A unique eukaryotic β -xylosidase gene from the phytopathogenic fungus *Cochliobolus carbonum*

Sigrun Wegener, Richard F. Ransom† and Jonathan D. Walton

Author for correspondence: Jonathan D. Walton. Tel: +1 517 353 4885. Fax: +1 517 353 9168.
e-mail: walton@pilot.msu.edu

Department of Energy –
Plant Research Laboratory,
Michigan State University,
East Lansing, MI 48824,
USA

The plant-pathogenic fungus *Cochliobolus carbonum* secretes one major β -xylosidase (Xyp1) when grown on xylan or maize cell walls. cDNA and genomic DNA encoding Xyp1 were isolated using PCR primers based on peptide sequences from the purified protein. XYP1 contains three introns, has 5' and 3' untranslated regions of 74 and 145 bp, respectively, and is predicted to encode a protein of 328 amino acids (*M*, 36 700) with four *N*-glycosylation sites. Although it is secreted, Xyp1 has no predicted signal peptide. Furthermore, Xyp1 appears not to be processed at the N-terminus because one of the peptides isolated from the mature protein is located only six amino acids downstream of the translational start methionine. The primary sequence of Xyp1 is unrelated to any known eukaryotic β -xylosidase but has 35% overall identity to two bacterial bifunctional β -xylosidase/ α -arabinosidases. Mutation of XYP1 by targeted gene replacement resulted in the loss of the major β -xylosidase activity corresponding to the product of XYP1, but a significant amount of secreted β -xylosidase activity (25% of wild-type) remained in the culture filtrates. The xyp1 mutant was still fully pathogenic on maize.

Keywords: *Cochliobolus* (*Helminthosporium*) *carbonum*, β -xylosidase, xylanase, plant pathogen, cell-wall-degrading enzyme

INTRODUCTION

A major long-standing question in plant pathology is the role of cell-wall-degrading enzymes in the process of pathogenesis. Plant-pathogenic bacteria and fungi make xylan-degrading enzymes, but evidence with regard to the role of most of these enzymes in ingress, ramification or the acquisition of nutrients is inconclusive. The major obstacle to answering this question is enzymic redundancy: most micro-organisms, including plant pathogens, make multiple xylan-degrading enzymes (Apel-Birkhold & Walton, 1996).

A variety of enzymes that can degrade xylans have been identified, and their genes cloned, from saprophytic prokaryotes and eukaryotes (Wong *et al.*, 1988). Many, perhaps most, microbial plant pathogens make xylan-

degrading enzymes (Maino *et al.*, 1974; Anderson, 1988; Dean *et al.*, 1989; Southerton *et al.*, 1993; Wu *et al.*, 1997). The role of xylan-degrading enzymes has been studied genetically in two plant-pathogenic fungi. *Cochliobolus carbonum*, which causes Northern corn leaf spot, attacks foliage, stems and ears of susceptible maize varieties. It makes at least two endo-1,4- β -xylanases as well as β -xylopyranosidase in culture (Apel *et al.*, 1993; Apel-Birkhold & Walton, 1996; Ransom & Walton, 1997). A strain of *C. carbonum* mutated in three of its four Family G endoxylanase genes (XYL1, XYL2 and XYL3) was fully pathogenic and grew at the wild-type rate on xylan or maize cell walls (Apel-Birkhold & Walton, 1996). *Magnaporthe grisea*, cause of blast of rice, makes at least six endoxylanases in culture and two xylanase genes, XYL1 and XYL2, have been cloned (Wu *et al.*, 1995, 1997). Strains of *M. grisea* mutated in XYL1 (Family G) and XYL2 (Family F) have reduced ability to grow on xylan as carbon source but are still fully pathogenic (Wu *et al.*, 1997).

The complete degradation of xylan requires the participation not only of endoxylanases but also β -

†Present address: Department of Pediatric Nephrology, University of Michigan Medical Center, Ann Arbor, MI 48109, USA.

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xylosidase (Xyp) (Wong *et al.*, 1988). Therefore, a full evaluation of the role of xylan degradation in the process of plant pathogenesis requires a genetic analysis of genes encoding Xyp as well as endoxylanases. *C. carbonum* makes one major extracellular Xyp, called Xyp1, when grown in culture on maize cell walls as a carbon source (Ransom & Walton, 1997). Xyp1 is glycosylated, has a low level of α -arabinosidase activity but no arabinanase activity, a pH optimum of 5.5–6.5, and a temperature optimum of 37 °C. Xyp1 prefers xylobiose as substrate but can also release reducing sugars from several complex plant xylans, including oat speltis xylan, birch wood xylan and wheat arabinoxylan. Xylose is the sole sugar released by Xyp1 from oat speltis xylan (Ransom & Walton, 1997).

