46	83	0.68/ -	[van der Veen, 1991 #292]
	B/Sec/P	3.5	3.8	56	67	0.52/ -	
niger 5-16	α-L-AFase/CA_s/P	3.5	4.0	60	67	ND/ -	[Kaneko, 1993 #278]
sojae no. 3	X-II-A/Sec/P	3.9	5.0	50	34/ n35	ND/ -	[Kimura, 1995 #504]
terreus VTT-D-82209	A/Sec/P	7.5	3.5-4.5	<20	39	ND/ -	[Luonteri, 1995 #505]
	B1/Sec/P	8.3	3.5-4.5	40	59	ND/ -	
	B2/Sec/P	8.5	3.5-4.5	40	59	ND/ -	

Table 1. (cont'd).

Organism	Enzyme/Location/ Degree of Purity[a]	pI[b]	Optimum pH	Optimum Temp (°C)	M_r[c] (kDa)	K_m (m*M*)/Other Hydrolytic Activities[d]	Reference
Aspergillus							
tubingensis NW756	*axhA** AxhA / - /P	3.6	ND	ND	32	ND/AXH	[Gielkens, 1997 #242]
Cochliobolus							
carbonum Nelson SB111	Arf/Sec/P	ND	3.5-4.0	50	63/ n62	ND/ -	[Ransom, 1997 #466]
Dichomitus							
squalens	- /Sec/P	5.1	3.5	60	n60	1.64/ -	[Brillouet, 1985 #469]
Monilinia							
fructigena	AFIII/Sec/P	6.5	4.0	ND	34/ n40	0.8/ -	[Kelly, 1987 #309]
Penicillium							
capsulatum	Ara I/Sec/P	4.2	4.0	60	65/ n61	0.18/ -	[Filho, 1996 #384]
	Ara II/Sec/P	4.5	4.0	55	63/ n61	1.3/ -	
Phytophthora							
palmivora CMI 63552	-/Sec/P	ND	4.0	50	n63	0.65/ -	[Akinrefon, 1968 #506]

Table 1. (cont'd).

Organism	Enzyme/Location/ Degree of Purity[a]	pI[b]	Optimum pH	Optimum Temp (°C)	M_r[c] (kDa)	K_m (mM)/Other Hydrolytic Activities[d]	Reference
Sclerotinia							
fructigena	AF3.0/Sec/pP	3.0	4.0-5	<30	n220	0.11/ -	[Laborda, 1973 #507]
	AF6.5/Sec/pP	6.5	5.0	<30	n40	1.75/ -	
	AF4.5/CA$_4$/pP	4.5	5.7-6	<30	n350	0.22/ -	
Sclerotinia							
sclerotiorum	α-L-ara/Sec/P	7.5	4.0-4.5	ND	62/ n64	ND/ -	[Baker, 1979 #470]
Thermoascus							
aurantiacus ATCC 26904	- /Sec/Cr	ND	4.0	70	ND	ND/ -	[Roche, 1994 #508]
Trichoderma							
reesei VTT-D-79125	- /Sec/P	7.5	4.0	ND	53/ n30	1.2/ -	[Poutanen, 1988 #468]
reesei Rut C-30	- /Sec/P	4.7	4.0	60	100/ n100	ND/XYLN	[Herrmann, 1997 #240]
reesei Rut C-30	*abf1** Abf1 / - /Cr	ND	ND	ND	49.1[f]	ND/ -	[Margolles-Clark, 1996 #249]
	*bxl1** Bxl1 / -/Cr	ND	ND	ND	80.4[f]	ND/XYL	

Table 1. (cont'd).

Organism	Enzyme/Location/ Degree of Purity[a]	pI[b]	Optimum pH	Optimum Temp (°C)	M_r[c] (kDa)	K_m (mM)/Other Hydrolytic Activities[d]	Reference
Termitomyces							
clypeatus	AF/Sec/pP	2.5-5.0	5.5	50	ND	ND/ -	[Sinha, 1995 #509]
Plant							
Daucus carota							
L. cv. Kintoki	- / - /P[g]	4.7	4.2	55	94/ n110	1.33/ -	[Konno, 1987 #472]
Glycine max							
L. cv. Akishirome	- / - /P[h]	ND	4.8	50	87	0.53/ -	[Hatanaka, 1991 #465]
Lupinus luteus							
L. cv. Weiko III	I/ - /P[i]	ND	4.4	ND	n70	16.6/ -	[Matheson, 1977 #471]
	II/ - /P[i]	ND	3.5	ND	n120	1.6/ -	
Pyrus serotina							
Rehd. var. *culta.*	α-L-Arafase/ - /P[j]	ND	5.0	ND	42	34.7/ -	[Tateishi, 1996 #510]

Table 1. (cont'd).

Organism	Enzyme/Location/ Degree of Purity[a]	pI[b]	Optimum pH	Optimum Temp (°C)	M_r[c] (kDa)	K_m (mM)/Other Hydrolytic Activities[d]	Reference
Raphanus sativus							
L. var. *hortensis*	- / - /P[k]	4.7	ND	ND	64/ n63	9.76/XYL	[Hata, 1992 #473]
Scopolia							
japonica	- /Sec/pP	8.0	4.8	<65	n62	6.7/ -	[Tanaka, 1978 #324]
Yeast							
Rhodotorula							
flava IFO 0407	- /Sec/P	ND	2.0	30	ND	9.1/ -	[Uesaka, 1978 #323]

[a]**Enzyme** = ARAF designation by authors; * = cloned. **Location** = cellular location (if known) or the fraction used for enzyme purification and/or assay: Sec = secreted; CA_s or CA_p = Cell-associated (CA) soluble (s) or particulate (p), respectively. **Degree of Purity** = purity achieved: P = purified; pP = partially purified; Cr = crude extract. A hyphen (-) indicates no information available.

[b]**pI:** Isoelectric point

[c]M_r: Subunit molecular mass determined by SDS-PAGE; n = native molecular mass.

Table 1. Footnotes continued from previous page.

[d] **K_m/ Other Hydrolytic Activities:** K_m determined with *p*-nitrophenyl-α-L-arabinofuranoside as substrate. Designation for other hydrolytic activities observed: XYL = β-xylosidase; AXH = arabinoxylan arabinofuranohydrolase; deb.ENDOX = debranching endoxylanase; XYLN = β-D-xylan xylanohydrolase; (-) no other hydrolytic activities detected.

[e] **ND:** Not determined

[f] Estimated molecular mass from the deduced amino acid sequence.

[g] Purified from carrot cell culture homogenate.

[h,i] Purified from germinated soybean (h) or lupin (i) seed (cotyledon) extract.

[j] Purified from solublized Japanese pear fruit cell walls.

[k] Purified from non-germinated radish seed extract.

Bacillus. Species of the genus *Bacillus* (Gram-positive endospore-forming bacteria) secrete a large number of industrially relevant enzymes, including cellulases and hemicellulases (Priest 1977). However, few have been examined for their xylanolytic enzymes (Coughlan and Hazlewood 1993). Of those studied thus far, all but one secrete their arabinofuranosidase enzymes. As examples, ARAF I purified from *Bacillus* sp. no. 430 was found in the spent growth medium and was able to liberate arabinose from *p*NP-AF, sugar beet arabinan, arabinogalactan, and arabinoxylan, thus placing it with the *A. niger* type of ARAFs (Yasuda, *et al.* 1983). The secreted AF166 purified from *B. polymyxa* was a 166 kDa polypeptide thought to consist of two 66 kDa and one 33 kDa subunits (Morales, *et al.* 1995). The enzyme had activity on *p*NP-AF and MU-AF substrates, but not on arabinoxylans, arabinans, or arabinogalactans, thus placing it with the *S. purpurascens* type ARAFs. *B. polymyxa* also contains a broad range of xylanases that have either been cloned and/or purified (Sandhu and Kennedy 1986, Yang, *et al.* 1988), along with a cloned endoxylanase (*xynD*) that contained arabinofuranosidase activity (Gosalbes, *et al.* 1991). The *xynD* gene product has recently been identified with the purified ARAFs (AF64 and AF53) of *B. polymyxa* (Morales, *et al.* 1995). These latter two ARAFs had identical N-terminal amino acid sequences that coincided with the deduced amino acid sequence from *xynD* after processing of the putative peptide signal sequence. In addition, the authors speculated that AF53 was a post-translationally modified product of AF64 created by removal of 100 amino acids from the C-terminal end. Both enzymes had activity on *p*NP-AF and MU-AF along with oat spelt- and wheat flour arabinoxylans, but neither was active on arabinoxylooligosaccharides, birchwood xylan, arabinan, or arabinogalactan. Studies with two purified xylanases from

B. polymyxa (Morales, *et al.* 1995), in combination with either ARAF, yielded greater overall xylanase activities, but no net increase in arabinose release.

B. subtilis F-11 secreted two ARAFs and an endoarabinase when grown on sugar beet residue (Weinstein and Albersheim 1979). One ARAF (II) was purified and shown to possess activity on *p*NP-AF, as well as an exo-activity on branched arabinans, but no activity upon a multitude of other synthetic or natural substrates (tested substrates included polysaccharides typically found in plant cell walls or synthetic mimics of their linkages). A secreted ARAF purified from *B. subtilis* 3-6 was specific for the furanoside linkage and released arabinose from arabinan and two of three highly purified arabinoxylooligosaccharides [*i.e.*, xylobiose (A_1X_2) and xylotriose (A_1X_3), but not xylotetraose (A_1X_4); (Kaneko, *et al.* 1994)]. The authors noted that the enzyme did not fit well into either of the two ARAF classes. They suggested additional research was needed with previously purified ARAFs to determine their activity on more defined substrates in order to establish a more refined ARAF classification scheme.

A thermophilic *Bacillus*, *B. stearothermophilus* L1, produced an ARAF that had a temperature optimum of 70°C, a native molecular mass of 110 kDa, and which consisted of 52.5 and 57.5 kDa subunits; and is only the second ARAF determined to consist of heterogeneous subunits (Bezalel, *et al.* 1993). When the ARAF from strain L1 was combined with a purified xylanase from strain L1, synergy was demonstrated on softwood kraft pulp by the 25% increase in the release of lignin, as compared to that released by the sum of the individual activities. *B. stearothermophilus* T-6 produced a mostly cell-associated ARAF with a native molecular mass of 256 kDa that was composed of four identical 64 kDa subunits (Gilead and Shoham 1995). The temperature

optimum for the T-6 ARAF was the same as that from *B. stearothermophilus* L1, but the first 50 N-terminal amino acids had 45.8% identity with an N-terminal region of an ARAF encoded by the *abfA* gene of *Streptomyces lividans* 66 (accession no. U04630). An unusual aspect of the T-6 ARAF was its weak activity on carboxymethylcellulose [(CMC), a soluble derivative of cellulose]. A T-6 xylanase exhibited synergy with the ARAF during hydrolysis of oat spelt arabinoxylan. About 60% of the ARAF activity was cell-associated, and only 9% of the cytoplasmic marker, isocitrate dehydrogenase, was found in the culture supernatant. Apparently, cell lysis was not the only explanation for the extracellular ARAF activity.

Bacteroides. The genus *Bacteroides* are Gram-negative anaerobes that have been studied mostly for their plant polysaccharide fermenting capabilities (Salyers, *et al.* 1977). Three xylan-degrading genes were cloned from *B. ovatus* V975 and found to cluster in a 3.8 kilobase region of the genome (Whitehead and Hespell 1990). A xylosidase with ARAF activity was partially purified from the cytoplasmic fraction of an *E. coli* JM83 clone. The enzyme was thought to be the first bifunctional xylan-degrading enzyme, *i.e.*, possessing both xylosidase and arabinofuranosidase activities.

Rumen bacteria. The rumen anaerobe, *Bacteroides xylanolyticus* X5-1, produces a mainly cell-associated ARAF that consisted of six identical 61 kDa subunits forming a 364 kDa native enzyme (Schyns, *et al.* 1994). The purified enzyme had activity on short arabinooligosaccharides, but not oat spelt arabinoxylan, arabinogalactan, or other aryl-glycosides. The anaerobe *Butyrivibrio fibrisolvens* GS113 produces an entirely cytoplasmic ARAF (Hespell and O'Bryan 1992) with a native molecular mass of 240 kDa and composed of eight identical 31 kDa subunits. The enzyme had activity on oat spelt-

and corn endosperm xylan, and sugar beet arabinan, but not arabinogalactan. The cytoplasmic location for ARAF suggested that its role was in removal of arabinose from arabinose-containing xylooligosaccharides generated by the action of other xylan-degrading enzymes and subsequently taken up into the cytoplasm.

A close phylogenetic relative of the *Bacteroides*, *Fibrobacter succinogenes*, is an anaerobic bacterium that produces a number of secreted xylan-degrading enzymes (Forsberg, *et al.* 1981). A purified debranching endoxylanase that contained ARAF activity had no activity on arabinans or synthetic ARAF substrates, but did release arabinose from oat spelt- and rye arabinoxylans and apparently did so before hydrolysis of the xylan backbone (Matte and Forsberg 1992). *Ruminococcus albus* 8 secretes a glycosylated ARAF (α-AF) of 305-310 kDa that consists of 75 kDa subunits (Greve, *et al.* 1984). α– AF was shown to act synergistically with a xylanase from *R. albus* 8 in hydrolyzing alfalfa cell walls.

Clostridium. The genus *Clostridium* includes anaerobic Gram-positive, endospore-forming, rod-shaped bacteria well known for their polysaccharide degrading capabilities (Bronnenmeier 1993, Hazelwood 1993). *C. acetobutylicum* ferments simple sugars to the solvents acetone, isopropanol, butanol, and ethanol. The organism also produces a variety of xylan-degrading enzymes including a secreted ARAF that consists of a single 94 kDa polypeptide (Lee and Forsberg 1987). The ARAF had no activity on arabinooligosaccharides with degrees of polymerization from 2 to 6. The enzyme released arabinose from arabinan, but had no activity on larchwood-, oat spelt-, and rye arabinoxylans, CMC, and arabinogalactan. Cooperativity with a xylosidase and/or

xylanase from the same strain was demonstrated with ARAF when used individually or in combination. A thermophilic *Clostridium*, *C. stercorarium*, produces a number of xylanolytic enzymes including two ARAFs encoded by genes *arfA* and *arfB* (Schwarz, *et al.* 1990). The *arfA* gene product was bifunctional, possessing both ARAF and xylosidase activities, whereas the *arfB* gene product was an ARAF specific for the furanoside configuration and was found to be the major ARAF activity in culture supernatants (Schwarz, *et al.* 1995).

Pseudomonads. An interesting feature of the ARAF encoded by *xynC* cloned from the soil-saprophyte *Pseudomonas fluorescens* subspecies *cellulosa* was that it contained a cellulose-binding domain [CBD; (Kellett, *et al.* 1990)]. Cellulose-binding domains have been described for a number of cellulases and xylanases (Ferreira, *et al.* 1993, Gilkes, *et al.* 1991, Kellett, *et al.* 1990), and although their role has not been entirely defined, CBDs are believed to enhance contact of the enzyme with cellulose. Although a number of organisms in Table 1 have been found to produce cellulases with cellulose-binding domains (*e.g.*, *Fibrobacter succinogenes*, *Thermomonospora fusca*, *Trichoderma reesei*, and *P. fluorescens* (Kellett, *et al.* 1990), the *xynC* gene product is the only ARAF so far described to contain a CBD. The author's speculate that the CBD could promote longer contact between the secreted ARAF and cellulose where it would have access to any nearby arabinose-containing polysaccharides.

