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Research Note

Cloning and Characterization of a Xylanase Gene from Corn Strains of *Erwinia chrysanthemi*

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The gene encoding a 42-kDa endoxylanase was cloned from *Erwinia chrysanthemi* strain D1. Sequencing of this gene, called *xynA*, showed that it encoded a primary protein product of 413 amino acids with an unusual and long (31 amino acid) leader peptide that was cleaved during secretion to the bacterial periplasm. This protein is distinct from xylanases in glycohydrolase families 10 and 11 and, instead, appears to be intermediate between families 5 and 30. The *xynA* gene is located downstream from a gene with high homology to ATP-dependent RNA helicases and the *Escherichia coli recD* gene. Large amounts of the mature xylanase were produced by *E. coli* cells carrying a T7 expression plasmid construct and the protein was isolated from the bacterial periplasmic fraction by chromatography on a CM Bio-gel column. Marker exchange mutagenesis of the *xynA* gene eliminated the ability of strain D1 to produce detectable extracellular xylanase activity but did not affect virulence on corn leaves.

Additional keyword: virulence factors.

Xylanases (endo-1,4-β-xylanases, EC 3.2.1.8) are currently found in two protein families (families 10 and 11 of glycosyl hydrolases) (Henrissat 1991) characterized by distinct folds (Davies and Henrissat 1995). They have been of interest because of the substantial xylan content in plant cell walls, particularly those of monocots (Cooper et al. 1988; Wong et al. 1988). Braun and Rodrigues (1993) purified an endoxylanase from strain SR120A of *Erwinia chrysanthemi*. This ca. 42-kDa protein was produced constitutively and extracellularly secreted by the bacteria. The purified protein acted synergistically with pectate lyase to promote maceration of plant tissues, most notably grasses. Further, Braun and Kelman (1987) showed that *E. chrysanthemi* strains isolated from grasses but not dicots produced significant xylanase activities. These observations led to the hypothesis that the xylanase might be an important virulence component of *E. chrysanthemi* strains attacking corn and other grass plants. To test this idea, we cloned the gene encoding the 42-kDa xylanase from strains SR120A and D1 and constructed a D1 marker exchange mutant strain deficient in the production of xylanase for pathogenicity testing.

Three clones were isolated from a strain D1 cosmid library and three from a strain SR120A library (Table 1) constructed in pLAFR5 (Keen et al. 1988). All of these clones in *Escherichia coli* DH5α produced clear zones surrounding colonies on remazol brilliant blue (RBB) xylan (Sigma, St. Louis, MO) plates (Vroemen et al. 1995). Restriction enzyme mapping disclosed that the insert DNAs of all positive cosmid clones from each strain were overlapping and all contained common *Nru*I, *Sph*I, *Pst*I, *Nsi*I, *Pml*I, and *Bam*HI restriction sites (Fig. 1). Outside of this ca. 5-kb region, however, the restriction maps of the clones from strain D1 had little in common with those from strain SR120A. Subcloning, according to Crouse et al. (1983), disclosed that the common regions from all the cosmid clones directed xylanase activity in *E. coli* cells on RBB xylan plates. Cosmid clone C68 from the D1 library was utilized to produce subclones pNTK103 and pNTK112 (Table 1; Fig. 1) and cosmid clone B11 from the SR120A library was used to construct subclone pNTK101 (Table 1). Further subcloning and deletion experiments showed that an internal 1.5-kb *Bam*HI fragment was required for xylanase activity (Fig. 1). pNTK107, carrying a ca. 3.3-kb *PstI/Nsi*I insert fragment, was selected for further characterization. This clone was more active than the same fragment in opposite orientation (data not shown), indicating that the xylanase gene reads from left to right as shown in Figure 1.

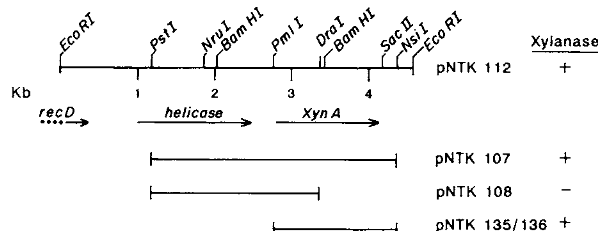

Fig. 1. Restriction map of the xylanase region of *Erwinia chrysanthemi* strain D1 and the xylanase activity of *Escherichia coli* DH5α cells carrying the noted deletion plasmids. Arrows denote position in pNTK112 of the *xynA* gene, as well as genes exhibiting high homology to *recD* and RNA helicases, as determined by DNA sequencing.

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Nucleotide and/or amino acid sequence data for *xynA* is to be found at GenBank as accession number U41750.

Some of the more active xylanase subclones were unstable in *E. coli* DH5α cells grown for several transfers on L ampicillin plates, but were more stable when the medium was supplemented with 0.2% glucose. Clones prepared from the unique *Pml*I site (Fig. 1) to the right-hand polylinker *Eco*RI site in pUC plasmids (e.g., pNTK135, Table 1) were xylanase active on RBB xylan plates but were unstable in *E. coli* DH5α, even after only one transfer of primary transformants.

