A modular cinnamoyl ester hydrolase from the anaerobic fungus *Piromyces equi* acts synergistically with xylanase and is part of a multiprotein cellulose-binding cellulase–hemicellulase complex

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A collection of clones, isolated from a *Piromyces equi* cDNA expression library by immunoscreening with antibodies raised against affinity purified multienzyme fungal cellulase–hemicellulase complex, included one which expressed cinnamoyl ester hydrolase activity. The *P. equi* cinnamoyl ester hydrolase gene (*estA*) comprised an open reading frame of 1608 nt encoding a protein (EstA) of 536 amino acids and 55 540 Da. EstA was modular in structure and comprised three distinct domains. The N-terminal domain was closely similar to a highly conserved non-catalytic 40-residue docking domain which is prevalent in cellulases and hemicellulases from three species of anaerobic fungi and binds to a putative scaffolding protein during assembly of the fungal cellulase complex. The second domain was also not required for esterase activity and appeared to be an atypically large linker comprising multiple tandem repeats of a 13-residue motif. The C-terminal 270 residues of EstA contained an esterase catalytic domain that exhibited overall homology with a small family of esterases, including acetylxyloxy esterase D (XYLD) from *Pseudomonas fluorescens* subsp. *cellulosa* and acetylxyloxy esterase from *Aspergillus niger*. This region also contained several smaller blocks of residues that displayed homology with domains tentatively identified as containing the essential catalytic residues

of a larger group of serine hydrolases. A truncated variant of EstA, comprising the catalytic domain alone (EstA'), was expressed in *Escherichia coli* as a thioredoxin fusion protein and was purified to homogeneity. EstA' was active against synthetic and plant cell-wall-derived substrates, showed a marked preference for cleaving 1 → 5 ester linkages between ferulic acid and arabinose in feruloylated arabino-xylo-oligosaccharides and was inhibited by the serine-specific protease inhibitor aminoethylbenzene-sulphonylfluoride. EstA' acted synergistically with xylanase to release more than 60 % of the esterified ferulic acid from the arabinoxylan component of plant cell walls. Western analysis confirmed that EstA is produced by *P. equi* and is a component of the aggregated multienzyme cellulase–hemicellulase complex. Hybrid proteins, harbouring one, two or three iterations of the conserved 40-residue fungal docking domain fused to the reporter protein glutathione S-transferase, were produced. Western blot analysis of immobilized *P. equi* cellulase–hemicellulase complex demonstrated that each of the hybrid proteins bound to a 97 kDa polypeptide in the extracellular complex.

Key words: dockerin, enzyme complex, esterase, *Piromyces*.

INTRODUCTION

Esterified hydroxycinnamic acids, notably ferulic and *p*-coumaric acids, occur widely in the cell walls of plants: ferulic acid for example accounts for up to 3 % of cell wall dry mass in maize bran [1], 1 % in sugar-beet pulp [2] and 1 % in wheat bran [3]. In graminaceous plants, hydroxycinnamic acids are mainly found esterified to the *O*-5 position of the arabinose substituents in arabinoxylan [4,5]. In dicotyledons, such as spinach and sugar beet, ferulic acid is esterified to the *O*-2 or *O*-3 position of arabinose and to the *O*-6 position of galactose in pectic polysaccharides [6–8]. Characterization of the products of chemical and enzymic hydrolysis of plant cell walls has established that hydroxycinnamic acids contribute to the structural integrity of the cell wall by cross-linking between hemicellulose chains and between lignin and hemicellulose [9–12]. In wheat straw for example, 93 % of *p*-coumaric acid residues are linked to lignin via ester bonds and 35–75 % of the ferulic acid residues are

linked to lignin via ether bonds [9]. Formation of diferulic acid dimers results in covalent cross-linking between hemicelluloses in cereal cell walls [10,13,14]. Cross-linking of maize cell wall polymers by esterified hydroxycinnamic acids, in particular by diferulate cross-linkages, has recently been proven to be a limiting factor during enzymic dissolution of plant cell walls [15]. Indeed, both the rate and extent of cell wall hydrolysis appear to decrease with an increased concentration of diferulate cross-links.