Actinomycetes. Various actinomycetes have been recognized and studied for their xylan-degrading enzymes. The genus *Streptomyces* contains filamentous prokaryotes found mainly in soils and decomposing vegetation that produce a wide variety of

hemicellulolytic enzymes (McCarthy 1987, McCarthy and Ball 1987, Zimmerman 1989). Of the many *Streptomyces* possessing ARAF activities, eight strains were studied in detail and most were found to produce multiple ARAFs. Ten ARAFs were purified and/or cloned, including one debranching endoxylanase (Table 1). *Streptomyces* sp. no. 17-1 produced a secreted ARAF, whereas *S. diastatochromogenes* 065 produced two ARAFs, one secreted and one intracellular [(Higashi, *et al.* 1983, Kaji, *et al.* 1981), respectively]. *S. diastaticus* produced an apparent multiplicity of secreted ARAFs, two of which were purified and characterized (Tajana, *et al.* 1992). Two ARAF-encoding genes (*abfA* and *abfB*) were cloned from *S. lividans* strain 1326 [(Manin, *et al.* 1994, Vincent, *et al.* 1997), respectively]. The cloned *abfA* gene, which lacked a deduced putative signal peptide, was expressed in *S. lividans* IAF10-164 (a cellulase-, xylanase-, and arabinosidase-negative mutant) and purified from the cytoplasmic fraction. It was found to be a 380 kDa polypeptide consisting of 69 kDa subunits. Its role was thought to remove arabinose from arabinose-containing xylooligosaccharides produced by the action of other xylan-degrading enzymes and taken up into the cytoplasm. The *abfB* gene was also expressed in the same host system as *abfA* and was secreted into the culture supernatant (Vincent, *et al.* 1997). The deduced amino acid sequence showed that the enzyme contained a catalytic domain and a xylan-binding domain (XBD). XBDs have only recently been described for xylanases and the *abfB* gene is the first ARAF determined to contain one (Black, *et al.* 1995). Other actinomycetes that also produced secreted ARAF activities include *Streptomyces massasporeus* [an *A. niger* type of ARAF; (Kaji, *et al.* 1982)], *S. purpurascens* [the largest reported ARAF of 495 kDa, consisting of 62 kDa subunits; (Komae, *et al.* 1982)], *S. roseiscleroticus* [a debranching

endoxylanase; (Grabski and Jeffries 1991)], and *Thermomonospora fusca* [producing multiple xylanases with cooperative/synergistic activities; (Bachmann and McCarthy 1991)].

Fungi. Fungi are by far the largest group of ARAF-producing organisms represented in Table 1. They have long been known for their hydrolytic activities, and in 1928, Ehrlich and Schubert reported the presence of arabinose-releasing activity in Takadiastase (Ehrlich and Schubert 1928). In 1967, the first ARAF was purified and characterized from *Aspergillus niger* (Kaji, *et al.* 1967). Since then, at least 32 cloned and/or purified ARAF enzymes have been described from a number of different fungi (Table 1). As was stated before, all but one of the ARAFs were secreted. Two ARAFs were purified from *A. awamori*, including the one described before for its inability to act on *p*NP-AF (Kormelink, *et al.* 1991). The second enzyme showed two isozymic forms by isoelectric focussing, acted synergistically with an endoxylanase purified from the same strain, and has the unique distinction of being the only known ARAF to release feruloyl- and *p*-coumaroyl-linked arabinose substituents from wheat straw arabinoxylan (Wood and McCrae 1996). Two strains of *A. nidulans* produced two different ARAFs. Strain WG096 produced an ARAF B that was antigenically similar to the ARAF B of *A. niger* N400 (Ramon, *et al.* 1993). Other species and strains of *Aspergillus* had ARAFs with a range of sizes and activities (Table 1). Comparison of some of these show striking similarities such as those between the AXH of *A. awamori* and the AXH of *A. tubingensis* [(Gielkens, *et al.* 1997, Kormelink, *et al.* 1993), respectively]. All the ARAFs produced by these fungi were secreted, except that of *A. niger* 5-16. The ARAF from this organism was found intracellularly and had activity on *p*NP-AF and A_1X_2, but not on gum arabic,

arabinoxylan, A_1X_3, or A_1X_4 (Kaneko, *et al.* 1993). Examination of Table 1 shows the enzyme also to have similar properties to the ARAF B of *A. niger* N400 and ARAF B of *A. nidulans* WG096. All the ARAFs from fungi showed acidic pH optimums and nearly all had acidic or neutral pIs with the exception of *A. terreus*. Three ARAFs were purified from *A. terreus* and all three were shown to possess basic pIs (Luonteri, *et al.* 1995).

Several ARAFs were purified and cloned from various fungi including a secreted ARAF from the plant pathogen *Cochliobolus carbonum* that, when purified, digested with a proteinase, and the resulting fragments sequenced, yielded two peptide fragments with amino acid sequence identities of 56 and 64% to a cloned xylosidase/arabinofuranosidase from *Bacteroides ovatus* (accession no. U04957; (Ransom and Walton 1997)). Other secreted ARAFs purified and characterized from fungi include those from the plant pathogens *Monilinia fructigena* (Kelly, *et al.* 1987), *Phytophthora palmivora* (Akinrefon 1968), *Sclerotinia fructigena* (Laborda, *et al.* 1973), and *Sclerotinia sclerotiorum* (Baker, *et al.* 1979), the white-rot fungus *Dichomitus squalens* (Brillouet and Moulin 1985), and a partially purified ARAF from the thermophilic fungus *Thermoascus aurantiacus* (Roche, *et al.* 1994).

Yeast. A secreted ARAF was purified and characterized from the yeast, *Rhodotorula flava*, and found to have activity on *p*NP-AF, branched and linear beet arabinans, and arabinoxylan (Uesaka, *et al.* 1978).

Plants. A few ARAFs have been purified from carrot cell cultures, germinating soybean and lupin cotyledons, Japanese pears, radish seeds, and partially purified from callus tissue. The polysaccharidases found in plants are thought to be involved with seed

germination, cell growth, expansion, and maturation, although their biological role has yet to be determined (Cleland 1981). The most thoroughly investigated ARAF was that purified from a cell homogenate of carrot cell culture (Konno, *et al.* 1987). The enzyme possessed arabinose-releasing activity on *p*NP-AF, arabinans, and carrot pectic polysaccharide fractions (containing arabinosyl residues), but not on larch arabinogalactan, and had physicochemical characteristics similar to ARAFs from other organisms (Table 1). Examination of the ARAF activity present in germinating soybean cotyledons revealed several isozymic forms and it was suggested that these isoenzymes may contribute to the metabolism of seed stored- or structural polysaccharides through their different characteristics and compartmentalization (Hatanaka, *et al.* 1991). One isoenzyme was purified and had substrate specificity towards *p*NP-AF, arabinan, and arabinose-containing soybean polysaccharides, but had no activity towards arabinogalactans or arabinoxylans. On germination of lupin seeds, the growth of cotyledons is associated with the depletion of intercellular- and cell wall polysaccharides [in particular, those containing arabinose and galactose constituents (Matheson and Saini 1977)]. Two cell-associated ARAFs (I and II) were purified from germinating seed extracts and found to be specific for the furanoside configuration of *p*NP-AF and MU-AF substrates and released arabinose from several lupin polysaccharide fractions. The cell-associated ARAF purified from extracts of Japanese pear fruits had the second highest K_m reported for all purified ARAFs (Table 1), and its activity increased dramatically during fruit ripening. The ARAF activity purified from radish seed extracts was a bifunctional xylosidase/ARAF that was thought to use a single catalytic site for both activities (Hata, *et al.* 1992). The only plant thought to secrete an ARAF was *Scopolia japonica*, since its

ARAF was partially purified from suspension culture medium of the calluses of the plant. ARAF activity was detected in the roots, stems, and leaves of the plant during flowering. The apparently secreted ARAF was in contrast to the other intracellular polysaccharidases and was thought to be involved in cell wall softening during cell growth.

From the above discussion, one can deduce that the substrate specificities of purified ARAFs are complex and varied. Comparisons of physical attributes of ARAFs have been relatively straight forward, but comparisons of their activities have been much more difficult. This is due in part to their varying substrate preferences, and also to the fact that different researchers rarely test purified ARAFs on exactly the same substrate(s).

In as much as little is known about complex, cell-associated xylanase systems, or about biopolymer-degrading enzymes in cytophagas (a group of bacteria widely recognized for such activity), the aim of the present study was to characterize some of the major components of the cell-associated xylanase system from *Cytophaga xylanolytica*. Herein I report on the isolation, characterization, and localization of a major α-L-arabinofuranosidase component (ArfI) and its interaction with partially-purified endoxylanase components. Also included is the amino acid sequence of ArfI, as deduced from the nucleotide sequence of the cloned gene (*arfI*) encoding it, as well as the deduced amino acid sequence of a second ARAF (ArfII) encoded by a gene (*arfII*) not expressed by *C. xylanolytica* under our growth conditions.

CHAPTER 2

PURIFICATION AND PROPERTIES OF AN α-L-ARABINOFURANOSIDASE FROM *Cytophaga xylanolytica*

ABSTRACT

An α-L-arabinofuranosidase (α-L-arabinofuranoside arabinofuranohydrolase; EC 3.2.1.55; referred to herein as ArfI) was extracted from cells of *Cytophaga xylanolytica* strain XM3 by using Triton X-100 and was purified 85-fold by anion-exchange and hydrophobic interaction column chromatography. The native enzyme had a pI of 6.1 and an apparent molecular mass of 160-210 kDa, and it appeared to be a trimer or tetramer consisting of 56 kDa subunits. With *p*-nitrophenyl-α-L-arabinofuranoside as substrate, the enzyme exhibited a K_m of 0.504 m*M* and a V_{max} of 319 μmol · min^{-1} · mg $protein^{-1}$, and it had optimum activity at pH 5.8 and 45°C. ArfI was relatively stable over a pH range of 4 to 10 and at temperatures up to 45°C, and it retained nearly full activity when stored at 4°C for periods as long as 24 months. The enzyme also released arabinose from 4-methylumbelliferyl-α-L-arabinofuranoside, as well as from rye-, wheat-, corn cob-, and oat spelt arabinoxylans and sugar beet arabinan, but not arabinogalactan. ArfI showed no hydrolytic activity towards a range of *p*-nitrophenyl- or 4-methylumbelliferyl-glycosides other than arabinoside, for which it was entirely specific for the α-L-furanoside configuration. ArfI interacted synergistically with three partially purified endoxylanase fractions from *C. xylanolytica* in hydrolyzing rye arabinoxylan. However, cell fractionation studies revealed that ArfI was largely, if not entirely, cytoplasmic, so its activity *in vivo* is probably most relevant to hydrolysis of arabinose-containing oligosaccharides small enough to pass through the cytoplasmic membrane. Antibodies prepared against purified ArfI also cross-reacted with arabinofuranosidases from other

freshwater and marine strains of *C. xylanolytica*. To our knowledge, this is the first α-L-arabinofuranosidase to be purified and characterized from any gliding bacterium.

INTRODUCTION

Xylans are included within the family of plant cell wall heteropolysaccharides referred to as hemicelluloses. They consist of a β-1,4-linked xylopyranose backbone, to which are often attached side groups of arabinose, (*O*-methyl-) glucuronic acid, ferulic or *p*-coumaric acid, and/or acetate, depending on the plant source (Aspinall 1959, Timell 1967). Next to cellulose, xylans are the most abundant polysaccharides on earth (Biely 1985), and it has been estimated that 10^{10} metric tons are recycled annually, with the degradative arm occurring largely through the action of microbes (Wilkie 1983).

In an effort to increase our understanding of the microbiology and biochemistry of xylan degradation, we initiated a study of the xylan-hydrolyzing multienzyme system (*i.e.*, the "xylanase system") of a new, anaerobic gliding bacterium, *Cytophaga xylanolytica* strain XM3 (Haack and Breznak 1993). Strain XM3 and other freshwater and marine strains similar to it were isolated from freshwater sediments on the basis of their ability to adhere to, and dominate the initial fermentation of, insoluble xylan particles in sulfidogenic and methanogenic enrichment cultures. Unlike the secreted xylanases found with most other organisms, the xylanase system of *C. xylanolytica* was almost entirely cell-associated, but it can be easily extracted from whole cells by using 0.2% Triton X-100. Such Triton extracts were shown to possess activities important for xylan hydrolysis, including endo-1,4-β-D-xylanase (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), and α-D- and β-D-glucopyranosidases (EC 3.2.1.20 and EC 3.2.1.21, respectively) (Haack and Breznak 1992). Triton extracts were remarkably stable, retaining full xylanolytic activities for more than 6 months when stored at 4°C; however, they exhibited no activity toward microcrystalline cellulose, ball-milled Whatman no. 1 cellulose filter paper, or carboxymethyl cellulose (CMC) (Haack and Breznak 1992). The latter observations were consistent with the inability of cells to grow on cellulose or CMC.

Efforts to resolve the nature and number of components of the xylanase system yielded several column chromatography fractions each enriched with multiple endoxylanase (ENDOX) activities, as well one fraction enriched with a single arabinofuranosidase (ARAF) activity. ARAFs are important, because they catalyze the hydrolysis of arabinofuranosyl residues from hemicellulosic polysaccharides (Kaji 1984). Such residues are typically attached by α-1,2- and/or α-1,3-linkages to the backbones of arabinoxylans, arabinans, and arabinogalactans (Biely 1985, Dekker and Richards 1976), and their removal by ARAFs usually facilitates the attack of the xylan backbone by ENDOXs (Greve, *et al.* 1984, Lee and Forsberg 1987, Puls and Poutanen 1989, Wong, *et al.* 1988). Here we report the purification and characterization of the apparently only ARAF (designated herein as ArfI) produced by *C. xylanolytica* XM3 when it is growing on arabinoxylan. Also, included are the results of studies performed to (i) determine the cellular location of ArfI, (ii) examine synergy between ArfI and ENDOXs of *C. xylanolytica*, and (iii) evaluate the occurrence of ArfI (or antigenically similar enzymes) in other freshwater and marine strains of *C. xylanolytica*. (A preliminary report of the findings has been presented previously [Renner and Breznak 1995]).

MATERIALS AND METHODS

Growth of cells and preparation of cell extracts. *C. xylanolytica* strain XM3 (= DSM 6779) and other freshwater strains were grown anoxically at 30°C in rubber-stoppered 2 L Pyrex bottles nearly filled with Na_2S-reduced, CO_2/bicarbonate-buffered freshwater mineral medium no. 2 containing 0.2% (wt/vol) oat spelt arabinoxylan (preextracted with 70% [vol/vol] ethanol) as the sole fermentable substrate (Haack and Breznak 1993). Marine strains were grown in a similar manner, except that oat spelt arabinoxylan was used as substrate in marine medium (Haack and Breznak 1993).

For enzyme purification, cells of strain XM3 were harvested from late-exponential- to early-stationary-phase batch cultures by centrifugation at 10,000 x *g* for 20 min at 4°C. The cell pellets were resuspended in 0.2% (wt/vol) Triton X-100 to 1/40th of the original culture volume and stirred for 2 h at 4°C. The treated cells were then removed by centrifugation at 10,000 x *g* for 30 min at 4°C, and the resulting supernatant fluid was further centrifuged at 100,000 x *g* for 2 h at 4°C. The supernatant fluid from the latter centrifugation step was placed into a 3500 molecular weight cut-off (MWCO) dialysis membrane and dialyzed against 50 m*M* *N*-[2-hydroxyethyl]piperazine-*N*'-2-ethanesulfonic acid (HEPES) buffer (pH 8.0) (ca. 4 L total) at 4°C. The dialyzed material, hereafter referred to as the "Triton extract," was stored at 4°C until use.

Sonicated cell extracts were prepared from cells harvested by centrifugation at 18,000 x *g* and then resuspended in 10 m*M* HEPES buffer (pH 6.8). Sonication was for eight 30 s bursts [output 5 and 50% duty from a 1/8" diameter tapered horn; Branson Sonifier model 450 (Danbury, CT)], followed by recentrifugation.