Genes encoding Xyp have been isolated from saprophytic bacteria and fungi and from at least one prokaryotic plant pathogen (Vroemen *et al.*, 1995), but to the best of our knowledge this is the first report of a Xyp gene, *XYP1*, from a plant-pathogenic fungus. The predicted protein sequence of *XYP1* has some similarity to two prokaryotic Xyp proteins but not to any known fungal Xyp.

METHODS

Fungal strains and growth. Methods used for storage and growth of *C. carbonum* and preparation of maize cell wall growth medium have been described previously (Sposato *et al.*, 1995).

Nucleic acid manipulations. DNA and RNA were isolated as described by Pitkin *et al.* (1996). The methods used for DNA blotting, probe labelling, hybridization and genomic and cDNA library screening have been described previously (Scott-Craig *et al.*, 1990; Murphy & Walton, 1996).

The *XYP1* gene was isolated using PCR primers corresponding to the Xyp1 amino acid sequences DIPWAT and DKPLGK (Ransom & Walton, 1997). The PCR primers had the sequences 5'-GAYATHCCNTGGGCNAC (96-fold degenerate) and 5'-TTNCCNARNGGYTTRTC (512-fold degenerate) (Y represents C or T; H represents A, T or C; R represents A or G; and N represents any nucleotide). Genomic DNA was used as template in PCR carried out in a Perkin-Elmer thermocycler under the following conditions: 3 min denaturation at 94 °C; 35 cycles of 1 min denaturation at 94 °C, 2 min annealing at 55 °C, and 3 min primer extension at 72 °C. The final step was 10 min primer extension at 72 °C. A 141 bp fragment was amplified, cloned, sequenced, and used to screen cDNA and genomic libraries. A 5.0 kb *Sall* genomic fragment that hybridized to an isolated 1.2 kb cDNA was subcloned into pBluescript II SK(–). The cDNA and the genomic DNA spanning the gene were sequenced on both strands. Sequencing was performed by automated fluorescent sequencing at the MSU-DOE-PRL Plant Biochemistry facility using an Applied Biosystems Catalyst 800 for *Taq* cycle sequencing and an Applied Biosystems 373A Sequencer for analysis of the products.

The transcription start site of *XYP1* was determined using a 5' RACE (Rapid Amplification of cDNA Ends) kit (Gibco) (Frohman *et al.*, 1988). Reverse transcription was primed with the oligonucleotide 5'-GGCTGGGAAGAAGAGGTA (the reverse complement of 5'-TACCTGTTCTTCCAGCC