Based on detailed studies of a few species, anaerobic chytridiomycete fungi, which inhabit the gastrointestinal tracts of ruminant and non-ruminant herbivores, are believed to digest plant cell walls by producing an extensive repertoire of extracellular enzymes that includes not only glycosyl hydrolases, which cleave the glycosidic linkages in cellulose and hemicelluloses, but also esterases, which release esterified acetic and hydroxycinnamic acids from cell walls [16,17]. Two feruloyl esterases (FAE-I and FAE-II) and an inducible *p*-coumaroyl esterase (CAE) have been purified from culture supernatant of

Abbreviations used: AEBSF, aminoethylbenzenesulphonylfluoride; Ara₂F, *O*-[2-*O*(E)-(1-feruloyl)- α -L-arabinofuranosyl]-(1 → 5)-L-arabinose; CAE, *p*-coumaroyl esterase; FAE, feruloyl esterase; GST, glutathione S-transferase; IPTG, isopropyl β -D-thiogalactopyranoside; LB, Luria–Bertani; FAXX, *O*-[5-*O*-(E)-feruloyl]- α -L-arabinofuranosyl]-(1 → 3)-*O*- β -D-xylopyranosyl-(1 → 4)-D-xylose; PAXX, *O*-[5-*O*-(E)-*p*-coumaroyl]- α -L-arabinofuranosyl]-(1 → 4)-D-xylose; PHA, polyhydroxyalkanoate.

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the anaerobic fungus *Neocallimastix* strain MC-2 [18–21]. CAE, a dimeric protein 11 kDa in size, released *p*-coumaric acid from *O*-{5-*O*-[(*E*)-*p*-coumaroyl]- α -L-arabinofuranosyl}-(1 $\rightarrow$ 4)-D-xylose (PAXX), and was 100 times more active against this cell-wall-derived substrate than against the corresponding feruloyl ester (FAXX) (*O*-{5-*O*-[(*E*)-feruloyl]- α -L-arabinofuranosyl}-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylose). Release of *p*-coumaroyl groups from finely ground plant cell walls by CAE was greatly enhanced by the addition of xylanase alone or a mixture of cell-wall-degrading enzymes [18]. The feruloyl esterases FAE-I and FAE-II were 69 kDa and 24 kDa respectively in size. Both enzymes released ferulic acid from FAXX, but were less active against finely divided plant cell walls; FAE-I was also active against PAXX [19].

In the wider context, biochemical analysis of the plant cell wall degrading enzyme systems of anaerobic fungi has shown that the multiple endoglucanases, xylanases and mannanases produced by these organisms form multiprotein cellulose-binding complexes analogous to the cellulosomes produced by *Clostridium thermocellum* and other anaerobic bacteria [21–25]. Structure–function analysis of individual cellulases and hemicellulases from *Neocallimastix patriciarum*, *Piromyces equi* and *Orpinomyces* MC-2 has revealed the following general features: (i) the enzymes exhibit modular architecture, being composed of discrete catalytic and non-catalytic domains joined by hinges or linkers; (ii) cellulose-binding domains, which are prevalent in cellulases and hemicellulases from aerobic microorganisms are, as a rule, absent from the fungal enzymes; (iii) a 40-residue non-catalytic domain, present in two or three copies, is highly conserved in the fungal enzymes and appears to facilitate formation of the multienzyme cellulase complex by binding to a 100 kDa scaffolding protein [26–30].

Although biochemical studies have shown that cinnamoyl ester hydrolases play a significant role in the hydrolysis of plant cell walls by some anaerobic fungi, it is not clear that they are produced by all species. It is also unclear whether they conform to the same pattern of architecture established for other fungal plant cell wall hydrolases, or if they are part of the multiprotein cellulose-binding cellulase complex which is characteristic of these organisms. To address these questions we have sequenced for the first time a cinnamoyl ester hydrolase gene from the anaerobic fungus *P. equi*, and we report here the properties and structure–function relationship of the encoded enzyme. We also show that a putative docking domain, normally found in multiple copies in cellulases and hemicellulases from anaerobic fungi, is present as only a single copy in the fungal esterase, but nevertheless binds to a putative scaffolding protein identified previously as an important component of the multienzyme fungal complex.

MATERIALS AND METHODS

Microbial strains, vectors and culture conditions

P. equi, isolated from horse caecum [31] and described by Munn [32], was cultured under anaerobic conditions in medium [33] containing soluble oat spelt xylan (0.1 %, w/v) and Sigmacell (0.5 %, w/v) (both from Sigma) as carbon sources. *Escherichia coli* strains JM83 [34], XL1-Blue (Stratagene) and BL21(DE3):plysS (Novagen) were cultured in Luria–Bertani (LB) broth. Antibiotics (ampicillin 100 μ g/ml, chloramphenicol 25 μ g/ml) were added as appropriate to select transformants, and isopropyl β -D-thiogalactopyranoside (IPTG; 0.5 mM) was used to induce expression of genes under the control of *lacO*. Plasmid vectors

used were as follows: pBluescript (Stratagene), pET32a (Novagen), pGEX-4T-2 (Pharmacia).