Purification of ArfI. Purification of ArfI from Triton extracts was done at room temperature (RT) by a low-pressure column chromatography procedure performed with an Econo System apparatus (Bio-Rad Laboratories, Hercules, CA) operating at a flow rate of 1 ml · min^{-1} (unless otherwise noted). Eluted fractions were monitored for protein content, by measuring the A_{280} and for ARAF activity by using a semi-quantitative microtiter plate assay (see below). Sequential column chromatography was performed as follows: **(i)** Triton extract (204 ml, 430 mg protein) was applied to a column of DEAE cellulose (2.5 x 50 cm) previously equilibrated with 50 m*M* HEPES buffer (pH 8.0). The column was washed with 1 L of equilibration buffer at a flow rate of 2 ml · min^{-1}, and elution of ArfI was achieved by using a linear gradient of 0 to 1 *M* NaCl in the same buffer. Fractions containing ArfI eluted from the column between 50 and 200 m*M* NaCl and were pooled. The pooled material was concentrated by ultrafiltration through a YM 10 membrane having a 10 kDa MWCO (Amicon Co., Danverse, MA), and in the process the buffer was changed to 20 m*M* HEPES (pH 8.0); **(ii)** The ArfI pool from step (i) (75 ml, 180 mg of protein) was applied to a DEAE Sephadex A-50 column (1.5 x 50 cm) previously equilibrated with 20 m*M* HEPES (pH 8.0), and elution was performed with linear gradient of 0 to 1 *M* NaCl in the same buffer. Fractions containing ArfI eluted from the column between 0.1 to 0.4 *M* NaCl: these were pooled, dialyzed, and concentrated as before, except that the dialysis buffer was 20 m*M* potassium phosphate (pH 6.5); **(iii)** The ArfI pool from step (ii) (75 ml, 74 mg of protein) was applied to a hydroxylapatite column (1.5 x 50 cm) previously equilibrated with 20 m*M* potassium phosphate buffer (pH 6.5), and chromatography was performed with a linear gradient of 20 to 300 m*M* potassium phosphate. Fractions containing ArfI eluted from the column between 170 to

225 m*M* phosphate and were pooled; **(iv)** A solution of 5 *M* NaCl was added to the ArfI pool from step (iii) (43 ml, 7.7 mg of protein) to achieve a final concentration of 1 *M* NaCl. The pool was then applied to a Phenyl-Sepharose CL-4B column (1.0 x 50 cm) previously equilibrated with 1 *M* NaCl in 5 m*M* 3-[*N*-morpholino]propanesulfonic acid (MOPS) buffer (pH 6.5). Elution of ArfI was performed with a simultaneously decreasing linear gradient of 1 to 0 *M* NaCl and an increasing pH gradient of 6.5 to 8.0 in 5 m*M* MOPS buffer. ArfI activity eluted between 250 to 0 m*M* NaCl and pH 7.5 to 8.0 and relevant fractions were pooled; **(v)** The ArfI pool from step (iv) was concentrated by ultrafiltration (as above), and the buffer was changed to 20 m*M* MOPS (pH 7.5). This concentrated pool of ArfI (74.5 ml, 1.7 mg of protein) was then applied to a Macro Prep 50Q column (1.0 x 50 cm) previously equilibrated with 20 m*M* MOPS (pH 7.5). Chromatography was performed with an increasing linear gradient of 0 to 1 *M* NaCl in the same buffer. Active fractions eluted between 50 to 100 m*M* NaCl and were pooled; **(vi)** The ArfI pool from step (v) was dialyzed, as before, against 20 m*M* MOPS (pH 8.0) containing 0.5 *M* NaCl, and the dialyzed pool (91 ml, 1.6 mg of protein) was rechromatographed on a column of Phenyl-Sepharose CL-4B (1.5 x 50 cm), but this time with a decreasing linear gradient of 0.5 to 0 *M* NaCl in the same buffer and a flow rate of 0.5 ml · min^{-1}. Active fractions eluted between 25 and 0 m*M* NaCl and were pooled. Ultrafiltration was then used to concentrate the final pool to 28.8 ml (1.2 mg of protein), which was stored at 4°C.

Partial Purification of ENDOXs. Components from three major electrophoretically-separable zones of ENDOX activity [*i.e.*, top, middle, and bottom zone after native polyacrylamide gel electrophoresis (PAGE) gels of Triton extracts (see Results)] were

partially purified from a Triton extract by using a four step procedure. The first three steps involved sequential column chromatography on the following matrices/eluants by using conditions as described above for ArfI: **(i)** DEAE cellulose/↑[NaCl]; **(ii)** Hydroxylapatite/↑[PO_4^{-3}]; and **(iii)** Macro Prep 50Q/↑[NaCl]. A significant amount of ENDOX activity in the major peak eluting from step (i) did not bind to the hydroxylapatite column of step (ii) and eluted in the void volume. This material (referred to as ENDOX II) was enriched with ENDOX components that migrated in the middle zone of native PAGE gels and was saved. Steps (ii) and (iii) were effective in removing some additional contaminating proteins from the remaining ENDOX activity (which consisted of components from the "top" and "bottom" zones of native PAGE gels), but did not resolve these. Therefore, an additional step (step **iv**) was included and involved preparative native PAGE of the remaining ENDOX activity through a 28 mm (inside diameter) Model 491 Prep-Cell column (Bio-Rad) containing a 2 cm (4% [wt/vol] polyacrylamide) stacking gel and 10 cm (10% [wt/vol] polyacrylamide) resolving gel (Davis 1964). Electrophoresis was done according to the manufacturer's recommendations, and fractions were screened for ENDOX activity by release of reducing sugar from xylan (see below) and by native PAGE slab gels subsequently overlaid with oat spelt arabinoxylan zymogram gels (see below). Step (iv) separated the remaining ENDOX components into a group that migrated furthest during native PAGE (referred to as ENDOX III) and a group that migrated least (referred to as ENDOX I).

Enzyme assays. ARAF activity was determined semi-quantitatively by a modification of the Bachmann and McCarthy method (Bachmann and McCarthy 1991), as follows: 1.0 µl portions of enzyme (*e.g.*, column chromatography fractions) were added to separate wells

of a 96 well microtiter plate (Corning Cell Wells; Corning Glass Works, Corning, NY) which also contained 90 µl of 4-methylumbelliferyl-α-L-arabinofuranoside (MU-AF) solution (10 µg · ml^{-1}) per well. After various periods of incubation (from 10 min to 24 h at RT, depending on the relative activity) the microtiter plates were placed on a UV transilluminator, and active fractions were identified by the intense fluorescence of liberated methylumbelliferone.

Quantitative assays for ARAF activity were performed as described by Greve *et al.* (Greve, *et al.* 1984) with 1 ml total volume reaction mixtures containing 1 m*M para*-nitrophenyl-α-L-arabinofuranoside (*p*NP-AF) in 50 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) plus enzyme. Reaction mixtures lacking enzyme were pre-warmed to 45°C, and reactions were started by the addition of 20 µl of appropriately diluted enzyme (0.24 to 42 mg of protein) also pre-warmed in the same buffer (the actual pH of the reaction mixture at 45°C was determined to be 5.8). After 1 min, the reaction was terminated by the addition of 2 ml of 1 *M* NH_4OH, and the A_{405} of the resulting solution was measured with a Bausch & Lomb Spectronic 20 colorimeter. Absorbance readings were converted to micromoles of *p*-nitrophenol by comparison to a standard curve. One unit of ARAF activity was defined as the amount of enzyme that produced 1 µmol of *p*-nitrophenol per min under the assay conditions. Catalytic constants of purified ArfI were determined in a similar way, but by using triplicate reaction mixtures each having a total volume of 4.5 ml and containing: 50 m*M* MES (pH 6.0); *p*NP-AF ranging in concentration from 25 µ*M* to 5 m*M*; and 0.06 Units (U) of ArfI in 50 m*M* MES [pH 6.0]. Periodically during incubation, 1 ml samples were removed and added to a separate tube containing 2 ml of 1 *M* NH_4OH, after which the A_{405} was determined as above.

Michaelis-Menten kinetic parameters were determined by using the method of Wilkinson (Wilkinson 1961).

Glycosidase activities other than ARAF, as well as acetyl esterase, were determined by using other *p*-nitrophenyl- or 4-methylumbelliferyl- derivatives as substrates and were assayed either quantitatively, as described above for *p*NP-AF, or semi-quantitatively by the microtiter plate assay as described above for MU-AF. The *p*-nitrophenyl- derivatives tested included (each at 2.5 m*M* final concentration): α-L- and β-L-arabinopyranoside; α-D- and β-D-glucopyranoside; α-D- and β-D-galactopyranoside; α-L-rhamnopyranoside; α-L-fucopyranoside; β-D-lactopyranoside; β-D-xylopyranoside; β-D-cellobioside; α-D-mannopyranoside; β-D-glucuronide; and acetate. The 4-methylumbelliferyl- derivatives (each at 5 m*M* final concentration) included: α-L-arabinopyranoside; α-D- and β-D-glucoside; α-D- and β-D-galactoside; β-D-glucuronide; β-D-cellobioside; and acetate.

The ability of purified ArfI to release arabinose from hemicellulose substrates was tested by incubating (40°C for 48 h) 0.52 U of enzyme in 1 ml reaction mixtures buffered with MOPS (10 m*M*, pH 6.5) and containing (10 to 25 mg per reaction): Lenzing beechwood-, rye-, wheat-, and oat spelt (arabino)xylans; three corn cob arabinoxylan fractions (CCXA [containing 5.6% arabinose, 87.7% xylose, 3% glucose, 0.1% galactose, and 3.6% other], CCXB [containing 15.3% arabinose, 77.1 % xylose, 2.7% galactose, and 4.9% glucose], and CCX [containing 34.8% arabinose, 57.3% xylose, 7.2% galactose, 0.5% glucose, and 0.2% other] all corn cob arabinoxylan fractions were prepared and analyzed at the Agricultural Research Service, U. S. Department of Agriculture, Peoria, IL); arabinogalactan; and sugar beet arabinan. Enzyme-free substrate

controls were also incubated under identical conditions. At the end of incubation, reaction mixtures were centrifuged at 11,000 x *g* for 15 min to sediment particulate material. The resulting supernatant fluids were then brought to 70% ethanol by the addition of 100% absolute ethanol, and recentrifuged. These second supernatants were removed to fresh microcentrifuge tubes and lyophilized. When dried, the samples were redissolved in 50 µl of H_2O, and 1-5 µl of each was spotted onto a 20 x 20 cm thin-layer chromatography plate pre-coated with a 250 µm layer of Whatman silica gel (K5) 150A. The plate was placed in a glass enclosed tank for chromatography with a solvent mixture of n-butanol: acetic acid: H_2O (2:1:1; vol/vol/vol). Lanes containing authentic arabinose, xylose, xylobiose, xylotriose, xylotetraose, and xylopentaose (10 µg each) were included as standards. After chromatography, the plate was air-dried, then sprayed uniformly with ca. 4 ml of aniline-diphenylamine reagent (Sigma) and developed by heating at 85°C for 20 min. Sugars gave blue-green or brown spots.

ENDOX (or any other enzyme activity capable of hydrolyzing xylan, or portions of xylan) was assayed by measuring the release of reducing sugar from Lenzing beechwood-, oat spelt-, and rye (arabino)xylan (0.5 mg · ml^{-1} [wt/vol] final concentration). Reducing sugar was quantified by the method of Nelson (Nelson 1944), as modified by Somogyi (Somogyi 1952), with xylose as standard. Samples were boiled for 30 min with the copper reagent for optimal detection of reducing sugars. One unit of activity is defined as the amount of enzyme liberating 1 µmol of reducing sugar equivalent (as xylose) per minute under the assay conditions. Synergy between ArfI and ENDOX pools was determined by using reaction mixtures containing rye arabinoxylan [2 mg · ml^{-1}, in 10 m*M* HEPES buffer (pH 6.8) containing 0.5 m*M* $CaCl_2$) and various

amounts of each enzyme (pool). Assays were run for 90 min, during which time aliquots were removed for determination of reducing sugar as described above.

Glyceraldehyde-3-phosphate dehydrogenase was determined by the method of Velick (Velick 1955), as modified by Hespell and Canale-Parola (Hespell and Canale-Parola 1970), by using 1 ml reaction mixtures containing: 150 m*M* HEPES (pH 8.4); 2 m*M* nicotinamide adenine dinucleotide (NAD); 10 m*M* D,L-glyceraldehyde 3-phosphate; 50 m*M* KH_2AsO_4; 10 μ*M* dithiothreitol (DTT); and appropriately diluted enzyme. The reaction (at 25°C) was started by the addition of enzyme and followed continuously for 2 min at 340 nm in a Perkin-Elmer Lambda 14 spectrophotometer (Norwalk, CT) equipped with a thermal jacketed cuvette holder. The level of reduced NAD was estimated using the molar extinction coefficient (1 cm light path) of 6.22 x 10^3 at 340 nm (Horecker 1948).

Determination of pH and temperature optima and stability. The pH optimum for ArfI activity was determined by using reaction mixtures buffered over the range indicated (in 0.5 pH increments) with: sodium citrate, pH 3 to 6; MES, pH 5.5 to 6.5; and MOPS, pH 6.5 to 8.0. Pre-warmed (45°C) reaction mixtures (4 ml, final volume) contained 3.575 ml of 50 m*M* buffer and 400 μl of 10 m*M* *p*NP-AF and were initiated by adding ArfI (25 μl containing 0.026 U in 2 m*M* MES buffer [pH 6.0], and 1 ml aliquots were removed at 2 min intervals for assay of *p*-nitrophenol (above). A similar procedure was used to determine the temperature optimum of ArfI (5 ± 0.2°C increments over the range of temperatures from 5°C to 70°C). Final concentrations of reactants in these 2.5 ml reaction mixtures were: 10 m*M* MOPS (pH 6.5); 2 m*M* *p*NP-AF; and 0.065 U enzyme.

To determine the stability of ArfI when exposed to various pHs, 50 μl of purified ArfI (0.26 U in 10 m*M* MES [pH 6.0]) was added to 50 μl of the following buffers (all at 50 m*M* concentrations and in 0.5 pH increments): acetate, pH 4.0 to 5.5; MES, pH 5.5 to 6.5; MOPS, pH 6.5 to 8.0; N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS), pH 8.0 to 9.0; and 2-[N-cyclohexylamino]ethanesulfonic acid (CHES), pH 9.0 to 10.0. Control tubes containing 100 μl of 10 m*M* MES buffer (pH 6.0) and 100 μl of each 50 m*M* test buffer were checked to ensure that ArfI was being exposed to the intended pH. Tubes were incubated at RT, and after 24 and 72 h residual activity was determined by the routine *p*NP-AF procedure (above). To determine the stability of ArfI to various temperatures, 400 μl samples of purified ArfI (0.52 U in 10 m*M* MES [pH 6.0]) were incubated for 1 h at temperatures from 25 to 65°C in 5 ± 0.2°C increments. After incubation, residual enzyme activity was determined by using the *p*NP-AF procedure (above).

Slab Gel Electrophoresis and Zymograms. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 4% (wt/vol) polyacrylamide stacking gels and 12.5% (wt/vol) polyacrylamide resolving gels (Laemmli 1970). Native PAGE (4% [wt/vol] polyacrylamide stacking gel; 10%, 12%, or 7.5 to 18% gradient [wt/vol] polyacrylamide resolving gel) was performed without SDS and 2-mercaptoethanol, and without pre-exposure of sample proteins to 100°C (Bachmann and McCarthy 1991). Unless otherwise noted, all native and SDS-PAGE gels were stained with a Silver Stain Plus kit (Bio-Rad) according to the manufacturer's instructions. Isoelectric focussing (IEF) of proteins was performed with a model 111 mini IEF cell (0.4 mm thick mini-gels; Bio-Rad), and pH 5 to 7 ampholytes

according to manufacturer's instructions. Unless otherwise noted, all electrophoresis gels were prepared with GelBond PAG films (for native and SDS-PAGE; FMC BioProducts, Rockland, ME) or Gel Support Films (for IEF; Bio-Rad) to facilitate handling.

Slab gels for detection of nucleic acids were prepared with 0.8% agarose in Tris-borate-EDTA (TBE) buffer. Electrophoresis conditions, and ethidium bromide staining, were performed according to previously published methods (Sambrook, *et al.* 1989).