Attempts to perform exonuclease III digestions with clone pNTK107/129 (Table 1) did not result in the recovery of a contiguous series of deletion clones. This may have been due to instability in *E. coli* of some of the deletion clones in the high copy pUC129 plasmid. Accordingly, the 3.3-kb DNA insert from pNTK107/129 was recloned into the low copy number plasmid, pCL1921, to generate pNTK107/1921 (Table 1). This plasmid contained polylinker restriction sites that permitted exonuclease III deletions on both DNA strands. *E. coli* DH5α cells carrying pNTK107/1921 consistently produced xylanase activity on RBB xylan plates after many sequential single colony transfers on L streptomycin plates. Exonuclease III digestions were accordingly performed on DNA of pNTK107/1921 and a series of deletion clones was recovered in both directions.

DNA sequencing showed that the 1,688-bp *Pml*I/*Nsi*I region of pNTK107 (Fig. 1) contained a long open reading frame (ORF) encoding the xylanase that read from left to right (Fig. 2), as predicted above by orientation to vector promoters. This gene has been assigned the designation *xynA*. Three possible ATG initiation codons were located at the 5′ end of the ORF, but only one of them, located at base 156, was positioned downstream from a stereotypic Shine-Dalgarno sequence. The validity of this start codon was supported by N-terminal sequencing of the mature XynA protein, discussed below, and by examination of exoIII deletion clones. For example, a 5′ deletion occurring 21 bp 3′ to the assigned ATG codon was totally inactive in *E. coli* cells plated on RBB xylan plates. A deletion 58 bp 3′ of this ATG was fully active, but was found to be in frame with the *lacZ* sequence of the cloning vector. The xylanase was therefore probably synthesized as a fusion protein. The *xynA* translational termination codon (TAA) determined by DNA sequencing occurred at base 1396 (Fig. 2). The validity of this terminator codon was supported by the phenotypes of exoIII deletions. Thus, a deletion 15 bases 3′ to the assigned terminator codon was fully active but another deletion occurring 122 bases before this codon was totally inactive in *E. coli* cells grown on RBB xylan

Table 1. Bacteria and plasmids used in this study

Name	Source	Reference
Erwinia chrysanthemi		
strain SR120A	Wild-type strain from corn	Braun and Rodrigues 1993
strain D1	Wild-type strain isolated from corn	Vroemen et al. 1995
D1 *bgx* Km	*bgxA* mutant strain; Km^r	Vroemen et al. 1995
D1 *xyn* Sm	*xynA* mutant strain; Sm^r	This work
D1 *bgx xyn* Km Sm	*bgxA xynA* mutant strain; Km^r; Sm^r	This work
Escherichia coli		
DH5α	Cloning strain	Gibco BRL, Gaithersburg, MD
B834(DE3)	T7 polymerase strain; Cm^r	Doherty et al. 1995
pUC128/129	*E. coli* cloning plasmids; Ap^r	Keen et al. 1988
pUC118	*E. coli* cloning plasmid; Ap^r	Vieira and Messing 1987
pWM529	*E. coli* cloning plasmid; Ap^r	Mandecki et al. 1990
pMTL22p	*E. coli* cloning plasmid, Ap^r	Chambers et al. 1988
pCL1921	Low copy number plasmid, Sm^r	Lerner and Inouye 1990
pLAFR5	Cosmid vector, Tc^r	Keen et al. 1988
pRSET5A	T7 expression plasmid; Ap^r	Schoepfer 1993
C68	pLAFR5 cosmid clone isolated from a strain D1 DNA library; xylanase positive	This work
B11	pLAFR5 cosmid clone isolated from a strain SR120A DNA library; xylanase positive	This work
pNTK101	ca. 6.2-kb *Sac*I/*Pst*I DNA fragment from B11 cloned into the same sites of pUC129; xylanase positive	This work
pNTK103	ca. 6.5-kb *Nsi*I/*Xho*I DNA fragment from C68 cloned into the same sites of pUC129; xylanase positive	This work
pNTK107	ca. 3.3-kb *Pst*I/*Nsi*I fragment from pNTK103, cloned into the *Pst*I site of pWM529; xylanase positive	This work
pNTK107/129	*Pst*I/*Xba*I insert fragment from pNTK107 recloned into the same sites of pUC129	This work
pNTK107/1921	*Nsi*I/*Sac*I insert fragment from pNTK107/129 recloned into the *Pst*I/*Sac*I sites of pCL1921	This work
pNTK108	ca. 2.1-kb *Pst*I/*Dra*I fragment from pNTK107 cloned into the *Pst*I/*Sma*I sites of pUC118; xylanase negative	This work
pNTK112	ca. 4.6-kb *Eco*RI fragment from C68 cloned into the same site of pMTL22p; xylanase positive	This work
pNTK123	pNTK112 partially digested with *Bam*HI to delete only a 1.5-kb fragment; the remaining ca. 6.2-kb plasmid was religated; xylanase negative	This work
pNTK125	Partial *Bam*HI deletion fragment used to construct pNTK123 was ligated with a 2.0-kb *Bam*HI fragment from pSmUC, encoding streptomycin resistance; xylanase negative; Sm^r	This work
pNTK129	*Eco*RI fragment from pNTK125 cloned into the same site of pRK415; xylanase negative; Tcr; Sm^r	This work
pNTK135	ca. 1.7-kb *Cla*I/*Xba*I fragment from pNTK107 cloned into the same sites of pUC129; strongly xylanase positive	This work
pNTK136	ca. 1.7-kb *Pml*I/*Eco*RI fragment from pNTK 107 was cloned into the *Pvu*II/*Eco*RI sites of pRSET5A; strongly xyn^+	This work