Library construction and screening

A cDNA library was constructed using mRNA extracted from *P. equi* grown in medium containing crystalline cellulose (Avicel) and soluble oat spelt xylan [24]. Recombinant phages were screened for the production of proteins immunoreactive with antibodies raised against the multiprotein cellulose-binding cellulase–hemicellulase complex produced by *P. equi* [24]. Antibody screening of phage plaques was carried out essentially as recommended in the manual accompanying the picoBlue™ immunoscreening kit (Stratagene), with the following modifications: IPTG (0.33 mM) was added directly to soft agar overlays containing recombinant λ ZAPII and host bacteria (*E. coli* XL1-Blue); plaques were lifted onto Hybond-C filters (Amersham); blocking solution contained dried milk powder (4 %, w/v) in place of BSA; anti-rabbit IgG (whole molecule) conjugated to horseradish peroxidase (Sigma) and diluted 1:4000 was used as secondary antibody; the colour development solution was 3,3'-diaminobenzidine (0.5 mg/ml) in 50 mM Tris/HCl buffer, pH 7.4, containing 5 mM hydrogen peroxide. Primary positives were subjected to successive rounds of plaque purification.

Other DNA methods

RNA was isolated from *P. equi* as described previously [26]. Plasmid and λ phage DNA were purified using Qiagen columns. Digestion of DNA with restriction endonucleases, ligation of DNA with T4 DNA ligase, agarose gel electrophoresis and transformation of *E. coli* were carried out as described [35]. DNA sequencing was carried out in an ABI 373 automated DNA sequencer, using the PRISM™ dye-deoxy terminator sequencing kit (Applied Biosystems Instruments). Nested deletions of the esterase cDNA in plasmid pBPA51 were generated by digesting with exonuclease III and S1 nuclease. Custom-made primers were used where necessary to complete the sequences of both strands. Northern hybridizations were performed as described previously [27,36].

High-level expression and purification of cinnamoyl ester hydrolase, EstA

The region of the fungal esterase cDNA encoding the catalytic domain of EstA was amplified by PCR using primers containing the appropriate restriction enzyme sites at the 5'- and 3'-ends respectively. The reaction (50 μ l) contained 50 mM Tris/HCl buffer, pH 9.0, 50 mM NaCl, 10 mM MgCl₂, 200 μ M of dNTPs, 50 pmol of each primer, 250 ng of template DNA (pBPA51) and 2 units of *Thermus aquaticus* DNA polymerase (MBI Fermentas, Munich, Germany). After overlaying with mineral oil, the reaction was subjected to 20 cycles of 30 s at 94 °C, 45 s at 50 °C and 1 min at 72 °C. The primers used to amplify the esterase gene were as follows: 5'-end: 5'-CGCGGATCCAACAGCGGTCC-AACTGTTG-3'; 3'-end: 5'-GCGAATTCTTATCTTATGGG-AGAGAG-3'. The amplified DNA was digested with *Bam*HI and *Eco*RI and ligated to *Bam*HI/*Eco*RI-restricted pET32a (Novagen Inc.) to generate pIF1.

E. coli BL21 (DE3):plysS, harbouring pIF1, was cultured to mid-logarithmic phase ($A_{600} = 0.5$) at 30 °C in LB broth containing ampicillin (100 μ g/ml) and chloramphenicol (25 μ g/ml). IPTG (0.5 mM) was added and, after a further 2 h incubation at 30 °C, cells were harvested, disrupted by ultrasonication in buffer

containing 50 mM NaH_2PO_4 , 10 mM Tris/HCl, pH 8.0, and 100 mM NaCl and a cell-free extract was obtained by centrifuging (20000 g; 20 min). Hybrid protein comprising the catalytic domain of EstA (EstA') appended to thioredoxin was purified by nickel affinity chromatography on TALONTM resin (Clontech Laboratories Inc.), according to the manufacturer's protocol. Bound fusion protein was cleaved from the affinity resin during 2 h at room temperature using restriction-grade thrombin (Sigma) at a concentration of 0.5 units per mg of fusion protein and under the recommended buffer conditions (Novagen Inc.). EstA' contained in the column eluent was dialysed against 10 mM Tris/HCl, pH 8.0, applied to a MonoQ column (1 ml; Pharmacia) which had been equilibrated with 10 mM Tris/HCl, pH 8.0, and the column was eluted at 1 ml/min with a linear gradient containing from 0 to 0.5 M NaCl in 10 mM Tris/HCl, pH 8.0. Purified EstA' with a molecular mass of 37 kDa was eluted at between 0.2 M and 0.25 M NaCl and was dialysed against 10 mM Tris/HCl, pH 8.0.