Zymograms were prepared by overlaying native PAGE or IEF gels with 7.5% polyacrylamide gels containing the desired substrate (Biely 1985, Royer and Nakas 1990). Zymogram gels for detecting ENDOX activity contained 1% (wt/vol) pre-extracted oat spelt arabinoxylan in 50 m*M* HEPES (pH 6.8) containing 0.5 m*M* $CaCl_2$ and were poured with GelBond Film. After an oat spelt arabinoxylan zymogram gel was placed on a native PAGE gel, both were wrapped together in Sealwrap (Borden Chemical, North Andover, MA) and incubated overnight at 40°C. After incubation, the zymogram gel was stained with Congo red (10 mg · ml^{-1}) for 2 h and destained for ca. 30 min with several changes of 1 *M* NaCl until bands of hydrolysis were visible and the destaining solution was fairly clear. The zymogram was then treated with 0.1% acetic acid, which converted Congo red-stained xylan to a dark purple color that enhanced the contrast of hydrolysis zones for photography.

Zymogram gels for detecting ARAF were prepared in a similar manner, but contained MU-AF (200 μg · ml^{-1}) instead of xylan and were incubated for only 20 min at room temperature before photography on a UV transilluminator.

Antibody Production and Western blotting. Polyclonal anti-ArfI antiserum was produced by injection of a female New Zealand White Rabbit with 100 μg of purified

enzyme emulsified in TiterMax (CytRx Corporation; Norcross, GA) according to the method of Harlow and Lane (Harlow and Lane 1988). A booster injection (100 μg, prepared as above) was administered at 28 days and serum was collected 14 days later. Anti-ArfI antiserum titer was determined by using 1 μg · well^{-1} of purified ArfI in a dot-blot apparatus (Bio-Rad) containing an Immobilon P^{SQ} nylon membrane (Millipore). Development was with a goat anti-rabbit IgG (H + L) alkaline phosphatase immun-blot assay kit (Bio-Rad) according to manufacturer's instructions. Western blots were performed by using 10% (wt/vol) polyacrylamide native PAGE gels, without GelBond film, blotted onto Immobilon P^{SQ} nylon membranes in a Trans-blot Cell apparatus (Bio-Rad) according to the method of Matsudaira (Matsudaira 1987). Detection of ArfI was performed as above with 1:20,000 diluted anti-ArfI antiserum. Preimmune rabbit serum (1:1000 dilution) was used to screen a duplicate blot for non-specific binding.

Fractionation of cells for ArfI localization. Cells from a 2 L culture were divided into equal portions and centrifuged at 10,000 x *g*, as above. After centrifugation, the supernatant (secreted enzyme fraction) was pooled and concentrated (10,000 MWCO membrane).

One cell pellet (containing cell-associated enzymes) was resuspended in 15 ml of 10 m*M* Tris buffer (pH 7.6) and subjected to sonication (above), followed by centrifugation (30,000 x *g* for 30 min at 4°C) to yield a particulate enzyme fraction and a soluble (*i.e.*, cytoplasmic + periplasmic) enzyme fraction.

A second cell pellet was subjected to an osmotic shock procedure similar to that described by Godchaux and Leadbetter (Godchaux and Leadbetter 1988). First, the pellet was thoroughly resuspended in 5 ml of ice cold 10 m*M* Tris buffer (pH 7.6) containing

0.3 *M* NaCl, and then rapidly warmed to 25°C in a 40°C water bath and incubated at 25°C for 5 min. The suspension was next chilled on ice and 0.75 mg of lysozyme (75 µl of 10 mg/ml stock solution) was added with rapid stirring. The lysozyme-treated cells were then subjected to a slow addition of 10 ml ice cold 10 m*M* Tris buffer (pH 7.6) and incubated for 2.5 min on ice. The suspension was then warmed as before to 25°C and kept at that temperature until >90% of cells were converted into spheroplasts (as determined by phase contrast light microscopy). Spheroplasts were centrifuged (30,000 x *g* for 30 min at 4°C) to yield a soluble periplasmic enzyme fraction (supernatant). The spheroplast pellet was then resuspended in 10 ml of 10 m*M* Tris buffer (pH 7.6), sonicated, and recentrifuged to yield a soluble cytoplasmic enzyme fraction (supernatant). A portion of the resulting pellet was treated with 0.2% Triton X-100 for 2 h at room temperature, then recentrifuged to yield Triton-extractable enzymes associated with particulate cell material.

Amino acid sequences of ArfI peptides. For partial amino acid sequence determination, purified ArfI (ca. 15 µg per lane) was electrophoresed on a 16% (wt/vol) polyacrylamide SDS-PAGE resolving gel with a 4% (wt/vol) polyacrylamide stacking gel and blotted onto Immobilon P^{SQ} membrane as described previously. After blotting, the membrane was rinsed with H_2O, soaked in 100% methanol, then stained with 0.2% Amido Black in 40% methanol for 30 seconds, and destained with multiple changes of H_2O. The band corresponding to the position of ArfI in each lane on the membrane was excised with a clean, sterile razor blade and placed in a sterile Eppendorf tube. The individual ArfI-containing membrane fragments were sent to the Worcester Foundation for Biomedical Research (Shrewsbury, MA) for sequence determination. Upon receipt, ArfI was digested

with trypsin and the oligopeptide fragments that were released were purified by reversed-phase high performance liquid chromatography (RP-HPLC). The N-terminal amino acid sequence of three such fragments was then determined by the Edman degradation method.

Alternatively, 48.0 μg of ArfI was digested with 3.125 μg of Endoproteinase Lys C (Boehringer Mannheim; Indianapolis, IN) at 37°C overnight according to the manufacturer's instructions. After digestion, sample was run on SDS-PAGE gel, blotted, and ArfI fragments excised, as above. ArfI-containing membrane fragments were sequenced, as above, at the Michigan State University Macromolecular Sequence Facility.

Examination for glycosylation. Glycosylation of purified ArfI was examined by using the Digoxigenin (DIG) Glycan Detection Kit according to the manufacturer's instructions (Boehringer Mannheim; Indianapolis, IN). Samples of ArfI (24 and 48 μg) were subjected to SDS-PAGE as described before, but without GelBond PAG film, then electroblotted onto an Immobilon P^{SQ} membrane (Millipore) by the method of Matsudaira (Matsudaira 1987). Positive (Transferrin) and negative (Creatinase) controls were included and were supplied by the manufacturer. The blotted membrane was washed with phosphate buffered saline (PBS), and then treated with sodium metaperiodate. The membrane was then washed again in PBS and treated with DIG-*O*-3-succinyl-ε-aminocaprioc acid hydrazide, washed with Tris-buffered saline (TBS), and stained with Ponceau S. After location of protein bands by the Ponceau S stain, the membrane was soaked in blocking solution (which removed Ponceau S stain) and washed with TBS. Next, the membrane was incubated with an anti-digoxigenin alkaline

phosphatase (AP) conjugated antibody. The membrane was then washed with TBS and glycosylated proteins were detected by incubation with AP substrate.

Other analytical procedures. The native molecular masses of purified ArfI and partially purified ENDOX pools were determined by size-exclusion chromatography with a Pharmacia fast protein liquid chromatography (FPLC) system equipped with either a Superose 6 (ArfI) or Superose 12 (ArfI and ENDOX pools) HR 10/30 columns (Pharmacia). The columns were pre-equilibrated with 20 m*M* MOPS (pH 6.5) containing 50 m*M* NaCl and run at a flow rate of 0.5 ml · min^{-1}. Column effluents were monitored for absorbance at 280 nm, as well as for enzyme activity by using the MU-AF plate assay (ArfI) or reducing sugar assay and native PAGE zymograms (ENDOX pools). Size-exclusion chromatography standards for Superose 6 included: blue dextran 2000 (2000 kilodaltons [kDa]); bovine thyroglobulin (670 kDa); ferritin (440 kDa); catalase (232 kDa); and aldolase (158 kDa). Size-exclusion chromatography standards for Superose 12 included: thyroglobulin (670 kDa); gamma globulin (158 kDa); ovalbumin (44 kDa); myoglobin (17 kDa); and vitamin B-12 (1.35 kDa).

Protein was determined as described by Smith (Smith, *et al.* 1985) using the Micro Bicinchoninic Acid (BCA) Protein Assay Reagent Kit (Pierce, Rockford, IL) or by the Bradford assay (Bradford 1976) with bovine serum albumin as standard.

Chemicals and other materials. Oat spelt arabinoxylan, arabinogalactan, xylose, *p*-nitrophenyl derivatives, 4-methylumbelliferyl derivatives, DEAE Sephadex A-50, nicotinamide adenine dinucleotide, D,L-glyceraldehyde 3-phosphate; KH_2AsO_4, dithiothreitol and bovine thyroglobulin were obtained from Sigma Chemical Co.

(St. Louis, MO). Phenyl-Sepharose CL-4B, blue dextran 2000, ferritin, catalase, and aldolase were obtained from Pharmacia Biotech (Uppsala, Sweden). Low molecular weight SDS-PAGE standards, IEF Standards, FPLC size-exclusion standards, pH 5 to 7 ampholytes, hydroxylapatite, and Macro-Prep 50Q were obtained from Bio-Rad Laboratories (Hercules, CA). DEAE cellulose was obtained from Whatman Specialty Products Inc. (Fairfield, NJ). Xylobiose, xylotriose, xylotetraose, xylopentaose, rye- and wheat arabinoxylans, and sugar beet arabinan were obtained from Megazyme, Inc. (Sydney, Australia). SPECTRA/POR molecularporous membrane tubing (45mm wide; 3500 molecular weight cutoff [MWCO]) was from Spectrum Medical Industries, Inc. (Los Angeles, CA). Lenzing beechwood xylan and three corn cob arabinoxylan fractions were kind gifts from Dr. Robert B. Hespell (Agricultural Research Service, U. S. Department of Agriculture, Peoria, IL). Other chemicals were of reagent grade and were obtained from commercial sources. All H_2O used was double distilled (Millipore Corp.; Bedford, MA).

RESULTS

Enzyme Activities in Crude Extracts. Triton extracts of *C. xylanolytica* contained a variety of enzymatic activities important for hydrolysis of xylans and other saccharides, including α-L-arabinofuranosidase (but not α-L-arabinopyranosidase), β-D-xylosidase, α-D- and β-D-glucosidases, α-D- and β-D-galactosidases, and acetylesterase (Figure 1). Likewise, native PAGE of Triton extracts revealed an array of electrophoretically separable proteins, including approximately 15 with ENDOX activity and distributed within a top (least migrating), middle, and bottom (furthest migrating) zones of the gel and detectable by zymograms with oat spelt arabinoxylan (Figure 2A and C; lane 1). By contrast, analogous zymograms with MU-AF revealed that ARAF activity was associated with only a single band, and this was referred to as ArfI (Figure 2B; lane 1). No apparent activity was seen for β-D-glucuronidase or β-D-cellobiosidase when assayed by using MU-linked substrates in microtiter plate wells (Figure 1).

Purification and Physicochemical Properties of ArfI. ArfI was purified 85-fold to homogeneity from Triton extracts by using anion exchange and hydrophobic interaction column chromatography (Table 2; Figures 2 and 3). With these procedures, 23.6% of the original activity in Triton extracts was recovered. A major step in the purification procedure was chromatography on hydroxylapatite, which alone yielded an 8-fold increase in purity. Although a slight decrease in specific activity was seen after Macro Prep 50Q chromatography (presumably owing to some inactivation of ArfI), this step

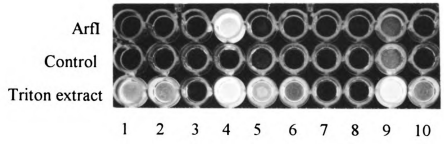


Figure 1. Microtiter plate assay comparing the activity of purified Arfl (1st row) to activities present in Triton extract (3rd row) on the following 4-methylumbelliferyl derivatives (all at 5 m*M* final concentration in 25 m*M* MES, pH 6.0): **1,** β-D-galactoside; **2**, α-D-galactoside; **3**, α-L-arabinopyranoside; **4**, α-L-arabinofuranoside; **5**, α-D-glucoside; **6**, β-D-glucoside; **7**, β-D-glucuronide; **8**, β-D-cellobioside; **9**, acetate; **10**, β-D-xyloside. Each well of purified Arfl contained 48 ng of protein (equivalent to 10.6 U of ARAF activity). Each well of Triton extract contained 4.2 μg of protein (equivalent to 0.011 U of ARAF activity). Control (2nd row) contained test substrate, but lacked enzyme. Incubation was for 12 h at room temperature.

Figure 2. Zymograms and a protein stain of native PAGE gels of ArfI after each purification step. **A. Oat spelt arabinoxylan** zymogram (stained with Congo red) of endoxylanase activity. **B. MU-AF** zymogram showing ARAF activity as a UV fluorescent band. **C. Native PAGE** (silver-stained) gel. Lanes 1-7 correspond to fractions obtained after each purification step listed in **Table 2** and contained 105; 123; 49; 9.1; 1.1; 0.9; and 1.2 μg of protein, respectively (B & C). The gel for panel A was loaded with 3x as much protein per lane. Arrow indicates position of ArfI. Note: Panels B & C are of higher magnification than A.

Figure 2. Zymograms of native PAGE gels of ArfI after each purification step.

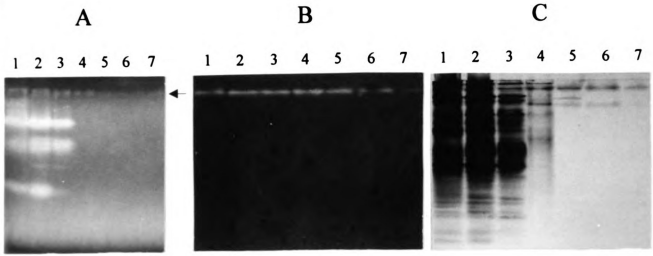


Table 2. Summary of Purification of α-L-arabinofuranosidase from *C. xylanolytica*.

Purification Step[a]	Total protein (mg)	Total activity (U)[b]	Sp act ($U \cdot mg^{-1}$)	Purification (fold)	Yield (%)
1. none (Triton Extract)	429.9	1,118	2.6	1.0	100.0
2. DEAE Cellulose	183.9	1,067	5.8	2.2	95.4
3. DEAE Sephadex	73.1	951	13.0	5.0	85.1
4. Hydroxylapatite	7.7	808	104.9	40.3	72.3
5. Phenyl Sepharose	1.7	347	204.1	78.5	31.0
6. Macro Prep 50Q	1.6	291	181.9	70.0	26.0
7. Phenyl Sepharose	1.2	264	220.0	84.6	23.6

[a] See Materials and Methods for details.

[b] As measured with *p*NP-AF as substrate.

nevertheless eliminated a significant amount of contaminating protein (compare lanes 5 and 6 of Figures 2C and 3).

Size-exclusion FPLC of purified ArfI yielded a single, symmetrical peak corresponding to a native molecular mass of 210 kDa with Superose 6 and 160 kDa with Superose 12 (not shown). However, SDS-PAGE revealed that ArfI consisted of subunits of 56 kDa (Figure 3, lane 7), implying that the native enzyme was a trimer or tetramer. IEF of purified ArfI yielded a single protein band with a pI of 6.1, which corresponded to the only ARAF activity in the IEF gel detectable by zymogram analysis (Figure 4). At no time during the purification of ArfI was more than a single activity region observed to elute from chromatographic columns, or more than a single band of activity observed on zymograms of native PAGE gels (Figure 2B). The major physical properties of ArfI are included as part of Table 3.

Enzymatic Activity and Stability of ArfI. With *p*-nitrophenyl-α-L-arabinofuranoside as substrate, ArfI had optimum activity at pH 5.8 and 45°C and exhibited a K_m of 0.504 m*M* and a V_{max} of 319 μmol · min^{-1} · mg $protein^{-1}$. ArfI was highly specific for the α-L-arabinofuranoside linkage, as no hydrolytic activity was seen with the following *p*NP derivatives: α-L- and β-L-arabinopyranosides; α-D- and β-D-glucopyranosides; α-D- and β-D-galactopyranosides; α-L-rhamnopyranoside; α-L-fucopyranoside; β-D-lactopyranoside; β-D-xylopyranoside; β-D-cellobioside; α-D-mannopyranoside; β-D-glucuronide; and acetate. This specificity was also seen when testing MU derivatives of many of these same compounds (Figure 1).