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   1                                                 CACGTGAAATATAAATTTCAT
  22 AAAATGACACATAAGTAAATTACTATTACAATGTAAGTTCTGGTGGGTTTATAGGCTGTCCCCATTA
  89 GTATCAGAAATAATAATTTGATATTTCCAATTATTTCCTAATGTTCATGTTATTAAGGATGGATGCT
     Met Asn Gly Asn Val Ser Leu Trp Val Arg His Cys Leu His Ala Ala Leu    17
 156 ATG AAT GGA AAT GTT AGT CTC TGG GTT CGT CAT TGT TTA CAT GCG GCT CTA
     Phe Val Ser Ala Thr Ala Gly Ser Phe Ser Val Tyr Ala Asp Thr Val Lys    34
 208 TTT GTT TCG GCT ACG GCA GGT TCT TTC TCT GTA TAT GCT GAT ACA GTA AAA
     Ile Asp Ala Asn Val Asn Tyr Gln Ile Ile Gln Gly Phe Gly Gly Met Ser    51
 259 ATT GAC GCA AAC GTT AAT TAT CAA ATA ATT CAA GGT TTT GGT GGA ATG AGT
     Gly Val Gly Trp Ile Asn Asp Leu Thr Thr Glu Gln Ile Asn Thr Ala Tyr    68
 310 GGG GTG GGG TGG ATC AAT GAT CTC ACC ACG GAA CAA ATT AAT ACT GCA TAC
     Gly Ser Gly Val Gly Gln Ile Gly Leu Ser Ile Met Arg Val Arg Ile Asp    85
 361 GGG AGT GGG GTT GGT CAG ATA GGG CTG TCG ATT ATG CGC GTC CGA ATT GAT
     Pro Asp Ser Ser Lys Trp Asn Ile Gln Leu Pro Ser Ala Arg Gln Ala Val   102
 412 CCA GAC TCC AGT AAA TGG AAT ATA CAG CTT CCG AGT GCA CGT CAG GCT GTT
     Ser Leu Gly Ala Lys Ile Met Ala Thr Pro Trp Ser Pro Pro Ala Tyr Met   119
 463 TCA CTG GGA GCC AAA ATA ATG GCT ACC CCC TGG TCA CCA CCC GCT TAT ATG
     Lys Ser Asn Asn Ser Leu Ile Asn Gly Gly Arg Leu Leu Pro Ala Asn Tyr   136
 514 AAA AGT AAC AAC AGC CTG ATA AAC GGC GGC CGT TTG TTG CCG GCA AAT TAT
     Ser Ala Tyr Thr Ser His Leu Leu Asp Phe Ser Lys Tyr Met Gln Thr Asn   153
 565 TCT GCC TAT ACT TCG CAC CTG CTG GAT TTC TCC AAA TAT ATG CAG ACT AAC
     Gly Ala Pro Leu Tyr Ala Ile Ser Ile Gln Asn Glu Pro Asp Trp Lys Pro   170
 616 GGT GCA CCG TTG TAT GCT ATT TCG ATA CAA AAT GAG CCA GAC TGG AAG CCG
     Asp Tyr Glu Ser Cys Glu Trp Ser Gly Asp Glu Phe Lys Ser Tyr Leu Lys   187
 667 GAT TAT GAA TCC TGC GAA TGG AGC GGG GAT GAA TTT AAA AGC TAT CTC AAA
     Ser Gln Gly Ser Lys Phe Gly Ser Leu Lys Val Ile Val Ala Glu Ser Leu   204
 718 TCG CAA GGA TCC AAG TTT GGC TCT CTC AAA GTC ATT GTC GCG GAG TCG CTA
     Gly Phe Asn Pro Ala Leu Thr Asp Pro Val Leu Lys Asp Ser Asp Ala Ser   221
 769 GGT TTT AAC CCG GCG TTA ACT GAC CCG GTA TTG AAA GAC AGT GAC GCA TCA
     Lys Tyr Val Ser Ile Ile Gly Gly His Leu Tyr Gly Thr Thr Pro Lys Pro   238