Assays and substrates

Esterase activity was routinely determined by measuring the release of 4-nitrophenol at 420 nm and 37 °C from 4-nitrophenyl acetate (1 mM; Sigma) in a 1 ml reaction volume containing 12 mM citric acid/50 mM Na_2HPO_4 buffer, pH 6.5. Activity against the 1-naphthyl esters of acetic, butyric, octanoic, hexanoic and dodecanoic acids (Sigma) was determined as described [37]. Release of acetic acid from chemically acetylated birchwood xylan was determined as described previously [38]. Activity against the methyl esters of hydroxycinnamic acids was determined as follows: methyl esters of 4-hydroxy-3-methoxycinnamic (ferulic), 3,4-dihydroxycinnamic (caffeic), 4-hydroxycinnamic (*p*-coumaric) and 3,5-dimethoxy-4-hydroxycinnamic (sinapic) acids at a final concentration of 1 mM in 100 mM Mops buffer, pH 6.0, containing 0.02% sodium azide, were incubated at 37 °C with purified EstA' (44 ng). Reactions were terminated by boiling and the free acid liberated was measured by reverse-phase HPLC [2]. Activity against substituted oligosaccharides containing ester-linked ferulic acid was determined as described [39]. FAXX and Ara₂F (*O*-[2-*O*(E)-(1-feruloyl)- α -L-arabinofuranosyl]-(1 → 5)-L-arabinose) were prepared from destarched wheat bran and sugar-beet pulp respectively according to published methods [8,40]. For measuring K_m and V_{max} , FAXX was used at concentrations varying from 3.7 μM to 49.2 μM and Ara₂F from 4.5 μM to 122.9 μM . Assays were carried out at 37 °C and pH 6.0 in 100 mM Mops buffer with 90 ng of purified EstA'. The release of ferulic acid was measured at 335 nm, as at this wavelength and pH 6.0 ferulic acid and ferulic acid esters have molar absorption coefficients of 14000 and 24000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ respectively [39]. The pH and temperature dependence of EstA' activity were measured with FAXX as substrate at a final concentration of 33 μM . pH was varied between 3 and 9 at a fixed temperature of 37 °C, using citric acid/ Na_2HPO_4 (pH 3–7) and KCl/boric acid (pH 8–9) buffers. The temperature was varied from 20 °C to 70 °C using a spectrophotometer with a temperature-controlled cuvette holder; assays were carried out at pH 6.0 in 100 mM Mops buffer. Cell-free extracts, prepared by sonication from recombinant strains of *E. coli* XL1-Blue containing *Piromyces* cDNAs cloned in pBlue-script, were screened for esterase activity using 4-nitrophenyl acetate as substrate. Qualitative measurement of esterase activity was carried out by spotting cell-free extract onto buffered agarose containing 4-methyl-umbelliferoyl (*p*-trimethylammonium cinnamate chloride) [MUTMAC (Sigma); 20 $\mu\text{g}/\text{ml}$] and moni-

toring the production of a fluorescent product under long-wavelength UV light [41].

Inhibition by aminoethylbenzenesulphonylfluoride (AEBSF)

EstA' (10 $\mu\text{g}/\text{ml}$) was treated with a specific inhibitor of serine hydrolases, AEBSF (up to 10 mM final concentration), in 100 mM Mops buffer, pH 6.0. After appropriate time intervals at 4 °C, aliquots of the treated enzyme were assayed for residual esterase activity against methyl ferulate, using the spectrophotometric method described above.

Generation of glutathione S-transferase (GST) fusion proteins

The region of the fungal esterase cDNA encoding the 40-residue docking domain was amplified by PCR using primers containing the appropriate restriction enzyme sites at the 5'- and 3'-ends respectively. Reactions were performed essentially as described above. After overlaying with mineral oil, the reaction was subjected to 30 cycles of 30 s at 90 °C, 45 s at 60 °C and 1 min at 72 °C. The primers used to amplify the dockerin domain from EstA were as follows: 5'-end: 5'-ACGCGTGCACCTTGTTGG-TCAGAAACATTAGC-3'; 3'-end: 5'-ATAAGAATGCGGC-CGCTTAGATACCACACAGTCATTATTTTCAAC-3'. The amplified DNA was digested with *NotI* and *SaI* and ligated into *NotI*/*SaI*-restricted pGEX-4T-2 to generate pGEX-1doc.

The region of a second fungal cDNA encoding three iterations of the 40-residue docking domain from a protein of unknown function was similarly amplified by PCR. Reaction conditions were identical with those above, except that annealing was performed at 25 °C for 90 s followed by a gradual increase in reaction temperature to 72 °C at a rate of 5 °C/min. The primers used to amplify the required region were as follows: 5'-end: 5'-TTTATAAGCCAAGCCGCGTGCTGGTCTGAATCT-CAGGGTTTTAAATG-3'; 3'-end: 5'-AACAGTTTCTGCGG-CCGCTTAAAGACCACCAATCACCATTTCGAAAC-3'. The amplified DNA was cloned into the PCR cloning vector pCR Script (Stratgene), digested with *SfiI* and *NotI* and ligated into *SfiI*/*NotI*-restricted pGEX-4T-2 (modified by Dr. P. J. White, Laboratory of Molecular Enzymology, the Babraham Institute, Cambridge, U.K., to incorporate restriction sites for *SfiI* and *NotI*) to generate pGEX-3doc. A recombinant plasmid (pGEX-2doc), directing the expression of a GST fusion protein containing two iterations of the dockerin domain normally present in *N. patriciarum* XylA, had been generated previously [28] and was used in this study.