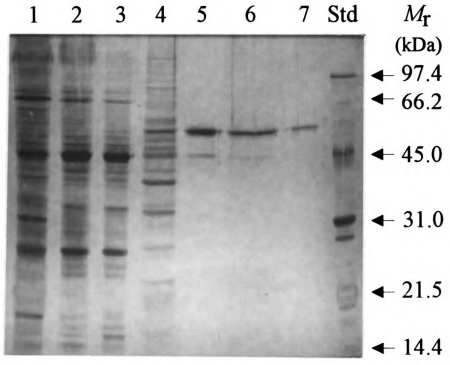


Figure 3. SDS-PAGE (silver-stained) gel of ArfI after each purification step. Lanes 1-7 correspond to fractions obtained after each purification step listed in **Table 2** and contained 10.5, 9.8, 4.9, 3.6, 2.3, 1.7, and 2.4 μg protein, respectively. Std, molecular weight markers in kilodaltons (kDa): 97.4, rabbit muscle phosphorylase B; 66.2, bovine serum albumin; 45.0, hen egg white ovalbumin; 31.0, bovine carbonic anhydrase; 21.5, soybean trypsin inhibitor; 14.4, hen egg white lysozyme.

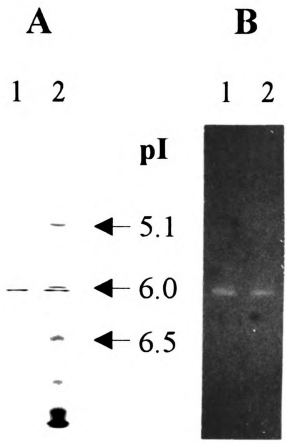


Figure 4. IEF gel of purified ArfI (1 μg protein per lane): stained with crocein scarlet (**A**); or overlaid with a MU-AF zymogram gel (**B**). **Lane: 1,** purified ArfI; **2**, purified ArfI plus IEF standards (pI standards: 5.1, β-lactoglobulin B; 6.0, bovine carbonic anhydrase; 6.5, human carbonic anhydrase).

Table 3. Properties of Purified α-L-arabinofuranosidase (ArfI).

M_r (kDa)	
Native protein	160-210
Subunits	56
pI	6.1
Glycosylation	not detected
K_m[a]	0.504 ± 0.034 m*M*
V_{max}[a]	319 ± 6.6 μmol · min^{-1} · mg^{-1}
Optima[a]	
pH (stability)[b]	5.8 @ 45°C (pH 4-10; 24 hr)
temperature (stability)[b]	45-50°C (≤ 50°C; 1 hr)

[a] As measured with *p*NP-AF as substrate.

[b] Retention of ≥80% activity, after exposure to conditions indicated.

Small amounts of reducing sugar were liberated when ArfI was incubated with oat spelt arabinoxylan, and this was due primarily to the release of arabinose (see below). However, bands of electrophoretically separated ArfI also coincided with a faint zone of Congo red non-binding that was present in zymogram gels designed to screen for ENDOX activity (Figure 2A, arrow). The basis for such band formation is not known, but does not appear to represent ENDOX activity associated with ArfI (see below).

A sensitive TLC assay was used to demonstrate the ability of ArfI to liberate arabinose from rye-, wheat-, corn cob-, and oat spelt arabinoxylans and sugar beet arabinan (Figure 5). Although the spot on TLC plates corresponding to arabinose was usually the most intense from all such digests, there was also a region of less intensely stained material below the arabinose spot in digests of corn cob- and oat spelt arabinoxylans (Figure 5; lanes 10, 12, 14, and 16). The nature of this material is unknown, but it migrated a distance between that of xylobiose and xylotriose standards and may be a branched oligosaccharide. The barely detectable arabinose spot seen with ArfI digests of corn cob arabinoxylan fraction A (Figure 5; lane 10) was not surprising, inasmuch as this substrate contained only 5.6% arabinose by weight. ArfI displayed no apparent hydrolytic activity on arabinogalactan or Lenzing beechwood xylan (not shown).

Whether present in Triton extracts or in purified form, ArfI was quite stable in solution. Essentially full ARAF activity was retained in Triton extracts when stored at 4°C for periods as long as 24 months. In fact, zymograms of native PAGE gels of Triton

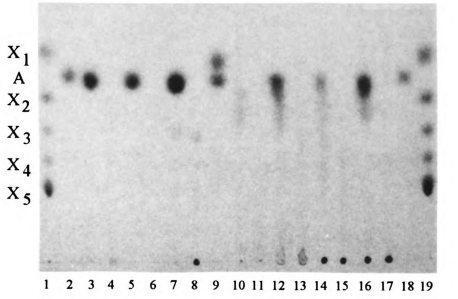


Figure 5. Thin-layer chromatogram of various arabinoxylans and an arabinan before (*i.e.*, controls) and after exposure to purified ArfI. **Lane: 1 and 19**, X_1-X_5 xylooligosaccharide standards; **2 and 18**, arabinose standard; **3**, rye arabinoxylan (RAX) + ArfI; **4**, RAX control; **5**, wheat arabinoxylan (WAX) + ArfI; **6**, WAX control; **7**, sugar beet arabinan (SBA) + ArfI; **8**, SBA control; **9**, A + X_1 standards; **10**, corn cob xylan A (CCXA) + ArfI; **11**, CCXA control; **12**, corn cob xylan B (CCXB) + ArfI; **13**, CCXB control; **14**, corn cob xylan (CCX) + ArfI; **15**, CCX control; **16**, oat spelt arabinoxylan (OSAX) + ArfI; **17**, OSAX control. Symbols: **A** = arabinose; $\mathbf{X_1}$ = xylose; $\mathbf{X_2}$ = xylobiose; $\mathbf{X_3}$ = xylotriose; $\mathbf{X_4}$ = xylotetraose; $\mathbf{X_5}$ = xylopentaose.

extracts stored at 4°C for up to 48 months had ARAF and ENDOX activities that were only slightly less than that seen with fresh extracts (not shown). Likewise, purified ArfI retained ≥ 80% of its activity when exposed at RT to pHs of 4 to 10 for 24 h, or to pHs from 6 to 10 for 72 h. Full activity was retained after exposure to temperatures up to 45°C for 1 h; however, activity began to decline sharply on exposure to temperatures >50°C and was completely lost at 65°C. The stability and catalytic properties of ArfI are included as part of Table 3.

Interaction of ArfI with Endoxylanases. The documented ability of many ARAFs to interact synergistically with ENDOXs (Bachmann and McCarthy 1991, Greve, *et al.* 1984, Kormelink, *et al.* 1991, Lee and Forsberg 1987, Manin, *et al.* 1994, Poutanen 1988, Vincent, *et al.* 1997, Wood and McCrae 1996), prompted us to explore this phenomenon with the analogous enzymes of *C. xylanolytica*. ENDOX components from three main zones separable by native PAGE of Triton extracts (Figure 2A) were partially purified and referred to as ENDOX I, ENDOX II and ENDOX III (Figure 6). The ENDOX II pool consisted of at least four ENDOX enzymes, including a small but significant amount of a component(s) present in ENDOX III. In contrast, the ENDOX I and ENDOX III pools each contained only one or perhaps two electrophoretically-resolvable ENDOX components. Each ENDOX pool, however, contained far less non-ENDOX Coomassie blue-stainable material than crude Triton extracts. Size-exclusion FPLC of each ENDOX pool yielded a single activity peak that eluted at a position corresponding to 130 kDa for ENDOX I, 63 kDa for ENDOX II, and 43 kDa for ENDOX III.

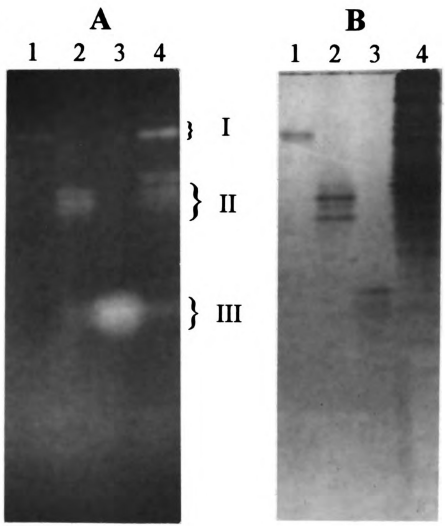


Figure 6. Zymogram and protein stain of native PAGE gels of partially purified ENDOX pools. **A**. **Oat spelt arabinoxylan** zymogram (stained with Congo red). **B**. **Native PAGE** (Coomassie blue-stained) gel. **Lane: 1**, ENDOX I; **2**, ENDOX II; **3**, ENDOX III; and **4**, Triton extract (0.24, 1.03, 1.30, and 110.9 μg of protein, respectively).

When ArfI was mixed with the ENDOX I, II, or III pool, the rate of hydrolysis of rye arabinoxylan was 22 to 46% greater than the sum of the rates for each component acting alone (Table 4). The increased rate of hydrolysis was apparently due to catalytic synergy, as opposed to enhanced stability of one enzyme component conferred by the presence of the other, because inclusion of an equivalent amount of bovine serum albumin (BSA) as a surrogate for any one of the enzyme components in the two-component reaction mixtures did not affect the rate of hydrolysis catalyzed by the remaining component acting alone.

Cellular Distribution of ArfI and ENDOX activities. The relatively high molecular weight of most arabinoxylans (Aspinall 1980, McNeil, *et al.* 1984), requires that they undergo hydrolysis to fragments small enough to pass through the cell membrane before dissimilation of the monomeric units can take place by cytoplasmic enzymes. Inasmuch as most of the enzymes of the xylanase system of *C. xylanolytica* are cell-associated, it therefore seemed likely that some or all of them, including ArfI, might be on or in the outer membrane or periplasm of this Gram-negative bacterium, where they would have access to the native substrate or to large fragments released from it. This notion was supported by the ready extractability of the xylanase system from whole cells with a low concentration (0.2%) of the detergent Triton X-100, a treatment which caused little or no obvious disruption of cells as assessed by phase-contrast and electron microscopy (Haack and Breznak 1992). However, Triton extracts were found to exhibit glyceraldehyde-3-phosphate dehydrogenase (GAP) activity (a typically cytoplasmic enzyme), and 0.8% agarose gel electrophoresis of Triton extracts revealed the presence of ethidium bromide

Table 4. Enhancement Between ArfI and ENDOX Fractions of *C. xylanolytica* in Hydrolysis of Rye Arabinoxylan.

Enzyme	Reducing sugar released[a]	Enhancement[b] (%)
ArfI	2.41	
ENDOX I	0.30	
ArfI + ENDOX I	3.42	26.2
ENDOX II	2.02	
ArfI + ENDOX II	6.45	45.6
ENDOX III	2.33	
ArfI + ENDOX III	5.79	22.2

[a] As μmoles of xylose equivalent · min^{-1} · ml^{-1}.

[b] Values represent the % increase in reducing sugar release above that expected from the action of ArfI alone + relevant ENDOX fraction alone. Controls for catalytic synergy consisted of an amount of bovine serum albumin equivalent in protein content to that of ArfI or the ENDOX fraction used in the reaction mixture.

stained material that migrated to a position typical of that of RNA (not shown). These observations suggested that Triton X-100 was in fact disrupting the cytoplasmic membrane enough to liberate cytoplasmic components. Therefore, a cell fractionation procedure was used to determine more precisely the cellular location of ArfI and other enzymes of the xylanase system.

Sonication of xylan-grown cells, followed by centrifugation, yielded a soluble supernatant fraction that contained almost all of the ArfI and GAP activities (Table 5). This indicated that ArfI resided in the cytoplasm and/or periplasm. When cells were subjected to an osmotic shock, little or no ArfI or GAP activities were released into the shock fluid (Table 5), suggesting that each was located primarily, if not entirely, in the cytoplasm. Unfortunately, a legitimate positive control could not be included in this experiment, as no authentic periplasmic enzymes have yet been documented for *C. xylanolytica*, and alkaline phosphatase (a typically periplasmic enzyme in many Gram-negative bacteria) was almost undetectable in this bacterium. However, the ability of exogenously added lysozyme to induce spheroplast formation during the osmotic shock procedure implied that the outer membrane was being disrupted enough to allow proteins such as lysozyme to pass through it. Therefore, if ArfI was located in the periplasm a significant amount of it should have been released into the post-shock extracellular fluid, but that was not the case. Rather, most of the ArfI and GAP activities from such spheroplasts were liberated into the soluble fraction following sonic disruption, a result entirely consistent with these enzymes being cytoplasmic. The relatively high proportion of GAP associated with the particulate fraction of spheroplasts (18.5%) is

Table 5. Distribution of ArfI and ENDOX Activities in Cells of *Cytophaga xylanolytica*.

Treatment/Cellular Fraction	Activity or Protein (% of total)[a]			
	ArfI	ENDOX	GAP	Protein
Sonicated Cells				
soluble fraction	96.6	60.3	99.9	79.7
particulate fraction	3.4	39.7	<0.1	20.3
Osmotic Shocked Cells				
extracellular shock fluid	1.8	8.7	<0.1	2.3
soluble (cytoplasmic) fraction	87.0	72.5	57.2	77.4
particulate fraction	2.6	19.7	18.5	13.1
Total recovery[b]	91.4	100.9	75.7	92.8

[a] Total amounts present in 1×10^{10} sonicated cells of *C. xylanolytica* are as follows: ArfI, 0.36 U; ENDOX, 0.36 U; GAP, 0.66 U; and protein 1.33 mg.

[b] Based on values given in footnote [a].

curious and may reflect a change in cells resulting from osmotic shock, as virtually all the GAP is routinely recovered in the soluble fraction of non-osmotically-treated cells.

In each cell fraction in which ArfI activity was detected, the activity could be attributed to the ArfI protein, based on zymograms prepared from native PAGE of homologous fractions (Figure 7A). In this experiment, virtually no ArfI (or any ARAF) activity was detected in concentrated samples of cell-free spent growth medium (Figure 7A; lane 5), although such samples contained a variety of proteins (Figure 7B; lane 5) including some ENDOX components (see below). However, a trace amount of ARAF activity observed in culture fluids in previous experiments eluted in the same position as ArfI during column chromatography and had similar pI values in IEF gels (not shown). Attempts to confirm further the primarily cytoplasmic location of ArfI, by immunogold-labelling followed by electron microscopy, were unsuccessful, presumably because the absolute amount of ArfI protein in the cytoplasm was too dilute to be detected in thin sections. However, the polyclonal anti-ArfI antiserum found use in screening other strains of *C. xylanolytica* for cross-reactive proteins in Western immunoblots (see below).

In contrast to ArfI, ENDOX activity was more widely distributed in *C. xylanolytica*, a result not surprising given the diversity of electrophoretically-separable ENDOX proteins produced by cells (*e.g.*, Figure 2A; lane 1). After sonication of whole cells, about 60% of the ENDOX activity was recovered in the soluble fraction (Table 5), which was rich in a variety of components representing the ENDOX I class, as well as two fairly discrete components representing the ENDOX II class (Figure 8; lanes 2 and 3). Triton X-100 extraction of the ENDOX activity associated with particulate fraction

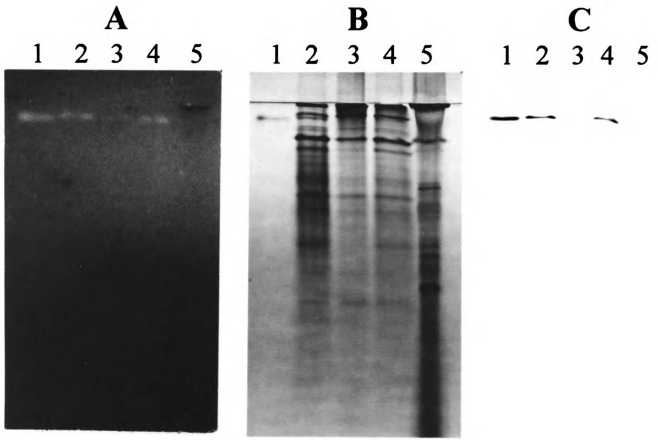
revealed that it also contained components from ENDOX I, as well as a band of activity from the ENDOX II region that appeared to be different from each of those present in the soluble fraction of sonicated cells (Figure 8; lane 4).