 820 AAA TAT GTG TCA ATC ATC GGT GGG CAC CTG TAT GGC ACG ACG CCT AAG CCC
     Tyr Pro Leu Ala Gln Asn Ala Gly Lys Gln Leu Trp Met Thr Glu His Tyr   255
 871 TAT CCG TTA GCA CAG AAT GCC GGT AAG CAA CTG TGG ATG ACC GAA CAC TAT
     Val Asp Ser Lys Gln Ser Ala Asn Asn Trp Thr Ser Ala Ile Glu Val Gly   272
 922 GTT GAT TCC AAA CAG TCG GCT AAT AAC TGG ACG TCG GCG ATT GAG GTG GGC
     Thr Glu Leu Asn Ala Ser Met Val Ser Asn Tyr Ser Ala Tyr Val Trp Trp   289
 973 ACT GAA CTG AAT GCC AGC ATG GTG TCA AAC TAT AGC GCT TAC GTC TGG TGG
     Tyr Ile Arg Arg Ser Tyr Gly Leu Leu Thr Glu Asp Gly Lys Val Ser Lys   306
1024 TAT ATT CGT CGT TCG TAT GGA TTA CTG ACA GAA GAC GGT AAA GTC AGT AAG
     Arg Gly Tyr Val Met Ser Gln Tyr Ala Arg Phe Val Arg Pro Gly Ala Leu   323
1075 CGG GGT TAT GTG ATG TCA CAA TAT GCC CGC TTC GTT CGC CCT GGT GCT CTG
     Arg Ile Gln Ala Thr Glu Asn Pro Gln Ser Asn Val His Leu Thr Ala Tyr   340
1126 CGT ATT CAG GCA ACG GAA AAT CCC CAG TCA AAT GTT CAT CTG ACT GCT TAT
     Lys Asn Thr Asp Gly Lys Met Val Ile Val Ala Val Asn Thr Asn Asp Ser   357
1177 AAG AAT ACC GAT GGA AAA ATG GTG ATT GTT GCG GTC AAT ACC AAT GAC TCC
     Asp Gln Met Leu Ser Leu Asn Ile Ser Asn Ala Asn Val Thr Lys Phe Glu   374
1228 GAC CAA ATG CTA TCG CTG AAT ATC AGT AAC GCC AAC GTG ACT AAA TTT GAG
     Lys Tyr Ser Thr Ser Ala Ser Leu Asn Val Glu Tyr Gly Gly Ser Ser Gln   391
1279 AAA TAC AGC ACG TCA GCA TCA CTG AAC GTT GAG TAT GGC GGC TCG TCC CAG
     Val Asp Ser Ser Gly Lys Ala Thr Val Trp Leu Asn Pro Leu Ser Val Thr   408
1330 GTT GAT AGT AGC GGC AAA GCA ACG GTA TGG CTG AAC CCG TTA AGT GTG ACA
     Thr Phe Val Ser Lys Stop                                              413
1381 ACG TTT GTC AGC AAA TAA TGTGGCGTATTAACCTGTATTAGTCACGACACTGTCTGACCAA
1442 TCAGTCTGAAAGCATGAGAGTTACCGATTGTGGTAGGGGACAAATACCCTGAAGTAAACATAAAAGG
1509 TAGCTCTCATCCCATTATGTAATACAACCCGCGGCGTATGCACCGGGTGATGCCAAAATGGCGTAGG
1576 GGAGCGCTGATTTCCCGCATGATGTTGTGAAAAGGCGGCTCCGGTGCTTTTGTGTATTGCAGTATCT
1643 CGACGGTTCTCAAAGCGTTACCTCTGTCAACGGCTTGTGTATGCAGGTCGAC
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Fig. 2. Sequence of the DNA insert fragment of pNTK 135/136 containing the *xynA* gene and its predicted translation product. Positions of various restriction enzymes are noted; the Shine-Dalgarno box is underlined and the signal peptide cleavage site is indicated by an arrow.