E. coli JM83, harbouring pGEX-1doc, pGEX-2doc or pGEX-3doc, were cultured to mid-logarithmic stage ($A_{600} = 0.5$; for *E. coli* JM83 harbouring pGEX-1doc) or late-logarithmic stage ($A_{600} = 1.0$; for *E. coli* JM83 harbouring pGEX-2doc or pGEX-3doc) at 37 °C in LB broth containing ampicillin (50 $\mu\text{g}/\text{ml}$). IPTG (1 mM) was added and, after a further 2 h, 0.5 h or 1 h incubation (pGEX-1doc, pGEX-2doc, pGEX-3doc respectively), cells were harvested, disrupted by ultrasonication in PBS and a cell-free extract was obtained by centrifugation (20000 g; 20 min). Hybrid proteins were purified by affinity chromatography on glutathione-Sepharose CL-4B (Pharmacia) according to the manufacturer's protocol. Bound fusion protein was eluted with reduced glutathione (10–50 mM in 10 mM Tris/HCl, pH 8.0). When necessary, Triton X-100 (0.1%, v/v) and NaCl (400 mM) were added to promote more efficient release. The hybrid proteins harbouring one, two or three iterations of the conserved 40 amino acid motif appended to the reporter construct GST were designated GST-1DOC, GST-2DOC and GST-3DOC respectively.

<i>N. patriciarum</i> CelBII	392	C F S	- - V N L G Y S C C	- N - G C E V E Y T D S D G E W G V E N G N W C G I K
<i>N. patriciarum</i> CelBII	437	C W S	- - E K L G Y P C C	Q N - T S S V V Y T D N D G K W G V E N G N W C G I Y
<i>P. equi</i> ManAI	492	C W S	- I - N L G Y P C C	I G - D Y - V V T T D E N G D W G V E N N E W C G I V
<i>P. equi</i> ManAII	311	C W S	- - E P L G Y P C C	V G - N T - V I S A D E S G D W G V E N N E W C G I V
<i>P. equi</i> ManAIII	570	C W A	- - E F L G Y P C C	V G - N T - V I S T D E F G D W G V E N D D W C G I L
<i>P. equi</i> XylAI	286	C P S	T I T S Q G Y K C C	S S - N C D I I Y R D Q S G D W G V E N D E W C G C G
<i>P. equi</i> XylAII	333	C P S	S I K N Q G Y K C C	S D - S C E I V L T D S D G D W G I E N D E W C G C G
<i>N. patriciarum</i> XylAI	524	C S A	R I T A Q G Y K C C	S D P N C V V Y Y T D E D G T Q G V E N N D W C G C G
<i>N. patriciarum</i> XylAII	567	C S S	K I T S Q G Y K C C	S D P N C V V F Y T D D D G K W G V E N N D W C G C G
<i>P. equi</i> EstA	22	C W S	- - E R L G W P C C	S D S N A E V T Y V D D D G D W G V E N N D W C G I Q

Figure 4 Comparison of the amino acid sequence (between residues 22 and 59) of EstA from *P. equi* with the sequence of a non-catalytic 40-residue domain reiterated in cellulase B and XYL A from *N. patriciarum* and MANA from *P. equi*

Regions containing identical residues or conservative substitutions are boxed.

source produced extracellular enzymes, albeit at an apparently low level, which released ferulic, caffeic and *p*-coumaric acids from the respective methyl esters (up to 22 m-units of esterase activity/mg of protein). Furthermore, these enzymes appeared to be located in the multienzyme cellulose-binding cellulase complex, which typically gave esterase activities varying from 15 m-units to 90 m-units per mg of protein, depending on the substrate used.