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1 TTAGTAATTCGCTTACTGACACAAGATAACGAGATCTCGCCTTCTCATTGGTACCATTCT
61 CCATTGCAATAACGCGTGCATCTCTACTTTGGCAGTCTGAAATGTTTGAACACACTC
121 GCGTCATCTTCCAATCTGCTGTTGTATGAGACGGCTGCTGCGCCACCTTCCGCGGGGA
181 CGATGTAAATCGGGCGGTGGAGGTCTGAATCTCGTGTCTATTTCAGGACATCACCAGATAA
241 TTGCACAAGTCCCGCAATCCCATCTCTTCTGCACCATTAAGATGGCCCATCTTAAACT
301 TCTCAGATTGCTTATCCCTTAAGATTAGGCTAAATTCGGGTGGGGACCGCCGATGTC
361 AGACACTAAATGACTTACTCAAGCTGCGCTATGAGGGCCTTAAAGGTTGCTGTCTCAC
421 GCGCTCTTGACAGCAGCCAGGCTTTCATTGCAACAAGTGAACCAAGCTTGCCAAACACT
#
481 CTTTCCTGTCGCGACTGCTGCTTTTCAACATGTCTGTTCAAGCCTACGACCCCGCTTA
M S V Q A Y D P P L
541 TCAGCACCTTCCACAGTCCGACCTGCGAGCCACGCTGGAAGCATGATCCCAACACTG
I S T F H Q C D P A A H V W K H D P N T
601 TATACATCTATGGATCCACGACTGGAAGTCTGACAGGCGAGCCCGGTGgtcagtattc
V Y I Y G S H D W N S T R Q P P V
661 aaggatttctattatgcgaatgcagggtgacaaagaacagCAATACGACATGAAGGATTA
Q Y D M K D Y
721 CTATGCTTACCAGACCGATTACACCAAGCTGCGACAGTCCGACCAAGGtatacca
Y V L T Q T D Y T K P A T V G P K
781 attctttagctagagattggaatttgaattgacagaaactagCTTTTGGACCTTGTTGG
L L D L V
841 ACATCCCATGGGCGACACAGCAACTCTGGGCTCTGATGCGACTGAGAAGGATGCAAT
D I P W A T Q Q L W A P D A T E K D G Q
901 ACTACCTGTTCTTCCAGCCAGGACCAAGGATGATGCTTCCGATCGGATGGCCCAAT
Y Y L F F P A K D K D D V F R I G V A K
961 CTGACAGCCGCGACGGAATAATCAAGCCGAGCCCAACCGATTGCGCGACCTACAGCA
S D K P D G K F K P E P N P I A G T Y S
1021 TTGACCCACAACTCTTGAAGACAATGGCACTACTACATGGTATGGCGGACTGTGGG
I D P T I L E D N G N Y Y M V W G G L G
1081 GTGGCGAGTCTCAAGCATGACCAACACAGCTTGTgaagccttcgcttcgacacaa
G G Q L Q A W T N N V L
1141 cccagctgttgtaagcgtgaccgaagcttaactaacgcgattttcagAACCATCCCTA
N T S L
1201 TTGCTTAACAATGCTCTTCTTCCGCGCCAGCACTCGGACCCGTTTCCGCAAGCTCTCG
F G N N A P T S G P A I G P R F A K L S
1261 GCTACCTCTACCTCTCTCGCCACCAAGCAAGTCTGCTATCTACGACAGGACGGC
A N L T S L A T T P K E L V I Y D Q D G
1321 AAGGTCATGACGAGGCGAGCAGCGCGCTTCTTCGAGGGCCATCTCTGAACAGGTC
K V M Q A G S T R F F E G P S L N K V
1381 GGCACCTCTATGCTCTCCAGTACTCTACCGGACCTCGCACACTGTTGAATCGCTACC
G N L Y V L Q Y S T G T S H T V E I A T
1441 GCTACCAAGCTGACGGTCTTACTACTGGAAGAGCAGCTTCTCGACCTGTCAAGGGA
A T K P D G P Y Y W K S T L L Q P V K G
1501 TGGACTACCCAGGATCGATTGTCAAGTTCAGAAATGACTGGCTTCTTACTACGCTAD
W T T H E S I V K F K N D W L L Y A G
1561 GCCTCGCTTCTGGCGTGATGATCTCCGCAACACCAAGGTCAGGAAGATCTTGTGTAG
A S L S G R D D L R N T K V R K I L F E
1621 AATGGCACCTTCAAGCTTGTCTGACGCTGACGCTGTCTTCTAGCTCTTAAAGCTATAAA
N G T F K L A Q P Q P V I P S S *
1681 TTGTTTATATCTACTCTACATATTTTGTACCGCTTAAGCTGTAATGTACACAGTTCGC
1741 TAGTTACTGAAAGCGTTGTTTCTACACATAGGATGCAAGCAATTAGATTGGACAATA
1801 TATTTGCCAAGATGTGAACGAGTCTCTGGGATCTACTGCTTACATGAGCATGTGTTAA
+
1861 ACCATTCCCTTGGTATAGGGGACAGTAAGCTGGTACAACATGTCTCTGTCAAAATCAA
1921 ATCAAGTAGGCAACGACTAATCATCTTGGTAAGACACCATGAAGCTGCCTATGAATCA
1981 AATAGCTATAGCTGTGAAGTCCGCTCCAGCTAGTAATTAAGCATGCTTGTATCATTAT
2041 CCTACCGATAGCATGGTAGCGGTTGCTCTGAATCTGTTGAAGACCTTA

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Fig. 1. DNA and deduced amino acid sequences of *XYP1*. Amino acids are shown below the corresponding codons. The two peptides used for the design of PCR primers are printed in bold type. The sequence in the promoter that is identical to the *xlnR*-binding site is underlined. The three introns are indicated by lower-case letters. The four potential glycosylation sites are doubly underlined. #, Transcriptional start site; *, stop codon; +, polyadenylation site (all symbols refer to the nucleotide directly above).