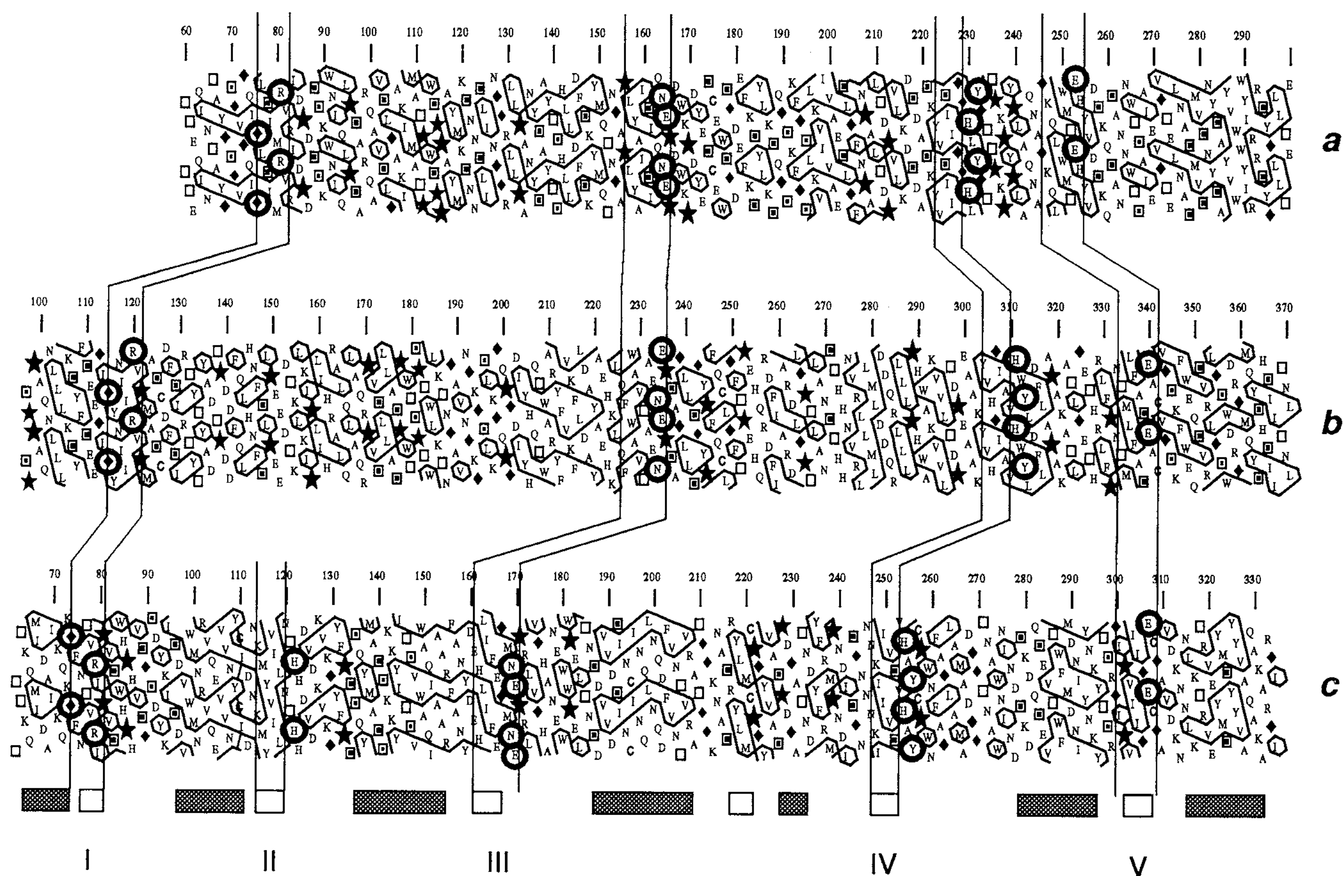

Fig. 3. Hydrophobic cluster analysis (HCA) of *Erwinia chrysanthemi* xylanase (**a**), human glucocerebrosidase (**b**), and *Clostridium cellulolyticum* endoglucanase A (**c**). In these bidimensional plots, produced with the HCA-plot program (Doriane, Le Chesnay, France), standard one-letter codes represent amino acids except for proline, glycine, serine, and threonine, which are represented by the solid star, solid diamond, open square with dot, and open square, respectively. Plot analysis followed published guidelines (Gaboriaud et al. 1987; Lemesle-Varloot et al. 1990; Henrissat et al. 1995). Gray and white boxes show respective locations of α helices and β strands as found in three-dimensional structure of the *C. cellulolyticum* endoglucanse A (Ducros et al. 1995). Vertical lines delineate conservation of glycosyl hydrolase family 5 segments I to V; conserved residues of that family are circled.

Table 2. Conserved residues in related families of glycosyl hydrolases

					Invariant residues[d] in segments I to IV[e]				
					I	II	III	IV	V
Family[a]	Enzyme[b]	Organism	EC no.	Accession no.[c]	(G to R)	(H)	(NE)	(H to Y)	(E)
	Xylanase	*Erwinia chrysanthemi*	3.2.1.8	This work	✓		✓	✓	✓
1	β-glucosidase	*Trifolium repens*	3.2.1.21	P26204			✓		✓
2	β–galactosidase Z	*Escherichia coli*	3.2.1.23	P00722			✓		✓
2	β–glucuronidase	Human	3.2.1.31	P08236			✓	✓	✓
2	β–mannosidase	Bovine	3.2.1.25	U17432			✓	✓	✓
5	Endoglucanase A	*Clostridium cellulolyticum*	3.2.1.4	P17901	✓	✓	✓	✓	✓
5	Exo-1,3-β-glucanase	*Saccharomyces cerevisiae*	3.2.1.58	P23776	✓	✓	✓	✓	✓
5	β-mannanase	*Trichoderma reesei*	3.2.1.78	L25310	✓	✓	✓	✓	✓
10	Xylanase	*Cellulomonas fimi*	3.2.1.8	P07986			✓		✓
17	β-1,3-1,4-glucanase	*Hordeum vulgare*	3.2.1.73	P12257			✓ (SE)		✓
30	Glucocerebrosidase	Human	3.2.1.45	P04062	✓		✓	✓	✓
35	β-galactosidase	Human	3.2.1.23	P16278			✓		✓
39	α-L-iduronidase	Human	3.2.1.76	P35475	✓		✓		✓
42	β-galactosidase	*Bacillus stearothermophilus*	3.2.1.23	P19668			✓		✓