Isolation and characterization of esterase cDNAs

A *P. equi* cDNA library comprising some 10^7 clones was screened for production of proteins immunoreactive with anti-cellulase complex antiserum. Two hundred positive clones were isolated from a pool of approx. 3×10^4 clones; for each positive clone, the fungal cDNA was excised and rescued as a recombinant of pBluescript SK⁻ and transformed into *E. coli* XL1-Blue. Cell-free extracts from all 200 clones were screened for esterase activity, and one was shown to produce activity that hydrolysed MUTMAC, but was inactive against carboxymethylcellulose, oat spelts, xylan, *p*-nitrophenyl β -D-cell obioside, *p*-nitrophenyl β -D-glucoside (all Sigma) or carob galactomannan (Megazyme). A restriction map of the cDNA cloned in the plasmid, designated pBPA51, is shown in Figure 1. In Northern blots, a probe comprising the 827 bp internal fragment between nt 784 and 1611 of the esterase cDNA (designated *estA*) hybridized to a 2300 nucleotide mRNA species produced during growth of *P. equi* in medium containing Sigmacell and soluble xylan (Figure 2).

Nucleotide sequence of *estA*

Translation of the nucleotide sequence of the *estA* cDNA revealed a single open reading frame of 1608 bp encoding a polypeptide of 536 amino acids with a predicted molecular mass of 55540 Da. The nucleotide sequence of *estA* and the deduced primary structure of the encoded protein, designated esterase A (EstA), are shown in Figure 3. Identification of the translation initiation codon was based on: (i) the presence of stop codons in all three reading frames upstream of the proposed ATG start codon; (ii) the absence of other ATG codons upstream of the open reading frame; and (iii) the observation that sequences 5' and 3' of the proposed protein-coding region were extremely rich in A and T nucleotides, a feature which is highly characteristic of the non-coding regions of anaerobic fungal cDNAs.

Structure of EstA

EstA from *P. equi* was modular in structure and conformed to a pattern of architecture described previously for the cellulases and

hemicellulases from anaerobic fungi. Examination of the primary sequence of EstA revealed a typical hydrophobic *N*-terminal signal sequence followed by a number of interesting and novel features, the first of these being a region of 38 amino acids spanning residues 22–59. A domain highly homologous with this region has been described previously in other plant cell-wall-degrading enzymes from anaerobic fungi (Figure 4). Tandem reiterated copies were present in xylanases from *N. patriciarum* [26], *P. equi* [28] and *Orpinomyces* strain PC-2 [30] and in endoglucanases from *N. patriciarum* [27] and *Orpinomyces* [30]; three iterations of the domain are present in a mannanase from *P. equi* [28]. Functional analysis of the duplicated domain from *N. patriciarum* XYLA confirmed that it was not required for catalytic activity and did not bind to cellulose. However, a fusion protein comprising the repeated domain joined to GST bound specifically to 97 kDa and 116 kDa polypeptides that are components of the cellulase complexes of *P. equi* and *N. patriciarum* respectively [28]. Following on from the putative protein docking domain, EstA contained 12 repeats of a 13-residue motif, NQGGGMPWGDFGG.

The remaining 270 residues of EstA between the middle domain and the C-terminus displayed 29.5% identity with a catalytically active fragment of the acetylxyylan esterase, XYLD, from *Pseudomonas fluorescens* subsp. *cellulosa* [42] (Figure 5). The same region of EstA displayed significant sequence identity with an acetylxyylan esterase from *Aspergillus niger* and an open reading frame of unknown function from *Mycobacterium leprae* and was assumed to constitute the catalytic domain of the fungal enzyme.

Homologies between EstA and the primary amino acid sequences of other phenolic acid esterases are less obvious. The distances between the active-site serine in the motif GHSLG and the putative aspartate and histidine catalytic residues of the *A. niger* ferulic acid esterase FaeA and a number of lipases were determined to be 55–61 and 53–60 residues respectively [43]. Although the putative serine-containing motif from EstA differs slightly (GFSMG), the distances separating the remaining triad components are comparable. The distances from S394 to D447 being 52 residues, and from D447 to H517, 70 residues. However, alignment of EstA with these sequences revealed no significant primary structure homology.

When the catalytic domain of EstA was screened against sequence databases using the BLAST algorithm [44], limited homology to a number of enzymes, including the xylanase XynZ from *C. thermocellum* [45], polyhydroxyalkanoate (PHA) depolymerases from *Pseudomonas lemoignei* [46] and *Alcaligenes faecalis* [47] was observed. These latter enzymes are members of a diverse family of Ser/Asp/His active-site hydrolases containing the cinnamoyl ester hydrolases CinA and CinB from *Butyrivibrio*