starting at nucleotide 903; see Fig. 1). PCR amplification was done using the primer sequence 5'-CCAGTCTGGGATC-CATAGAT (the reverse complement of 5'-ATCTATGGAT-CCCACGACTGG starting at nucleotide 606; see Fig. 1) and the 'abridged anchor' primer supplied with the RACE kit. Three independent RACE products were sequenced. Two products started at nucleotide 436, and one product started at nucleotide 448 (see Fig. 1).

Transformation-mediated gene replacement. The central part of the coding region of *XYP1* was excised from the 5.0 kb genomic *Sall* fragment by cutting with *Bgl*II, and replaced with the *Escherichia coli hph* gene (encoding hygromycin phosphotransferase) driven by the *Aspergillus nidulans trpC* promoter from plasmid pCB1003 (Carroll *et al.*, 1994). In this way, the complete coding region of *XYP1* except for 60 bp of the 3' end was removed. The fragment used for transformation was released from the vector by cutting with *Sall*. Protoplast isolation and transformation have been described previously

(Scott-Craig *et al.*, 1990; Apel *et al.*, 1993). The transformants were subjected to two rounds of single-spore isolation to ensure nuclear homogeneity.

Protein purification and enzyme assays. *C. carbonum* wild-type and mutant strains were grown in 1 l flasks containing 125 ml modified Fries' medium (Van Hoof *et al.*, 1991) containing sucrose at 0.2% and maize cell walls at 1% (w/v). Xyp1 was purified as described by Ransom & Walton (1997). β -Xylosidase activity was determined using *p*-nitrophenyl β -D-xylopyranoside (Sigma N2132) (Ransom & Walton, 1997).

RESULTS AND DISCUSSION

Isolation and characterization of *XYP1*

A 141 bp PCR product was obtained using PCR primers based on two internal peptides from purified Xyp1 (Ransom & Walton, 1997). The PCR product was used to screen cDNA and genomic DNA libraries and both cDNA and genomic copies of the encoding gene, *XYP1*, were sequenced (Fig. 1). The predicted amino acid sequence of *XYP1* contains the two peptides previously reported from purified Xyp1 (Ransom & Walton, 1997). Peptide 1 (LLDLVDIPWATQQLWA) is a perfect match, but peptide 2 contains three discrepancies, including one in the sequence upon which one of the PCR primers was designed. The predicted sequence of *XYP1* also contains three additional peptides derived from the purified protein by trypsin or Asp-N digestion, namely FFEGPSLNK, TKPATVGP and LLDLVDIP. The third of these peptides overlaps with peptide 1.

XYP1 contains three introns of 51, 52 and 73 bp. *XYP1* has a 5' untranslated region of 74 bp and a 3' untranslated region of 145 bp. In the promoter there is a sequence (GGCTAAA; underlined in Fig. 1) identical to the binding site for the product of *xlnR*, a zinc binuclear Cys₆ transcription factor that regulates endo-1,4- β -xylosidase and β -xylosidase gene expression in *Aspergillus niger* (van Peij *et al.*, 1998).

XYP1 is predicted to encode a protein of 328 amino acids with an M_r of 36700, which is slightly smaller than the M_r (42000) estimated by SDS-PAGE (Ransom & Walton, 1997). Xyp1 is a glycoprotein (Ransom & Walton, 1997), and the amino acid sequence has four potential *N*-glycosylation sites (Fig. 1). Therefore, the difference between the calculated and the experimentally determined sizes is probably due to post-translational glycosylation.

Xyp1 contains no apparent signal peptide

All of the 14 known extracellular cell-wall-degrading enzymes of *C. carbonum* (Görlach *et al.*, 1998; Scott-Craig *et al.*, 1998) contain canonical eukaryotic signal peptide sequences according to the SignalP program (Nielsen *et al.*, 1997), which is based on the rules of von Heijne (1986). Calculated signal peptide lengths for the *C. carbonum* enzymes range from 15 to 19 amino acids with a mean of 18. The most highly conserved positions are Ala at -2 (12 of 14) and Pro at +2 (12 of 14).