[a] Families defined in Henrissat (1991) and Henrissat and Bairoch (1993).
[b] Only one or a few representatives of each family are shown.
[c] Swiss-Prot or EMBL/GenBank sequence data banks.
[d] Invariant residues of glycosyl hydrolase family 5 unambiguously identified in the related glycosyl hydrolase families.
[e] Conserved sequence segments (I to V) of glycosyl hydrolase family 5. See Figure 3.

plates (Fig. 2). Limited sequencing of the strain SR120A *xynA* gene in pNTK101 showed greater than 90% homology at the nucleotide level to the gene from strain D1. This is consistent with the conservation of restriction sites noted above and indicates that the genes are highly homologous.

Sequencing of the DNA region upstream of *xynA* in clone pNTK112 showed homology to two other genes. One ORF was located ca. 200 bp upstream of *xynA* (Fig. 1) and its translation product was homologous with the *E. coli* ATP-dependent RNA helicase gene, *dbpA* (Iggo et al. 1990). The 3′ end of a gene with 45% amino acid identity to the *E. coli recD* gene was identified further upstream (Finch et al. 1986).

The *xynA* ORF encoded 413 amino acids but did not include a typical leader peptide sequence (von Heijne 1985). N-terminal amino acid sequencing of the protein secreted to the periplasm of *E. coli* cells expressing *xynA* disclosed the sequence DTVKIDA, identical to positions 32 to 38 of the pre-protein in Figure 2 as deduced by DNA sequencing. This established that an unusual leader sequence occurs in *xynA* that, in addition to being long (31 amino acids), contains at least two polar amino acids (serine and threonine) in the hydrophobic domain.

A data base search (Blast Program [PDB, SwissProt, SPupdate, PIR, GenPept, GPUpdate], 11 December 1995) indicated that the *xynA* peptide product had significant homology to vertebrate cerebrosidase sequences (e.g., O'Neill et al. 1989), but not to other glycosidases. Glucocerebrosidases form family 30 in a general classification of glycohydrolases based on sequence similarities (Henrissat 1991). They have recently been shown to be distantly related to a superfamily grouping several other families of glycohydrolases that share the same catalytic machinery and probably the same $(\beta/\alpha)_8$ fold (Henrissat et al. 1995). Hydrophobic cluster analysis, a sensitive sequence comparison method (Gaboriaud et al. 1987; Lemesle-Varloot et al. 1990), was thus conducted to compare the *xynA* gene product with the above superfamily. The analysis identified two regions in XynA that displayed similarity with the two regions around the catalytic residues of these glycohydrolases (Fig. 3). Residues E165 and E253 in XynA are therefore likely to be the catalytic proton donor and nucleophile, respectively. In addition to glucocerebrosidases, the glycohydrolase sequences that appeared to be most closely related to XynA belonged to family 5 (Henrissat 1991), which contains eight conserved residues in five conserved stretches corresponding to five of the eight β strands of the $(\beta/\alpha)_8$ structure found in this family (Dominguez et al. 1995; Ducros et al. 1995). Four of the five stretches with seven of the eight invariant residues of family 5 are found in XynA and in the glucocerebrosidases (see Figure 3 and Table 2). The *E. chrysanthemi* xylanase sequence thus appears to represent an intermediate between glycosidase families 5 and 30 and accordingly substantiates their grouping into the same superfamily.

The *xynA* gene could be expressed to high level in *E. coli* with a transcriptional fusion (pNTK136, Table 1) in the T7 plasmid, pRSET5A. This construct was stable after several transfers in *E. coli* strains DH5α or B384(DE3) on L ampicillin plates. Following isopropyl-β-D-thiogalactopyranoside (IPTG) induction (added to 0.6 mM at a cell density of ca. 0.5 at 600 nm in L ampicillin broth and cultures grown at 28°C), a strong new sodium dodecyl sulfate (SDS) gel band at the position of the mature xylanase (ca. 42 kDa) was observed at 4 h after induction (Fig. 4). Another strong band at about 3 kDa higher mass was assumed to be the pre-protein since high level T7 promoter expression of other secreted proteins has been observed to result in relatively large accumulation of pre-proteins (N. Kita and N. Keen, manuscript in preparation). The relatively large mass difference between the mature and pre-xylanase bands is consistent with the relatively long leader peptide determined by N-terminal sequencing of the mature XynA protein. The mature xylanase was present at high amounts in the periplasmic fraction (prepared according to Witholt et al. 1976) from induced B834(DE3) cells carrying pNTK136 (Fig. 4).