A

<i>A. niger</i> AxeA	49	Y	I	Y	V	-	P	N	N	-	L	A	S	N	P	-	G	I	V	V	A	I	H	Y	C	T	G	T	G	P	G	Y	S	A	S	P	Y	A	T	L	S	E	Q	Y	G	F	I	V	I	Y	
<i>M. leprae</i> ORF	43	V	L	H	V	P	P	G	-	-	-	-	S	P	V	G	L	V	L	N	L	H	G	G	G	T	G	A	G	Q	Q	G	L	T	D	F	D	A	V	A	D	L	N	N	L	L	V	A	Y		
<i>P. fluorescens</i> XYLD	347	Y	I	L	D	A	P	G	N	Y	N	N	K	P	Y	R	L	V	I	G	Y	H	W	R	G	T	A	Q	D	V	H	T	G	Q	T	V	Q	R	D	A	W	A	H	Y	G	M	K	K	L		
<i>P. equi</i> EstA	294	V	I	V	K	F	P	S	G	F	T	G	D	K	A	A	P	L	L	I	N	Y	H	P	I	M	G	S	A	S	Q	W	E	S	G	S	Q	T	A	K	A	A	L	N	D	G	A	I	V	A	F

<i>A. niger</i> AxeA	P	S	S	P	Y	S	-	-	-	-	-	-	-	-	-	-	-	G	G	C	W	D	V	S	S	Q	A	T	L	T	H	N	G	G	N	S	N	S	I	A	N	M	V	T	W	T	I				
<i>M. leprae</i> ORF	P	D	G	Y	D	K	S	W	A	D	G	R	G	A	S	P	A	D	R	R	H	V	D	D	V	G	F	L	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. fluorescens</i> XYLD	A	G	D	S	T	I	F	V	A	P	Q	G	I	G	N	G	W	G	N	G	G	A	D	V	T	F	T	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. equi</i> EstA	M	D	G	A	Q	G	P	M	G	Q	A	W	N	V	G	P	C	-	C	T	D	A	D	D	V	Q	F	T	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

<i>A. niger</i> AxeA	S	E	Y	G	A	D	S	S	K	V	F	V	T	G	S	S	S	G	A	M	L	T	N	V	M	A	A	T	Y	P	E	L	F	A	A	A	T	V	Y	S	-	G	V	S	A	G	C	F	Y	-
<i>M. leprae</i> ORF	S	D	F	G	V	A	P	G	H	V	F	V	T	G	M	S	N	G	G	F	M	S	N	K	L	A	C	D	R	A	D	M	F	A	A	I	A	P	V	-	A	G	T	L	G	V	D	V	A	-
<i>P. fluorescens</i> XYLD	T	E	L	C	I	D	E	S	R	V	F	A	N	G	F	S	F	G	G	G	M	S	Y	A	I	A	C	A	R	A	N	V	F	K	A	V	A	V	Y	S	A	G	V	I	S	G	C	-	-	-
<i>P. equi</i> EstA	S	K	A	C	V	D	P	K	R	I	Y	A	A	G	F	S	M	G	G	G	M	S	N	Y	A	G	C	Q	L	A	D	V	I	A	A	A	A	P	S	A	F	D	L	A	K	E	I	V	D	G

<i>A. niger</i> AxeA	-	-	S	N	T	N	Q	V	D	G	W	N	S	T	C	A	Q	G	D	V	I	T	T	P	E	H	W	A	S	I	A	E	A	M	Y	S	G	Y	S	G	S	R	P	R	M	Q	I	Y	H	G	
<i>M. leprae</i> ORF	-	-	C	H	P	S	Q	P	V	S	V	M	D	A	H	G	T	D	D	P	L	V	P	F	N	G	G	D	V	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. fluorescens</i> XYLD	-	-	S	G	A	N	S	P	I	A	Y	F	G	A	H	G	L	G	D	S	V	L	G	I	S	G	G	R	S	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. equi</i> EstA	G	K	C	K	P	A	R	P	F	P	I	L	N	F	R	G	T	Q	D	N	V	V	M	Y	N	G	G	L	S	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

<i>A. niger</i> AxeA	T	L	H	T	T	L	Y	P	Q	N	Y	E	T	C	K	Q	W	S	G	V	F	G	Y	D	Y	S	A	P	E	K	T	E	A	N	T	P	Q	T	N	Y	E	T	-	-	-	-	-	-	-		
<i>M. leprae</i> ORF	-	-	-	G	R	G	V	S	H	V	S	V	T	N	M	V	E	K	W	R	T	A	D	G	C	Q	G	D	P	S	Q	Q	E	L	P	D	V	G	D	G	T	-	F	V	R	R	F	D			
<i>P. fluorescens</i> XYLD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>P. equi</i> EstA	-	-	-	V	V	Q	G	K	P	I	T	F	M	G	A	K	N	N	F	K	E	W	A	K	M	N	G	C	T	G	E	P	K	Q	N	T	P	G	N	-	-	-	-	-	-	-	-	-	-	-	-