In contrast to all other known *C. carbonum* extracellular enzymes, however, the SignalP program did not predict any cleavage site for Xyp1 within the first 50 amino acids. In addition, the first 20 amino acids of Xyp1 are not rich in hydrophobic residues: whereas 41% of the amino acids of the known *C. carbonum* signal peptides are Ala or Leu, only 15% of the first 20 amino acids of Xyp1 are. There are no other Met codons or any predicted signal peptide in any reading frame between the experimentally determined transcriptional start and the proposed Met start codon (Fig. 1). The length of the 5' untranslated region is consistent with other *C. carbonum* genes, and the context of the predicted Met start codon agrees with the consensus for filamentous fungi (CAACATGTC vs the consensus CAMMATGNC, where M is A or C, and N is any nucleotide) (Edelmann & Staben, 1994). Therefore, the apparent lack of a signal peptide is probably not due to misidentification of the putative translational start site.

Additional evidence against Xyp1 being processed during secretion is that another peptide sequence obtained experimentally from Xyp1 by Asp-N digestion had the sequence DPKLISTFHQKA. Starting at amino acid 7 (Fig. 1) is the predicted sequence DPpLISTFHQcd, which agrees in nine of twelve positions (discrepancies are indicated in lower case). Assuming that the discrepancies are due to errors in the peptide sequencing, the presence of this peptide in mature Xyp1 indicates that, if Xyp1 is at all processed post-translationally, it loses no more than six amino acids.

Other proteins have been described that are secreted but lack a cleaved hydrophobic signal peptide (Muesch *et al.*, 1990). Chicken ovalbumin and human plasminogen activator inhibitor-2 contain uncleaved internal signal sequences that are located near their N-termini, which allow these proteins to be secreted by the normal secretory pathway through the endoplasmic reticulum (ER) and the Golgi apparatus (Meek *et al.*, 1982; Ye *et al.*, 1988). The fact that Xyp1 is glycosylated and contains four potential *N*-glycosylation sites speaks for a secretion mechanism through the classical ER/Golgi pathway. However, it is not known if Xyp1 is truly *N*-glycosylated as opposed to *O*-glycosylated. In contrast to ovalbumin, Xyp1 does not contain a stretch of hydrophobic residues near its N-terminus or anywhere else in the protein that could plausibly function as an uncleaved internal signal peptide. On the other hand, several other proteins that lack a secretory signal sequence are instead secreted by pathways other than the ER/Golgi pathway. These proteins include annexins (Siever & Erickson, 1997), mammalian basic fibroblast growth factor (Mignatti *et al.*, 1992), interleukin-1 β (Rubartelli *et al.*, 1990) and the mating α -factor of *Saccharomyces cerevisiae* (Kuchler *et al.*, 1989). The secretion of α -factor is dependent on *STE6*, which encodes a plasma membrane P-glycoprotein. *S. cerevisiae* also has protein secretory pathways that are independent of either the classical secretory pathway or *STE6* (Cleves *et al.*, 1996). Thus, the lack of a signal peptide in Xyp1 is unusual but not unprecedented. It