When cells were subjected to osmotic shock, about 9% of the total ENDOX activity was released into the shock fluid (Table 5) and presumably represented ENDOX components present in the periplasm. Zymograms of this material (Figure 8; lane 5) were similar to that of Triton-extracted particulate cell material obtained after sonication of whole cells (Figure 8; lane 4) suggesting that some of the ENDOX activity associated with the latter (Table 5) was due to periplasmic ENDOXs that were not completely liberated by sonication of cells and, hence, remained entrapped within the particulate fraction. Most of the ENDOX activity remaining in the lysozyme-induced spheroplasts formed during the osmotic shock procedure (ca. 73% of total) appeared to be of cytoplasmic origin, as it was released as soluble activity following sonication of the spheroplasts (Table 5). As expected, its zymogram profile (Figure 8; lane 6) was virtually identical to that obtained in the soluble fraction of sonicated whole cells (Figure 8; lane 2). Triton X-100 extraction of the particulate fraction of spheroplasts contained about 20% of the total activity and yielded a zymogram pattern (Figure 8; lane 7) similar to that of material extracted with Triton from the particulate fraction of whole cells and that released by osmotic shock (Figure 8; lanes 4 and 5, respectively).

Although cell-free spent growth medium generally contained $\leq$ 5% of the total ENDOX activity of cultures, zymograms of this material after native PAGE (Figure 8;

Figure 7. Specificity of anti-ArfI antiserum. **A. MU-AF** zymogram of native PAGE gel (viewed by UV light). **B. Native PAGE** (silver-stained) gel. **C. Western blot** with anti-ArfI antiserum. **Lane: 1**, purified ArfI; **2**, soluble cell-free extract (obtained by sonication and centrifugation); **3**, Triton extract of particulate cell material (obtained by sonication and centrifugation); **4**, Triton extract of whole cells; and **5**, concentrated cell-free growth medium (0.6, 75.0, 72.5, 60.0, and 72.0 μg of protein, respectively).

Figure 7. Specificity of anti-ArfI antiserum.



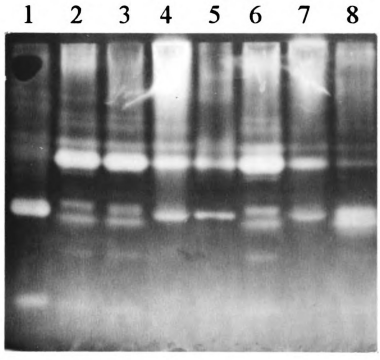


Figure 8. Oat spelt arabinoxylan zymogram of native PAGE gel showing the distribution of ENDOX components in cells and culture fluids of *C. xylanolytica* strain XM3. **Lane: 1**, concentrated cell-free growth medium; **2**, soluble cell-free extract; **3**, soluble cell-free extract plus Triton X-100; **4**, Triton extract of particulate cell material; **5**, osmotic shock fluid (= cell periplasm fraction); **6**, soluble extract of osmotically shocked cells (= soluble, cytoplasmic fraction); **7**, Triton extract of particulate fraction from osmotically shocked cells; **8**, Triton extract of untreated cells (0.014, 3.7, 3.7, 2.8, 0.094, 5.1, 1.8, and 4.4 mg protein, respectively).

lane 1) revealed activities of similar electrophoretic mobility to those seen in cell-associated ENDOXs including component(s) from ENDOX II (in particular, the ENDOX II component released by osmotic shock and by Triton extraction of particulate cell material (Figure 8; lanes 4, 5 and 7), and a component from ENDOX III that was seen in cytoplasmic contents (Figure 8; lanes 2 and 6).

ArfI-like Enzymes in Other Strains of *C. xylanolytica*. Polyclonal antiserum produced against purified ArfI was specific for ArfI, as judged by Western immunoblot analysis of native PAGE gels containing purified ArfI, or various cell fractions containing other proteins as well (Figure 7C). As with the zymograms, Western immunoblots failed to demonstrate ArfI in cell-free spent culture fluids.

The antiserum was used to determine whether ArfI-like proteins were present in other freshwater and marine strains of *C. xylanolytica*. Zymograms of native PAGE gels containing soluble, cell-free extracts from sonicated cells of various freshwater and marine strains of *C. xylanolytica* (Haack and Breznak 1993) revealed that most of the strains possessed an ARAF, which appeared as a single band of activity (Figure 9A). However, three marine strains did not appear to express any ARAF activity (Figure 9A; lanes 11-13), and the ARAF activity bands for the two other marine strains were very faint (Figure 9A; lanes 9 and 10). Zymograms prepared from Triton extracts of the particulate fraction of sonicated cells yielded patterns that were essentially identical to those seen for the soluble cell fraction (not shown). Western immunoblots showed that ARAFs produced by various freshwater (but not marine) strains cross-reacted with the anti-ArfI antiserum (Figure 9B), although some of these did so only weakly (*e.g.*, Figure

9B; lanes 4-6). The immunoblots also showed that some of the freshwater and marine strains had additional cross-reactive bands that were not associated with any ARAF activity detectable by zymogram analysis (Figure 9B; lanes 7-10).

Internal peptide sequences and glycosylation of ArfI. We were unable to obtain an N-terminal amino acid sequence of purified ArfI, as the protein appeared to be blocked. However, treatment of ArfI with trypsin (a protease that cleaves after a lysine or arginine residue) followed by reversed phase-high pressure liquid chromatography (RP-HPLC) yielded three fragments with the following amino acid sequences: EAAQWVEYITSNNPSPMTNLR, YFCIGNENWGCGGHMTPEYYADLYR, and DALVAGINLNIFNNNADR (Fragments no. 1-3, respectively). Analysis of fragments 1, 2, and 3 by using the program BLASTp (Altschul, *et al.* 1990) revealed that fragment no. 1 most closely resembled ARAFs from *Bacteroides ovatus* strain V975 (*asdII* gene product; GenBank accession no. U15179), *Clostridium stercorarium* (*arfB* gene product; Genbank accession no. AF002664), and *Bacillus subtilis* (arabinosidase; Genbank accession no. Z75208) in having % identity/% similarity ratios of 66/80, 47/66, and 38/71. By contrast, fragment no. 2 resembled the same three ARAFs as fragment no. 1, but in the order of *C. stercorarium*, *B. subtilis*, and *B. ovatus* as having % identity/% similarity ratios of 84/96, 84/92, and 76/84. Fragment no. 3 closely resembled only the ARAF from *C. stercorarium*, with a % identity/% similarity ratio of 77/94. No evidence was obtained for glycosylation of ARAF (not shown).

Treatment of ArfI with Endoproteinase Lys C (a protease that cleaves after a lysine residue) followed by blotting and sequencing of resulting fragments yielded three peptides with identical N-terminal sequence of GSSIVFDEQSxHQILRHxLR. Analysis

of the fragment sequence (designated fragment no. 4) using the program BLASTp revealed similarity to the ARAF from *C. stercorarium* (above), with a % identity/% similarity ratio of 35/50.

The four peptide fragments were compared to the cloned ARAFs from *C. xylanolytica* (Appendix; Figures 10 and 11). The three tryptic peptide fragments matched the deduced amino acid sequence of *arfI* (Appendix; Figure 12). Fragment four produced by Endoproteinase Lys C digestion matched the deduced amino acid sequence of *arfI* except for an Isoleucine (I) instead of Tyrosine (T) at position number 14 of the peptide fragment. Two amino acids of fragment no. 4 were undetermined (denoted by x).

Figure 9. ArfI-like proteins and enzyme activity of various freshwater and marine isolates of *C. xylanolytica* after growth in freshwater (FW) or marine (MN) medium. **A. MU-AF** zymogram of native PAGE gel showing ARAF activity (viewed by UV light). **B. Western immunoblot** of native PAGE gel with polyclonal anti-ArfI antiserum. **Lane: 1**, purified ArfI; **2**, strain XM3 (FW); **3**, strain XM3 (MN); **4**, strain MA3 (FW); **5**, strain EW1 (FW); **6**, strain SL1 (FW); **7**, strain EPFW (FW); **8**, strain EPFW (MN); **9**, strain EPA (MN); **10**, strain EPB (MN); **11**, strain OP2E (MN); **12**, strain OP2F (MN); **13**, strain PR2L (MN) (0.6, 91.0, 142.0, 138.0, 121.0, 166.0, 146.0, 68.0, 226.0, 201.0, 85.0, 130.0, and 59.0 μg of protein, respectively). Lanes 2-13 contained soluble cell-free extract produced after sonification and centrifugation. Note: Panel B is of higher magnification than A.

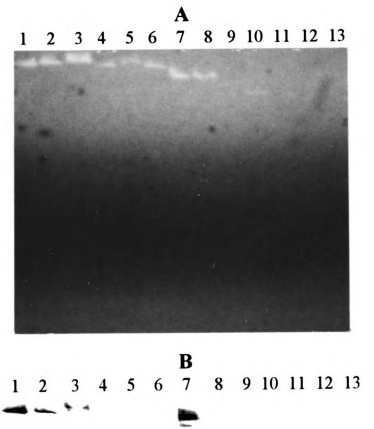


Figure 9. Arfl-like proteins and enzyme activity of various freshwater and marine isolates of *C. xylanolytica* after growth in freshwater (FW) or marine (MN) medium.

DISCUSSION

A cytoplasmic α-L-arabinofuranosidase (ArfI) from *C. xylanolytica* was purified 85-fold by column chromatography and was judged to be a single protein species on the basis of SDS-PAGE, native PAGE, and IEF analyses, all of which yielded a single protein band (the latter two retaining ARAF activity in zymograms). No evidence was obtained that the ArfI existed in multiple forms, as had been observed with some endoxylanase and arabinofuranosidase enzymes produced by other organisms (Komae, *et al.* 1982, Matte and Forsberg 1992, Van Laere, *et al.* 1997, Vincent, *et al.* 1997).

ArfI accounted for virtually all of the ARAF activity in cultures of *C. xylanolytica*, and it appeared to be the only ARAF produced by this bacterium under our growth conditions. A recent study (Van Laere, *et al.* 1997) suggested that some ARAFs may go undetected owing to their inability to cleave *p*NP-AF, the substrate commonly used to assay ARAF activity. However, MU-AF was also used as a substrate in the present study, and again the only MU-AF-hydrolyzing activity observed was that attributable to ArfI and seen as a single electrophoretically-separable band in various fractions produced throughout the purification procedure (Figure 2B). ArfI also appeared as single bands with identical pIs in IEF gels prepared previously from both xylan- and xylose-grown cells (Haack and Breznak 1992). The pI of ArfI reported in that preliminary study [*i.e.*, 5.85; (Haack and Breznak 1992)] was slightly lower than that reported herein for the purified protein (pI = 6.1), but the former was determined by IEF zymogram analysis of crude Triton extracts and may have been underestimated. The trace amount of extracellular ARAF activity occasionally observed in cell-free culture fluids of

xylan- and xylose-grown cells (Haack and Breznak 1992) is probably the result of lysis of some cells in the population, since the protein responsible for that activity has properties similar to that of ArfI. However, although ArfI was the only ARAF detected in this present study, it is apparently not the only ARAF that cells are capable of producing. Indeed, two different ARAF-encoding genes have been cloned from the genome of *C. xylanolytic*: *arfI*, which encodes ArfI; and *arfII*, which encodes a putative ARAF (ArfII) expressed by *E. coli*, but which has not yet been observed in cells or culture fluids of *C. xylanolytica* (Appendix; Figures 10 and 11, respectively). In any case, the single protein (ArfI) accounting for ARAF activity was in marked contrast to the multiplicity of endoxylanase activities, which segregated into three major zones (ENDOX pools) during native PAGE and were associated with ca. 15 individual bands. Such multiplicity of endoxylanases is not uncommon among xylanolytic systems (Dekker 1985, Wong, *et al.* 1988).

Some of the properties of ArfI from *C. xylanolytica* were similar to those of ARAFs from various other organisms, *e.g.*, subunit molecular mass (which is usually within 30-95 kDa), an acidic pH optimum (typically lying between pH 2.5 - 6.9), and the ability to release arabinose from *p*NP- and MU-α-L-arabinofuranosides, arabinoxylans, and arabinan (but with an otherwise narrow substrate specificity). However, the apparent native molecular mass of 160-210 kDa, which was consistent with that of a trimer or tetramer composed of 56 kDa subunits (Table 3), was higher than that of many other ARAFs (Table 1). Among purified ARAFs, ArfI bears perhaps the closest resemblance to the ARAF of *Butyrivibrio fibrisolvens* GS113 in terms of physical parameters (similar pIs; identical subunits of 56 kDa vs. 31 kDa, respectively), location (both cytoplasmic,

see below), catalytic properties (similar *Km* values and temperature and pH optima), and narrow substrate specificity (*i.e.*, specific for the α-L-furanoside configuration and no activity on arabinogalactan [Hespell and O'Bryan 1992]).

One curious property of the ArfI of *C. xylanolytica* was its ability to induce Congo red non-binding to oat spelt arabinoxylan present in zymogram gels (Figure 2A, arrow). However, we are reluctant to attribute true endoxylanase activity (hence bi-functionality) to this enzyme because (i) little or no reducing sugar was liberated (above that expected for arabinose alone) when ArfI acted on oat spelt arabinoxylan, (ii) neither a significant amount of reducing sugar, nor spots on TLC plates corresponding to xylooligomers, were liberated by ArfI from an essentially arabinose-free Lenzing beechwood xylan (data not shown), and (iii) no apparent xylooligomers accompanied the release of arabinose from rye or wheat arabinoxylan (Figure 5). Nevertheless, it is conceivable that a small amount of xylan-depolymerizing activity might be associated with ArfI, akin to that seen with certain endoxylanases of *Clostridium thermocellum* (which liberate too little reducing sugar to be detected by conventional colorimetric assays, but can effect enough depolymerization of the substrate to be detected in Congo red-stained zymograms (MacKenzie, *et al.* 1989]) or with the ARAF of *Streptomyces lividans* (which hydrolyzed the xylan backbone after prolonged incubation (Vincent, *et al.* 1997]). Such activity might also be responsible for the small amount of saccharide trailing the arabinose spot in TLC of ArfI digests of corn cob- and oat spelt arabinoxylans (Figure 5). However, the ArfI of *C. xylanolytica* bears little resemblance to the arabinose-releasing endoxylanases that release arabinose side groups before attacking the xylan backbone (Matte and Forsberg 1992). Such debranching endoxylanases do not act on

MU-AF and *p*NP-AF substrates, whereas the ArfI of *C. xylanolytica* has significant activity on these and very little activity on oat spelt arabinoxylan.

An osmotic shock method, originally developed for the isolation of inner and outer membrane components from several *Cytophaga* species (Godchaux and Leadbetter 1988), was employed as part of a cell fractionation procedure that inferred an almost entirely cytoplasmic location for ArfI. Even though no validated periplasmic enzyme markers are known for cytophagas, the activity of an endoxylanase present in the shock fraction suggested a possible periplasmic location (Figure 9, lane 5). Our assessment of a periplasmic location for at least one ENDOX component is a first approximation, however, since so little is known about complex cell-associated xylanolytic enzyme systems. Other researchers have shown that the distribution of xylanase activities found within xylan-degrading microorganisms can be affected by the amount and type of growth substrate, the culture conditions employed, the phase of growth at cell harvest, and/or some combination of these (as examples, see [Lee, *et al.* 1993, Lee and Zeikus 1993, McDermid, *et al.* 1990, Rodriguez, *et al.* 1985]). In addition, research has shown that intrinsic differences in the substrates used for detection of xylanases, and the ability or inability of enzymes to act on them, can make it difficult to determine the distribution of xylanase activities (*e.g.*, the different specificities of ARAFs for artificial and natural substrates).