The xylanase was purified by dialysis of the periplasmic fraction against 5 mM tris-HCl, pH 8.0, and fractionation on a CM Bio-gel column equilibrated with the same buffer (Fig. 5). The xylanase adsorbed to the column matrix and, following washing with the tris buffer, was not eluted with the same buffer containing 0.25 M NaCl. The protein was then eluted with 0.5 M NaCl in the tris-HCl buffer (Fig. 5) and this preparation ran as a single Coomassie R staining band on SDS gel electrophoresis (Fig. 4). Peak fractions from the CM Bio-gel column were pooled and dialyzed against distilled water at 4°C, and the protein stored at –20°C or lyophilized. No significant activity loss was detected during these manipulations. Ten milligrams or more of lyophilized protein was obtained per liter of culture and determined to be >99% pure when various amounts were examined on SDS gel electrophoresis (data not shown).

A xylanase-deficient marker exchange mutant strain of strain D1 was constructed as well as a double mutant strain defective in both the *xynA* gene and the previously studied *bgx*A gene encoding a xylosidase (Vroemen et al. 1995). pNTK112, containing the cloned *xynA* gene, was restricted with *Bam*HI and re-ligated, deleting a 1.5-kb fragment con-

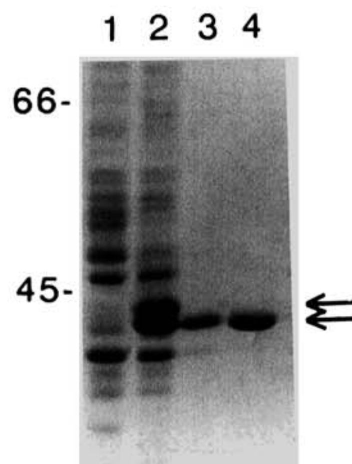

Fig. 4. Sodium dodecyl sulfate gel electrophoresis of *Escherichia coli* B834(DE3)[pLysS] cells or periplasmic fractions carrying expression plasmid pNTK136. Lane 1, cells carrying cloning plasmid, pRSET5A only, induced for 4 h with 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG); lane 2, cells carrying pNTK136 and induced for 4 h with 0.6 mM IPTG; lane 3, periplasmic fraction from induced cells carrying pNTK136; lane 4, xylanase from periplasmic fraction in lane 3 after purification on a CM Bio-gel column. Cells grown at 28°C in L ampicillin broth. Size standards on left; position of the xylanase mature and pre-proteins denoted by arrows.

taining most of the *xynA* gene (pNTK123, Fig. 1, Table 1). A ca. 2.0-kb *Bam*HI fragment from pSmUC (Murillo et al. 1994) was ligated into the *Bam*HI site of pNTK112, forming plasmid pNTK125 (Table 1). As expected, this construct did not direct xylanase activity in *E. coli* on RBB xylan plates. The DNA insert from pNTK125 was re-cloned into pRK415 to yield pNTK129 (Table 1). This plasmid was introduced into *E. chrysanthemi* strains D1 or D1 *bgx* Km by conjugation and the plasmid cured as described by Vroemen et al. (1995). The resulting streptomycin-resistant marker exchange mutant strains were confirmed by Southern blots (data not shown). The mutant strains gave negligible xylanase activity when grown on RBB xylan plates compared with the starting strains, which yielded clear halos surrounding the colonies (Fig. 6). The two mutant strains also yielded no detectable xylanase activity when grown in liquid medium and the culture fluids assayed for xylanase activity as reported by Braun and Rodrigues (1993). Based on the detection limits of this reducing sugar assay, the mutant strains therefore produced less than 0.5% of the wild-type xylanase level. Attempts to construct a SR120A marker exchange strain failed because the pRK415 construct analogous to pNTK129 could not be introduced into strain SR120A either by conjugation or electroporation.