<i>C. carbonum</i>	M S V Q A - Y D P R I S T F H Q C D P A A H V K K H D P N T V Y I Y G S H D W	39
<i>B. ovatus</i>	M K T E K R Y - - - V P G D Y M A D P A V H V F D - - - G K L Y I Y P S H D W	34
<i>P. ruminicola</i>	M K A - - K Y - - - V F P S D Y M A D P A A N V F D - - - G K L Y I Y P S H D Y	32
<i>C. carbonum</i>	N S T - - - R Q P P Y Q Y D M K D Y Y V L T Q T Q Y T K P A T Y G P - - K L I D	74
<i>B. ovatus</i>	E S G I A E N D N G D H F N M K D Y H V Y S M D - D V M N G E I K D H G V V L S	73
<i>P. ruminicola</i>	D S G E C F D D D G G H F Q M K D Y H V L C I D G D P M E Q D A K D C G Q F G	72
<i>C. carbonum</i>	L V D I P W A T Q Q I W A P D A T E K D G Q Y Y L F F P A K D K D D V F K I G V	114
<i>B. ovatus</i>	T E D I P W A G R Q I W D C D V V C K D G K Y Y M Y F P L K D Q N D I F R I G V	113
<i>P. ruminicola</i>	I E D I P W V E K Q I W D N D C V E K D G K Y Y I Y S A K D Y T G V F H L G V	112
<i>C. carbonum</i>	A K S D K P D G K F K P E P N P I A G T Y S I D P T I L E D - N G N Y Y N V W G	153
<i>B. ovatus</i>	A V S D K P Y G P P I E A N P M K G S Y S I D P A V W D D G D G N Y Y I Y F G	153
<i>P. ruminicola</i>	A V A D K P E G P P V P E A D P T R G S Y S I D P C V F K D D D G E I Y V Y F G	152
<i>C. carbonum</i>	G L W G G Q L Q A W T N N - V L N T S I F G N N A P T S G P A I G P R F A K L S	192
<i>B. ovatus</i>	G L W G G Q L Q R Y R N K A E S A I L P E G E E E - - - A T P S R V A R L S	190
<i>P. ruminicola</i>	G I N G G Q L Q W Y K D N K M I K A E H L P E G K E D - - - P L P S R V A R M T	189
<i>C. carbonum</i>	A N L T S I A T T P K E L V I Y D Q D G K V M Q A G S T - H R F F E G P S L N K	231
<i>B. ovatus</i>	E D M M E F A E E P R A V V I L D E D G K P L T A G D T E R R P F E A S W M H K	230
<i>P. ruminicola</i>	G D V K Q F A E A P R A V I T V D E T G K P L P A D D P - H R F F E A S W M H K	228
<i>C. carbonum</i>	Y N G N L Y V L O Y S T G T S H T V E I A T A T K P D G P Y Y W K S T I L O P V K	271
<i>B. ovatus</i>	Y N G K Y Y F S Y S I G D T H L L C Y A I G D N P Y G P F T Y Q G V I T P V V	270
<i>P. ruminicola</i>	Y N G K Y Y F S Y S I G D T H L L C Y A V G D N P Y G P F T Y Q G V I E P V V	268
<i>C. carbonum</i>	G W T T H S I V K F K N D N L L Y A D - A S L S G R D L R N T K V R K I L	310
<i>B. ovatus</i>	G W T T H H A I V E F K G K W Y L F H H D C V P S E G K T W I R S L K V C E L Q	310
<i>P. ruminicola</i>	G W T T H S I V E Y K G K W Y L F H H D C V P S N D T T W I R S L K V A E L E	308
<i>C. carbonum</i>	F E - N G T E K L A Q P Q P V I P S S *	329
<i>B. ovatus</i>	Y D A D G R I T I E G K D E *	326
<i>P. ruminicola</i>	Y D A E G H I K T V K *	320

Fig. 2. Comparison of the deduced amino acid sequence of Xyp1 to two related bacterial β -xylosidase sequences. Sequence references: *Bacteroides ovatus*, GenBank accession no. U04957, Whitehead (1995); *Prevotella ruminicola*, GenBank accession no. Z49241, Gasparic et al. (1995). Amino acids in the other two enzymes that are identical to Xyp1 are shaded. Dashes indicate introduced sequence gaps; asterisks indicate stop codons.

would be interesting to determine if alternative protein export mechanisms exist in filamentous fungi.

Xyp1 is related to bacterial but not fungal xylosidases

By BLAST analysis (Altschul et al., 1990), Xyp1 is not related to any of the six fungal β -xylosidases in the current database. Xyp1 is related in both size and primary sequence to two bacterial β -xylosidases, those of *Bacteroides ovatus* (35% overall identity) and *Prevotella* (formerly *Bacteroides*) *ruminicola* (30% identity) (Whitehead & Hespell, 1990; Whitehead, 1995; Gasparic et al., 1995) (Fig. 2). Interestingly, neither of these two bacterial xylosidases has an obvious signal peptide. On this basis, the β -xylosidase of *P. ruminicola* was presumed to be located in the cytoplasm, although this has apparently not been experimentally tested (Gasparic et al., 1995).