In light of the largely (if not entirely) cytoplasmic nature of ArfI, it seems reasonable to assume that the major role of this enzyme in *C. xylanolytica* is to remove α-L-arabinofuranosyl residues from arabinose-containing xylooligosaccharide fragments

that pass through the cytoplasmic membrane and whose presence might otherwise impede the action of cytoplasmic ENDOXs and or β-xylosidases. This interpretation is consistent with the ability of ArfI to release arabinose from various arabinoxylans (Figure 5) and to exhibit synergy with various ENDOX components (Table 4), including those present in the cytoplasmic fraction of cells (Figure 8, lanes 2 and 6). Such synergy is not unusual and has been reported for the xylanolytic enzyme systems of other organisms (Bachmann and McCarthy 1991, Matte and Forsberg 1992). Although one might assume that xylooligosaccharides capable of passing through the cytoplasmic membrane are restricted to short oligomers, preliminary evidence for the uptake of large oligosaccharides (up to seven glucose units in length) has been obtained for *Bacteroides thetaiotaomicron*, a bacterium closely related to cytophagas phylogenetically (Salyers, *et al.* 1996).

The comparison of four peptide fragments from ArfI to other proteins in the database gave an unexpected result. Although the order of similarity of fragments no. 1 and 2 were different, the fact that fragment no. 3 and 4 had similarity only to an ARAF from *Clostridium stercorarium* was unusual. Perhaps these fragments are from a less conserved region of ARAFs than the regions containing fragments no. 1 and 2.

Further studies on the nature and cellular location of specific ENDOXs, as well as on the mechanisms and limits of saccharide uptake by cells, would undoubtedly refine our concept of arabinoxylan degradation by cells. Hence, we consider this study as but one step in dissecting the complex xylanase system of this fascinating gliding bacterium.

APPENDIX

APPENDIX

α-L-ARABINOFURANOSIDASE-ENCODING GENES FROM *Cytophaga xylanolytica*

PREFACE

I specifically want to acknowledge the work of Kwi Kim (cloning and sequencing) and the efforts of Dr. Tim Lilburn (sequence searches and PCR design) for the information contained in this Appendix (**Kim, K. S., T. G. Lilburn, M. J. Renner, and J. A. Breznak.** *arfI* and *arfII*: two genes encoding α-L-arabinofuranosidases in *Cytophaga xylanolytica*).

Figure 10. Nucleotide sequence of *arfI* and the deduced amino acid sequence of ArfI from *C. xylanolytica*. The sequence of both DNA strands was determined. The ORF (residues 232 to 1761, encoding 509 amino acids) corresponds to *arfI*. Encoded amino acids are indicated below the nucleotide sequence. Numbers to the left of the figure designate the first nucleotide in each row. Plus symbols (+) designate every 10 nucleotides in sequence. Bold and underlined portions designate the amino acid sequence of tryptic peptides used to design degenerate primers for PCR. Asterisk represents proposed termination codon.

Figure 10. Nucleotide sequence of *arfI* and the deduced amino acid sequence of ArfI from *C. xylanolytica*.

```
  1 TGGTCGTTGGTGAAATACAGCGGTTTGTGTTTGGAGACTCAGCATTTCCC
            +         +         +         +         +

 51 CGACTCGCCCAATAAGGCTAATTTTCCCTCGGTGATTCTTAATCCCGGCG
            +         +         +         +         +

101 AGAAATATACTTCGACTACCATTATGAAATTTGGGGTAAAGTAACCCAAT
            +         +         +         +         +

151 CTTTAATCAGGAGGCACCGCTCGTTGAATAATCAACTTGGGTGCCTCTTT
            +         +         +         +         +

201 TGTTTGGTCATTATTAACTTTTGATATACTGATGAATATGAAAAGAATTT
            +         +         +         +         +
                                  M   N   M   K   R   I   S

251 CTATTGCCTTTTTGCCGACGCTTGCCTTATTGCCTTTGTCGGCGCAAACC
            +         +         +         +         +
      I   A   F   L   P   T   L   A   L   L   P   L   S   A   Q   T

301 AAAATTACTTTGCAGGCCGATCAGGCCAAGGAGCGCATTAATCCTGAAAT
            +         +         +         +         +
    K   I   T   L   Q   A   D   Q   A   K   E   R   I   N   P   E   I

351 TTATGGTCATTTTGCCGAGCACCTTGGGCGATTGATTTATGGTGGAATTC
            +         +         +         +         +
     Y   G   H   F   A   E   H   L   G   R   L   I   Y   G   G   I   L

401 TGGTCGAGCCGGGTTCTCAAATTGACAATATTCGGGGCTTCCGTACCGAT
            +         +         +         +         +
     V   E   P   G   S   Q   I   D   N   I   R   G   F   R   T   D

451 GTGATTGGAGCCCTCCGCGACATGAAAGTGCCCATTTTGCGTTGGCCCGG
            +         +         +         +         +
    V   I   G   A   L   R   D   M   K   V   P   I   L   R   W   P   G

501 CGGTTGTTTTGCCGATACTTACAACTGGCGCGATGGCATTGGTCCCAAGC
            +         +         +         +         +
     G   C   F   A   D   T   Y   N   W   R   D   G   I   G   P   K   Q

551 AAAATCGTCCCTCTATTGTGAATATCCACTGGGGTGGGGTCACCGAAGAT
            +         +         +         +         +
     N   R   P   S   I   V   N   I   H   W   G   G   V   T   E   D
```

Figure 10. (cont'd).

```
601 AACAGTTTTGGAACGCATGAGTTTTTCGATTTTTGTGAACTGATTGGTGC
          +         +         +         +         +
    N  S  F  G  T  H  E  F  F  D  F  C  E  L  I  G  A

651 CGAACCATACGTGAATCTGAACGTAGGCTCAGGAACTGTGCGCGAAGCTG
          +         +         +         +         +
     E  P  Y  V  N  L  N  V  G  S  G  T  V  R  E  A  A

701 CTCAGTGGGTGGAGTACATTACTTCTAACAACCCCAGCCCGATGACCAAT
          +         +         +         +         +
      Q  W  V  E  Y  I  T  S  N  N  P  S  P  M  T  N

751 TTGCGCAAAGAAAACGGTCGTGATAAACCCTGGGATATCAAGTATTTTTG
          +         +         +         +         +
    L  R  K  E  N  G  R  D  K  P  W  D  I  K  Y  F  C

801 CATTGGTAATGAAAACTGGGGATGTGGTGGACACATGACGCCTGAGTATT
          +         +         +         +         +
     I  G  N  E  N  W  G  C  G  G  H  M  T  P  E  Y  Y

851 ATGCCGATTTGTATCGTAATTATGCAACCTATTGTGGTGGTGTGAAATAT
          +         +         +         +         +
      A  D  L  Y  R  N  Y  A  T  Y  C  G  G  V  K  Y

901 AAAATCGCCGGCGGTCCCAATGTGGATGATTATCGTTGGACGGAAGTGTT
          +         +         +         +         +
    K  I  A  G  G  P  N  V  D  D  Y  R  W  T  E  V  L

951 GATGAATAAAACGCAGCATCACAAGTTCCTCATGCAGGGATTGTCACTTC
          +         +         +         +         +
     M  N  K  T  Q  H  H  K  F  L  M  Q  G  L  S  L  H

1001 ATTATTATACGTTCCCCGGTCGTTGGGAAGATAAGGGTTCTTCTATTGTT
          +         +         +         +         +
      Y  Y  T  F  P  G  R  W  E  D  K  G  S  S  I  V

1051 TTTGATGAACAGTCGTGGCATCAAACCTTGCGTCATACCTTGCGCATGCA
          +         +         +         +         +
    F  D  E  Q  S  W  H  Q  T  L  R  H  T  L  R  M  Q

1101 GGAACTCATCAGCAAGCATGGCGAAATTATGGACAAATATGACCCCAAAA
          +         +         +         +         +
     E  L  I  S  K  H  G  E  I  M  D  K  Y  D  P  K  K
```

Figure 10. (cont'd).

```
1151 AGCAAATCGGCTTGATTGTTGACGAATGGGGAACCTGGTACGATGTGATG
          +         +         +         +         +
       Q  I  G  L  I  V  D  E  W  G  T  W  Y  D  V  M

1201 CCCGGTACGAACCCCGGCTTTTTGTATCAGCAAAATACGTTACGTGATGC
          +         +         +         +         +
     P  G  T  N  P  G  F  L  Y  Q  Q  N  T  L  R  D  A

1251 TCTGGTGGCGGGGATCAATCTTAATATCTTCAACAACAACGCTGATCGGG
          +         +         +         +         +
      L  V  A  G  I  N  L  N  I  F  N  N  N  A  D  R  V

1301 TGAAAATGGCTAATATTGCCCAAATGGTCAATGTGTTACAGGCTGTAATT
          +         +         +         +         +
       K  M  A  N  I  A  Q  M  V  N  V  L  Q  A  V  I

1351 CTCACTCAGGGCGATGAAATGATTCTGACGCCCACTTACTATGTCTTTAA
          +         +         +         +         +
     L  T  Q  G  D  E  M  I  L  T  P  T  Y  Y  V  F  K

1401 AATGTACAATGTGCACCATGGTGCTACGTTGATTCCCATGAACATTCAGA
          +         +         +         +         +
      M  Y  N  V  H  H  G  A  T  L  I  P  M  N  I  Q  T

1451 CGCAAGATTATGTGATGGGAGATCAAAAAATTCCGATGGTCAATGCCTCT
          +         +         +         +         +
       Q  D  Y  V  M  G  D  Q  K  I  P  M  V  N  A  S

1501 GCCTCCATCAAGGATAAAACGGTGAGTGTAACCTTGTGCAACCTTCACGC
          +         +         +         +         +
     A  S  I  K  D  K  T  V  S  V  T  L  C  N  L  H  A

1551 ATCCCAATCAACCAAAGTTGAAATTGATGTGACTGGTTTTGAAGGCAAAA
          +         +         +         +         +
      S  Q  S  T  K  V  E  I  D  V  T  G  F  E  G  K  T

1601 CGGCTACCGGTCAAATCATCACCGGCGAAAAAATTACCGATTACAACGAT
          +         +         +         +         +
       A  T  G  Q  I  I  T  G  E  K  I  T  D  Y  N  D

1651 TTTGGTAAGCCTGAAATGGTTGGTCTCAAGGCTTTTAATGTGGCCAAGCC
          +         +         +         +         +
     F  G  K  P  E  M  V  G  L  K  A  F  N  V  A  K  P
```

Figure 10. (cont'd).

```
1701 TAAAGGCAATAAACTGACTGTTGAAATCCCCGCGAAGTCAGTTGTGTTAG
           +         +         +         +         +
      K  G  N  K  L  T  V  E  I  P  A  K  S  V  V  L  V

1751 TGCAAATGAAGTAATTTCAATTTCTTTTCCCTTATAAAGCCGTTGCCCTA
           +         +         +         +         +
       Q  M  K  *

1801 TAGCAGCGGCTTTTTTTGTGTTCGGTGGGAGCGATTTGTC
           +         +         +         +
```

Figure 11. Nucleotide sequence of *arfII* and the deduced amino acid sequence of a putative α-L-arabinofuranosidase from *C. xylanolytica*. The sequence of both DNA strands was determined. The ORF (residues 294 to 1895, encoding 534 amino acids) corresponds to *arfII*. Encoded amino acids are indicated below the nucleotide sequence. Numbers to the left of the figure designate the first nucleotide in each row. Plus symbols (+) designate every 10 nucleotides in sequence. Asterisk represents proposed termination codon.

Figure 11. Nucleotide sequence of *arfII* and the deduced amino acid sequence of a putative α-L-arabinofuranosidase from *C. xylanolytica*.

```
  1 GAATTCATGGCCGGTGATGTACGTTTCGCAAGGACTCTTTTTTCTCTTAA
            +         +         +         +         +
 51 GGAAACGATATGGATTGTTGATTCAATGTCGTTAAGTTAGTGCGATTCAC
            +         +         +         +         +
101 AAAAGAGACAAGAAGATGATGAGACTAAAGACAGATAGATTAGACTGAAG
            +         +         +         +         +
151 AGACGAAGTTCACGGATCTAAATATCTGTTTTTTCATCTCATCATCTGTC
            +         +         +         +         +
201 TCTTCGTCTGACATCTTTTTCTCTTAACTAAACGACATTGATTGTTGATT
            +         +         +         +         +
251 TGTCGTTTGATGATTGATTTGAAATAGTATAAATTGAAATAGTATGAAGA
            +         +         +         +         +
                                              M  K  K

301 AACGAATGAGTATTCAAAAGCGAATTGCTTTCAATGGAGCCTTGGTGTTG
            +         +         +         +         +
      R  M  S  I  Q  K  R  I  A  F  N  G  A  L  V  L

351 GCATTTATGTTGACCATGTGCACCCCAAAACCTCCTCAGAATGAGGATGT
            +         +         +         +         +
    A  F  M  L  T  M  C  T  P  K  P  P  Q  N  E  D  V

401 CGTAAAAATGTCCGTCGACGTCACGCAAAGTGGCCCTGAAATCAACCGTC
            +         +         +         +         +
     V  K  M  S  V  D  V  T  Q  S  G  P  E  I  N  R  H

451 ACATCTTTGGCCAGTTTGCCGAACATTTGGGCACGGGCATTTACGGCGGC
            +         +         +         +         +
      I  F  G  Q  F  A  E  H  L  G  T  G  I  Y  G  G

501 ATTTGGGTGGGACCCGAATCGGAAATCCCCAATACCCGCGGGATTCGTAA
            +         +         +         +         +
    I  W  V  G  P  E  S  E  I  P  N  T  R  G  I  R  N

551 TGATGTGGTGAAAGCTTTAAAGGCCATCAAAGTGCCCAACGTGCGCTGGC
            +         +         +         +         +
     D  V  V  K  A  L  K  A  I  K  V  P  N  V  R  W  P

601 CGGGCGGTTGTTTTGCCGATCAGTACCACTGGCGCGATGGCATCGGTGCA
            +         +         +         +         +
      G  G  C  F  A  D  Q  Y  H  W  R  D  G  I  G  A
```

Figure 11. (cont'd).

```
651 CCCGAGGAACGCAAAAGTCGCATCAATGTCAGCTGGGGCGGTAGTCCCGA
          +         +         +         +         +
    P  E  E  R  K  S  R  I  N  V  S  W  G  G  S  P  E

701 AGCCAACACCTTTGGCACGCATGAATATTTCGATTTCATTTCTCAAATTG
          +         +         +         +         +
     A  N  T  F  G  T  H  E  Y  F  D  F  I  S  Q  I  G

751 GTTCCGAAGCCTTTATTTCGGCCAATGTGGGATCGGGTACTGTGCAGGAA
          +         +         +         +         +
      S  E  A  F  I  S  A  N  V  G  S  G  T  V  Q  E

801 TCGGCTGATTGGCTGGAGTATCTCACGGCTTCAGGGTCGACATTGGCTCT
          +         +         +         +         +
   S  A  D  W  L  E  Y  L  T  A  S  G  S  T  L  A  L

851 CGAGCGTGCCCGAAACGGTCACCCCGACCCCTACGATGTTGCCTTTTGGG
          +         +         +         +         +
     E  R  A  R  N  G  H  P  D  P  Y  D  V  A  F  W  G

901 GCGTGGGCAACGAAGTGTGGGGATGCGGCGGTCCTTTTACCCCGCAGGAA
          +         +         +         +         +
      V  G  N  E  V  W  G  C  G  G  P  F  T  P  Q  E

951 TATATCACCGAGCTTAAAAAGTTTGCCACTTTTACCAATAACTACAATGC
          +         +         +         +         +
    Y  I  T  E  L  K  K  F  A  T  F  T  N  N  Y  N  A

1001 CAACGTCAATACTCAGTTTGTTGCGGTGGGCCCCGACTCGTTTGATGAAT
          +         +         +         +         +
     N  V  N  T  Q  F  V  A  V  G  P  D  S  F  D  E  Y

1051 ACAGCTTTGGCTATCCCGAAGCCATCATGGAGGCCTGGGCAAAGAAAACC
          +         +         +         +         +
     S  F  G  Y  P  E  A  I  M  E  A  W  A  K  K  T

1101 TGGACCTGGGATATTCAAGGGCTTTCGTTGCACATGTACACGCGTGGCGG
          +         +         +         +         +
   W  T  W  D  I  Q  G  L  S  L  H  M  Y  T  R  G  G

1151 CTGGCCGGCAGTGATTCCTGCTACCGGTTTCAACGAATCGGGATACGCAG
          +         +         +         +         +
     W  P  A  V  I  P  A  T  G  F  N  E  S  G  Y  A  A
```