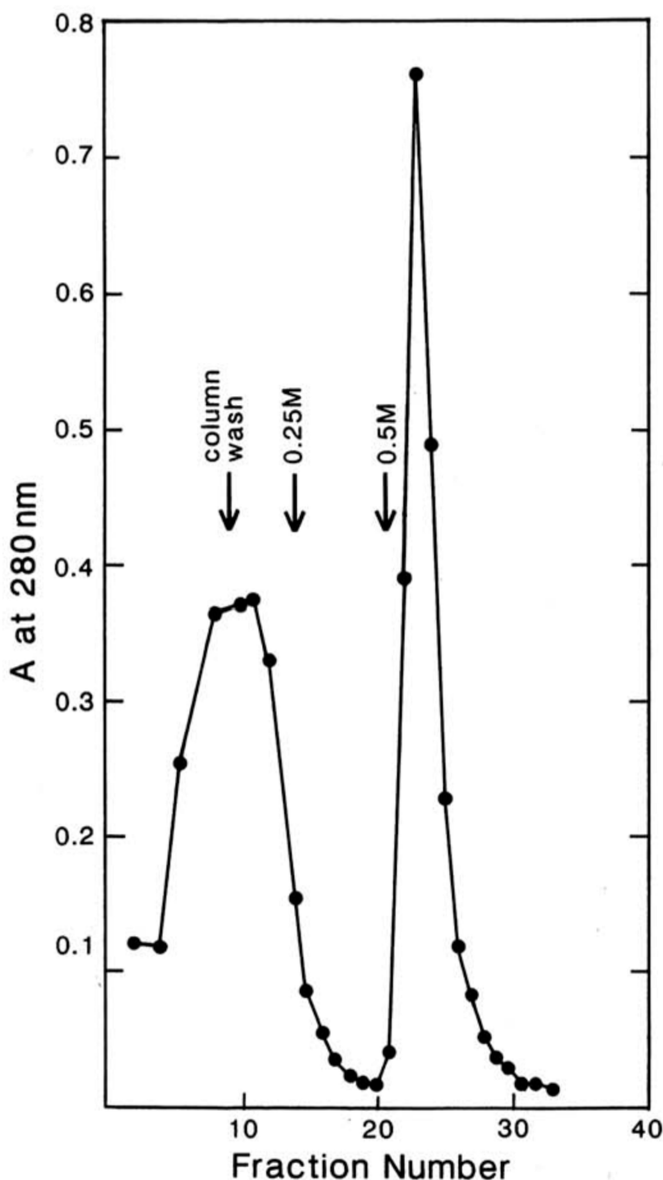

Fig. 5. Purification of xylanase expressed in *Escherichia coli* B834(DE3)[pLysS] cells carrying pNTK136 and induced with isopropyl-β-D-thiogalactopyranoside as described in Figure 4. Following dialysis against 5 mM tris-HCl, pH 8.0, the protein was applied to a 1.2 × 20 cm column of Bio-gel CM (Bio-Rad) in the same buffer. Following application of the sample, the column was washed with the tris-HCl buffer only, as shown by arrow, then with 0.25 M NaCl in the same buffer but little additional protein eluted. Finally, the xylanase was eluted when the column was eluted with 0.5 M NaCl in the tris-HCl buffer. This preparation was dialyzed against 2 mM tris-HCl, pH 8.0, and lyophilized or stored frozen at –20°C.

Pathogenicity tests on corn leaf sections (sweet corn cv. Golden Bantam) were performed according to Vroemen et al. (1995) and the lengths of bacterial lesions were measured at several times after inoculation. Experiments with various inoculum levels ranging from 10^4 to 10^8 cells/ml did not yield detectable differences in virulence with either the *xynA* single mutant or the *xynA bgxA* double mutant strains compared with the wild-type D1 strain. Incubation temperatures ranging from 18 to 30°C caused large differences in the rapidity of symptom formation, but again no reproducible differences were seen in plants inoculated with the mutant or wild-type strains.

The failure to observe detectable changes in the pathogenicity of the D1 marker exchange mutants is similar to results seen with a fungal mutant deficient in xylanase (Apel et al. 1993). Thus, despite substantial suggestive evidence implicating xylanases as virulence factors in pathogens of grasses (Braun and Kelman 1987; Cooper et al. 1988), our results do not support this possibility for *E. chrysanthemi* D1. However, interpretation of negative data resulting from such experi-

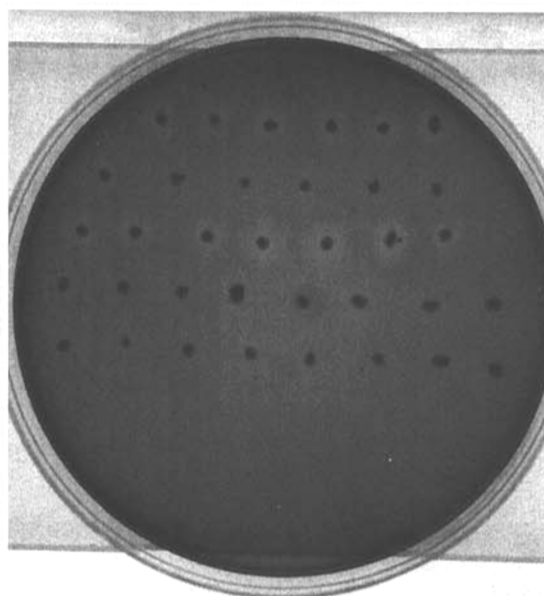

Fig. 6. Remazol brilliant blue xylan plate with colonies of *Erwinia chrysanthemi* D1 wild-type and mutant strains. Clear halos surrounding colonies indicate xylanase activity. Row 1, D1 *bgx* Km; row 2, D1 *bgx xyn* Km Sm; row 3, D1 wild type; rows 4 and 5, two independently selected strains of D1 *xyn* Sm.

ments should be treated with caution (Keen 1995), and it is possible that differences might be detected under other experimental conditions.

Its moderate size, coupled with the ability to overexpress *xynA* in *E. coli* and readily purify the protein from the periplasmic fraction, encourages structural work with XynA. XynA is an interesting protein for structure-function relationship studies since it is the first xylanase that does not belong to glycohydrolase families 10 or 11 and has, instead, an interesting position between families 5 and 30.

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