Both of the related bacterial β -xylosidases have been assigned to Family 43 of glycosylhydrolases (Henrissat & Bairoch, 1996). The *B. ovatus* enzyme is a bifunctional β -xylosidase/ α -arabinosidase, and the *P. ruminicola* enzyme has in addition exoxylanase activity, defined as the ability to release free xylose from xylan. Xyp1 resembles the *P. ruminicola* enzyme in having exoxylanase activity, but it cannot be considered to be bifunctional because its α -arabinosidase activity is only 12% of its β -xylosidase activity (Ransom & Walton, 1997). The sole fungal enzyme currently placed in Family 43 is an endoarabinanase from *A. niger*, but this enzyme and Xyp1 show no overall amino acid similarity, the largest region of identity being composed of five amino acids, LWAPD (Flipphi et al., 1993). Xyp1 is not active against sugar-beet arabinan (Ransom & Walton, 1997). Xyp1 is therefore unusual in two respects. First, it is related to bacterial β -xylosidases but not to any other

known fungal β -xylosidase. Secondly, although it is clearly secreted, the predicted protein lacks any typical signal peptide and, based on peptide sequences obtained from the mature protein, is probably not post-translationally cleaved.

Gene replacement of XYP1

To test that XYP1 encodes the enzymically characterized Xyp1 protein, and to analyse the role of Xyp1 in pathogenesis, we constructed a strain of *C. carbonum* in which XYP1 was disrupted by gene replacement (Fig. 3a). Two Tox2⁺ (HC-toxin-producing) strains of *C. carbonum* (367-1a and 367-2A) were transformed with a vector in which the majority of the XYP1 coding sequence had been replaced by a gene, *hph*, conferring hygromycin resistance. A total of 36 hygromycin-resistant transformants were obtained, and one transformant of each strain (T661-1 and T666-2) was further analysed. The restriction map of the wild-type XYP1 locus and the predicted map resulting from homologous integration of a single copy of the replacement vector are shown in Fig. 3(a). The patterns of hybridization with the *Bgl*II fragment of XYP1, the complete *Sal*I genomic fragment of XYP1, or the *hph* gene indicate that the XYP1 gene had been deleted in both transformants (Fig. 3b).

Growth of the mutant T661-1 on maize cell walls, birchwood xylan or oat spelts xylan was not significantly different from wild-type (data not shown). Total extracellular β -xylosidase activity in culture filtrates of T661-1 grown on maize cell walls was reduced by 75% compared to wild-type. β -Xylosidase activities from wild-type and T661-1 were purified in parallel through cation-exchange HPLC, as described by Ransom & Walton (1997). The *xyp1* mutant completely lacked the major peak of xylosidase activity, but contained a small

Fig. 3. DNA blot analysis of wild-type and *XYP1* disruption mutants. (a) Genomic restriction maps of the wild-type *XYP1* locus (top) and of the *XYP1* locus after transformation with the replacement vector (bottom). The region that was sequenced (Fig. 1) is in black. The position and direction of transcription of the *XYP1* ORF is shown by the arrow. S, *SalI*; B, *BglII*; H, *HindIII*; P, *PstI*. (b) DNA blot comparing wild-type and two *XYP1* replacement transformants (T661-1 and T666-2). DNA was cut with *PstI* or *HindIII* as indicated. The blots were probed with the *XYP1* *BglII* fragment (panels on left-hand side), a cDNA copy of *hph* (middle panels) or the complete *XYP1* 5.0 kb *SalI* genomic fragment (panels on right-hand side). Fragment sizes are indicated on the left.

Fig. 4. Cation-exchange HPLC fractionation of culture filtrates from wild-type (367-1a) and the *XYP1* mutant T661-1. Columns were run at pH 5.0 (a) or pH 4.0 (b). ●, 367-1a; △, T661-1. One unit of xylosidase activity is defined as the amount producing 1 μ g *p*-nitrophenol $\text{min}^{-1} \text{ml}^{-1}$.

peak of β -xylosidase activity in the void fraction (Fig. 4a). When the cation-exchange column was run at pH 4 rather than pH 5, the residual activity was retained on the column and was eluted as two small peaks, 7 and 10 min before the major activity attributable to Xyp1 (Fig. 4b). We conclude that *C. carbonum* must have at least one additional gene that encodes another extracellular enzyme with β -xylosidase activity.

Pathogenicity of the *xyp1* mutant

We compared wild-type and mutant strains on susceptible maize cultivars (genotype *hm1/hm1*) in the greenhouse. Lesion size, lesion morphology and rate of lesion development were examined daily for 10 d. No difference in lesion morphology could be detected between the wild-type and two independent transformants tested. Thus we conclude that *XYP1* is neither essential for pathogenicity nor a major virulence factor. Since *C. carbonum* has at least one additional gene encoding a β -xylosidase, it is not possible to conclude conclusively that β -xylosidase has no role in pathogenicity or virulence.

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