Figure 11. (cont'd).

```
1201 CAGTCATTAAGGAAACTCTGGAAATGGACAAATTTATCAGCGACAATCGC
          +         +         +         +         +
       V  I  K  E  T  L  E  M  D  K  F  I  S  D  N  R

1251 GCCATCATGGATAAGTTCGACCCCGAACACAAAGTGTCGATCATGGTGGA
          +         +         +         +         +
    A  I  M  D  K  F  D  P  E  H  K  V  S  I  M  V  D

1301 TGAGTGGGGCACCTGGTATGCACCCACCGAAGGGACCAATCCCGGTTTTC
          +         +         +         +         +
       E  W  G  T  W  Y  A  P  T  E  G  T  N  P  G  F  L

1351 TGCAACAGCAAAACTCACAACGCGATGCTGTGTTGGCGGCTTTGAATTTC
          +         +         +         +         +
       Q  Q  Q  N  S  Q  R  D  A  V  L  A  A  L  N  F

1401 AACATCTTTATTCGTCATGCCGAGCGGGTGAAAGGGGCTAACATTGCCCA
          +         +         +         +         +
     N  I  F  I  R  H  A  E  R  V  K  G  A  N  I  A  Q

1451 GATGATCAATGTGTTGCAAGCCATGATTCTGACCGAAGGTGAGAAGATGG
          +         +         +         +         +
       M  I  N  V  L  Q  A  M  I  L  T  E  G  E  K  M  V

1501 TGCTCACACCCACTTATCACACTTTCCGCATGTACGTGCCTTTTCAGGAT
          +         +         +         +         +
       L  T  P  T  Y  H  T  F  R  M  Y  V  P  F  Q  D

1551 GCTACGCGTTTGCCAATCAATTTCAATAAAGGCTTTTATAAAGAAGGAAC
          +         +         +         +         +
     A  T  R  L  P  I  N  F  N  K  G  F  Y  K  E  G  T

1601 CATTGAATTGCCGCGTGTGGATGCCATTGCTGCCAAAGGAAGAGATGGCA
          +         +         +         +         +
       I  E  L  P  R  V  D  A  I  A  A  K  G  R  D  G  K

1651 AAATTTACGTGGCCATCACCAATATCGACCCCAAAAACAACGCTTCGTTG
          +         +         +         +         +
       I  Y  V  A  I  T  N  I  D  P  K  N  N  A  S  L

1701 GATCTCTCGTTTGATGAGCAAACCATCAAGGATGTGAAGGGGGAAACGCT
          +         +         +         +         +
     D  L  S  F  D  E  Q  T  I  K  D  V  K  G  E  T  L
```

Figure 11. (cont'd).

```
1751 GTATGCTTCGGCCATTGATGCTGTTAACACATTTGACAACCCTAACAATG
           +         +         +         +         +
      Y  A  S  A  I  D  A  V  N  T  F  D  N  P  N  N  V

1801 TTGCTCCTACATCCATTAACGCAAGTTTAACGGGCAGCAATGTGTCGGTA
           +         +         +         +         +
       A  P  T  S  I  N  A  S  L  T  G  S  N  V  S  V

1851 ACGGTGCAGCCTCAGTCGGTCACGGTGCTCGAAATTGTATCGGAGTGATT
           +         +         +         +         +
     T  V  Q  P  Q  S  V  T  V  L  E  I  V  S  E  *

1901 GCGTTTGGAGGTTTTGGTTTATAGCATCCACCTGTTCTGTGAATTC
           +         +         +         +
```

Figure 12. CLUSTAL W comparison of the deduced amino acid sequences of *arfI* and *arfII* to similar ARAFs from other organisms. A multiple alignment was performed with the deduced amino acid sequences of *arfI* and *arfII* from *C. xylanolytica* to ARAFs from *Bacteroides ovatus* strain V975 (accession no. U15179), *Clostridium stercorarium* (accession no. AF002664), and *Bacillus subtilis* (accession no. Z75208). Boxes around amino acid sequences designate regions of sequence identity. The four shaded regions represent the amino acid sequences of three trypsin- and Endoproteinase Lys C-generated peptides of ArfI. Dashes indicate gaps in the sequence. Numbers to the left designate the number of the first amino acid in each row and numbers to the right designate the last amino acid in each row. Designations: **ArfI** = purified arabinofuranosidase from *C. xylanolytica*; **ArfII** = putative arabinofuranosidase from *C. xylanolytica*; ***B. ovatus*** = *Bacteroides ovatus asdII* gene product; ***C. sterc.*** = *Clostridium stercorarium arfB* gene product; and ***B. subtilis*** = *Bacillus subtilis* gene product. Dashes indicate gaps in the sequence.

Figure 12. CLUSTAL W comparison of the deduced amino acid sequences of *arfI* and *arfII* to similar ARAFs from other organisms.

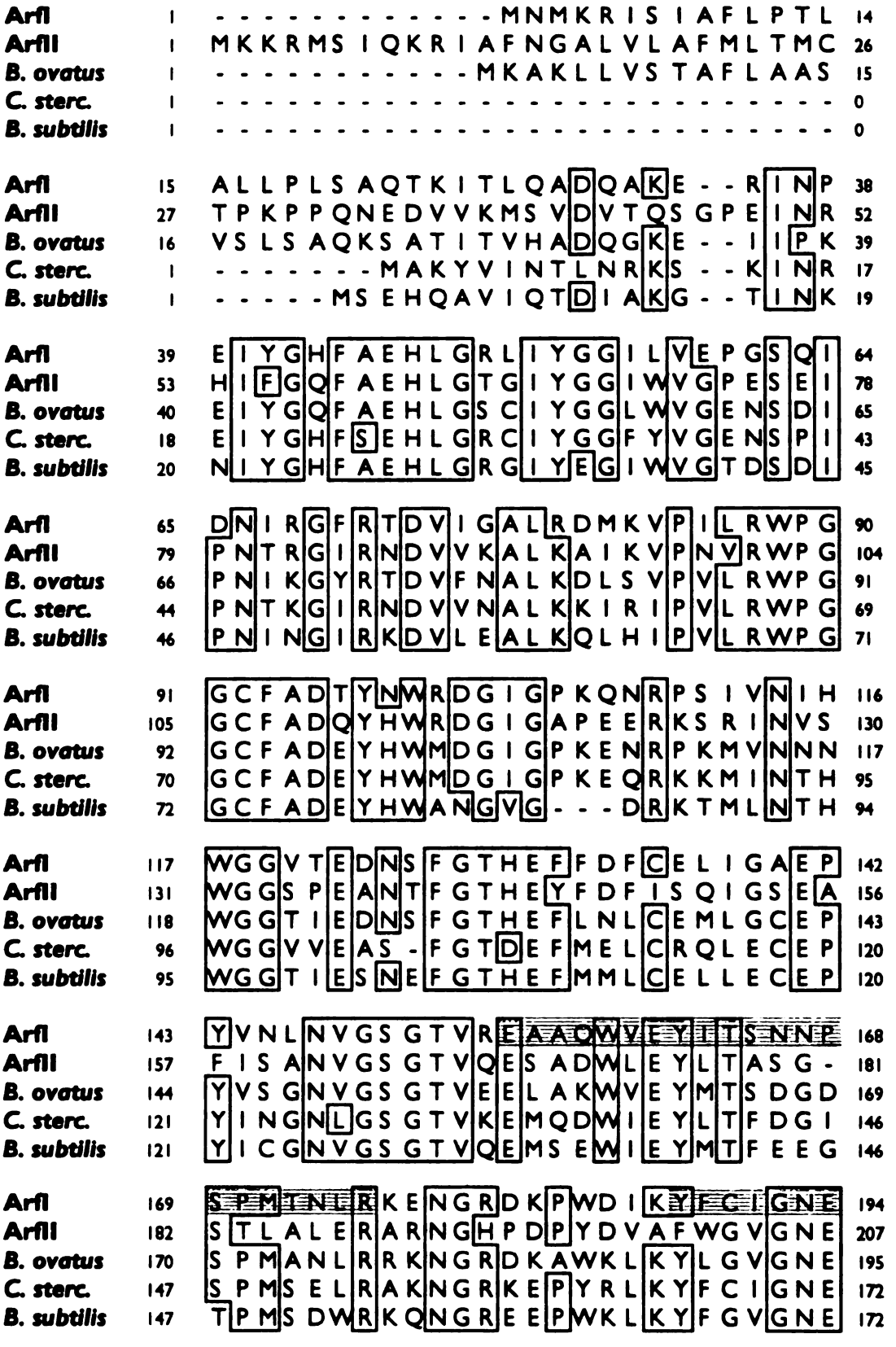


Figure 12. (cont'd).

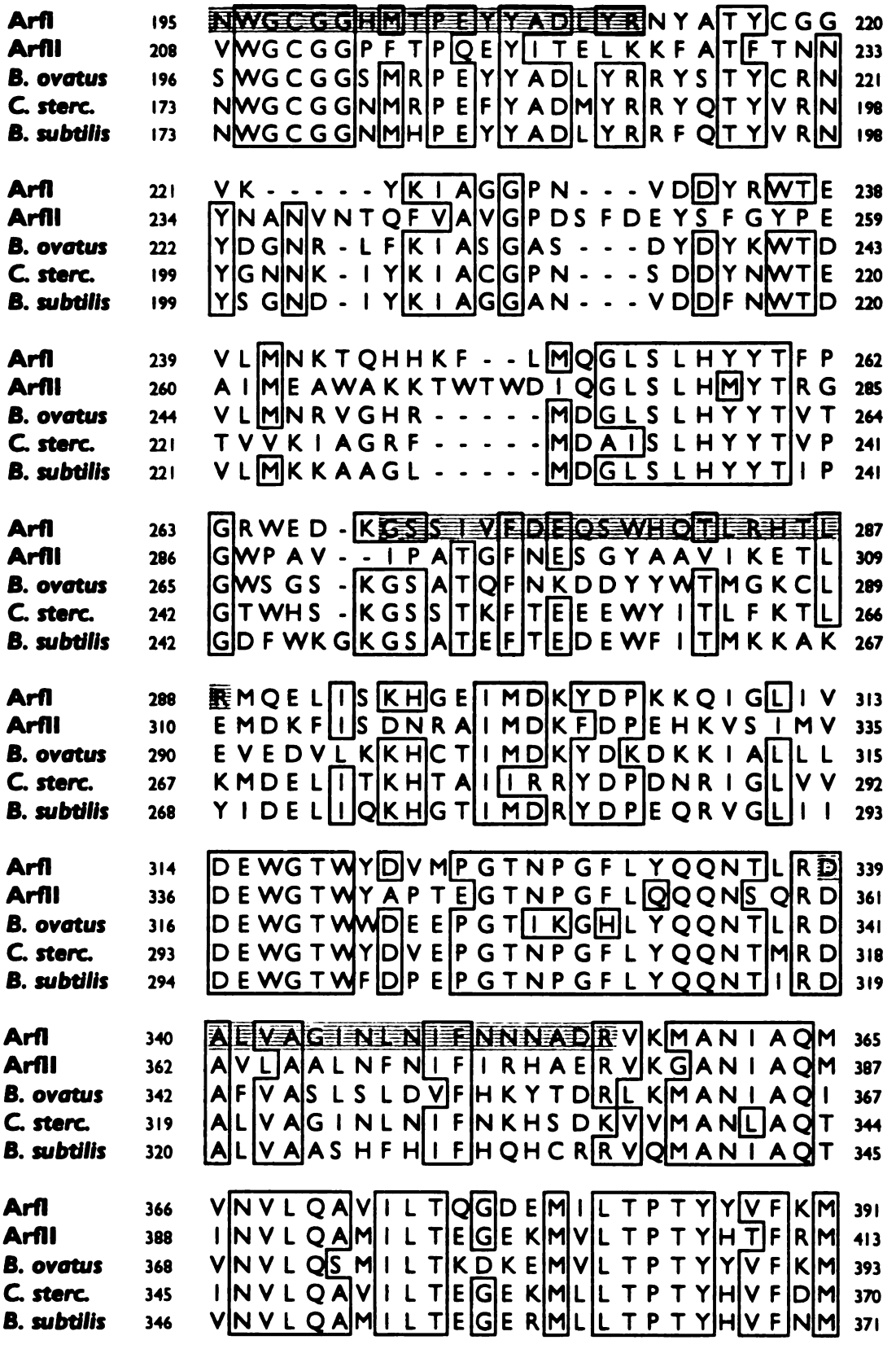
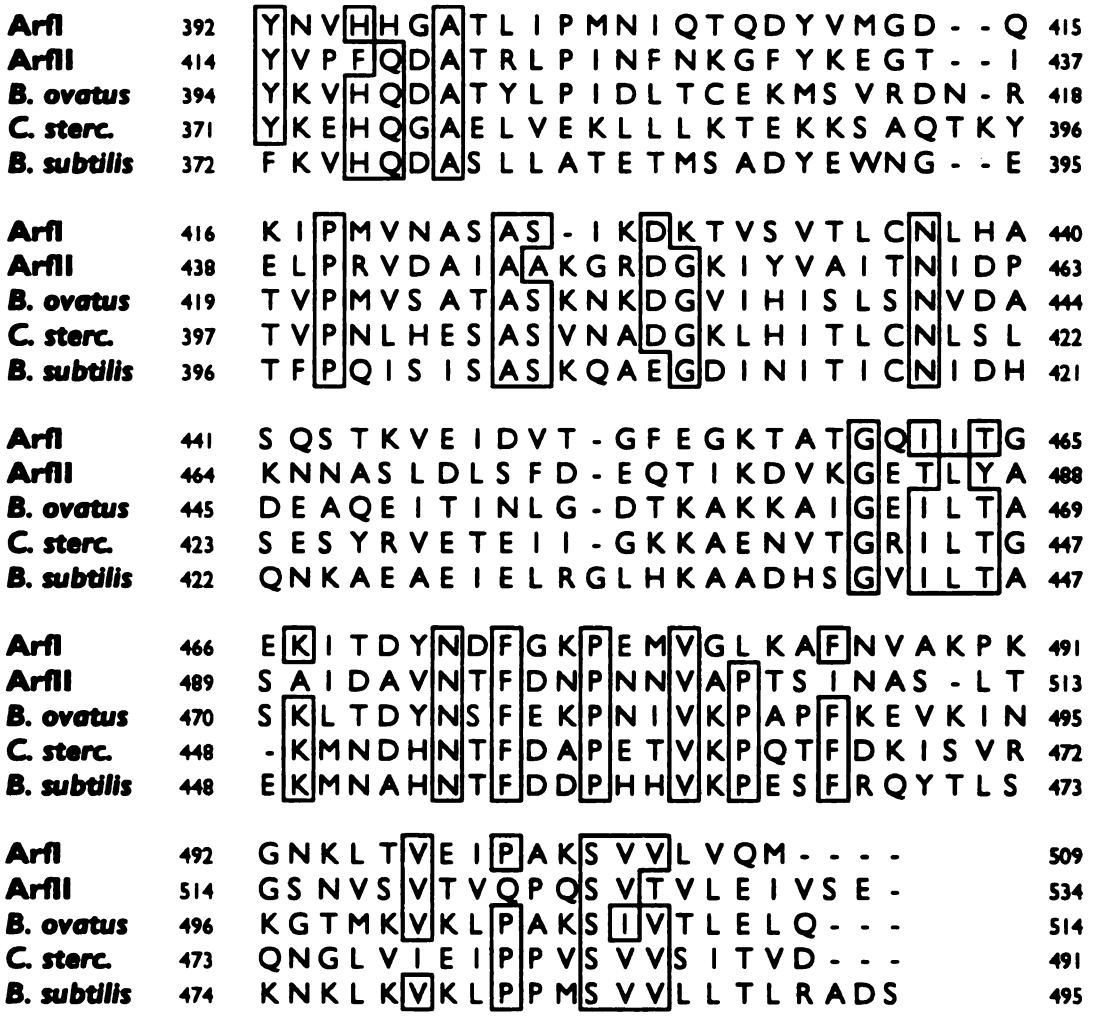


Figure 12. (cont'd).



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