<i>A. niger</i> AxeA	-	-	-	T	I	W	G	D	S	L	Q	G	I	F	A	T	G	V	G	H	T	V	P	I	H	G	D	K	D	M	E	W	F	G	F	A	304
<i>M. leprae</i> ORF	Y	T	V	C	A	N	S	A	E	V	V	F	Y	K	I	N	K	G	G	H	T	W	P	G	G	K	Q	Y	L	P	K	V	V	I	G	S	281
<i>P. fluorescens</i> XYLD	S	Y	V	C	P	N	R	Q	Y	P	V	R	W	C	A	F	D	G	G	H	D	A	S	P	L	D	N	G	V	G	Q	K	G	Y	T	W	571
<i>P. equi</i> EstA	Y	E	N	C	K	G	V	K	V	G	L	C	T	I	N	G	G	H	A	E	G	D	G	K	M	G	W	D	F	V	K	Q	F	S	534		

B

<i>B. pickettii</i> PHA	75	L	V	L	V	L	H	G	C	L	Q	T	A	S	G	(75)	V	Y	V	A	G	L	S	S	G	G	G	M	T	(60)	A	G	A	V	W	G	T	S	D	Y	T	V	(50)
<i>P. lemoignei</i> PHAC	67	L	V	V	A	L	H	G	C	T	Q	T	A	A	A	(67)	V	Y	V	T	G	L	S	A	G	A	F	M	T	(63)	I	S	I	W	H	G	S	S	D	Y	T	V	(49)
<i>A. fecalis</i> PHA	71	L	V	L	V	L	H	G	C	V	Q	T	A	S	G	(75)	V	Y	V	A	G	L	S	S	G	G	G	M	T	(60)	A	G	A	V	W	G	T	S	D	Y	T	V	(62)
<i>M. tuberculosis</i> ORF	44	L	V	I	S	L	H	G	G	G	T	G	A	G	(65)	V	F	A	T	G	M	S	N	G	G	F	M	S	(34)	V	L	E	A	H	G	T	A	D	P	L	V	(68)	
<i>C. thermocellum</i> XynZ	83	V	L	Y	L	L	H	G	I	G	S	E	N	D	(68)	R	A	T	A	G	L	S	M	G	G	G	Q	S	(43)	L	F	I	A	C	G	T	N	D	S	L	I	(21)	
<i>A. niger</i> AxeA	62	I	V	V	A	T	H	Y	C	T	G	T	G	P	G	(66)	V	F	V	T	G	S	S	S	G	A	M	L	T	(34)	W	N	S	T	C	A	Q	G	D	V	I	T	(82)
<i>M. leprae</i> ORF	54	L	V	L	N	L	H	G	G	G	T	G	A	G	(65)	V	F	V	T	G	M	S	N	G	G	F	M	S	(35)	V	M	D	A	H	G	T	D	D	P	L	V	(68)	
<i>P. fluorescens</i> XylID	363	L	V	I	G	Y	H	W	R	G	T	A	Q	D	(65)	V	F	A	N	G	F	S	F	G	G	G	M	S	(33)	Y	F	G	A	H	G	L	G	D	S	V	L	(50)	
<i>P. equi</i> EstA	310	L	L	I	N	Y	H	P	I	M	G	S	A	S	Q	(64)	I	Y	A	A	G	F	S	M	G	G	G	M	S	(38)	I	L	N	F	R	G	T	Q	D	N	V	V	(62)

Block 1

Block 2

Block 3

<i>B. pickettii</i> PHA	V	S	G	M	A	H	A	W	P	A	308
<i>P. lemoignei</i> PHAC	I	T	G	M	G	H	G	T	P	V	294
<i>A. fecalis</i> PHA	V	S	G	M	A	H	A	W	P	A	304
<i>M. tuberculosis</i> ORF	I	D	N	G	G	H	T	W	P	G	259
<i>C. thermocellum</i> XynZ	I	Q	G	G	G	H	D	F	N	V	264
<i>A. niger</i> AxeA	A	T	G	V	G	H	T	V	P	I	292
<i>M. leprae</i> ORF	I	N	K	G	G	H	T	W	P	G	269
<i>P. fluorescens</i> XylID	A	F	D	G	G	H	D	A	S	P	559
<i>P. equi</i> EstA	T	N	G	G	G	H	A	E	G	D	521

Block 1

Block 2

Block 3

Block 4

Figure 5 Comparison of the amino acid sequence (between residues 294 and 534) of EstA from *P. equi* with other esterases

(A) EstA is aligned with the sequences of AxeA from *A. niger*, an unidentified open reading frame from *M. leprae* and XYLD from *P. fluorescens* subsp. *cellulosa*. In (B) the four blocks of conserved sequence in esterases, which contained residues that have been previously demonstrated to be components of the catalytic triad or the oxyanion hole [46,49–51] are compared with the corresponding sequences in EstA. The esterases were PHA depolymerase from *Burkholderia pickettii* [57], PHA depolymerase from *Pseudomonas lemoignei* [46], PHA depolymerase from *A. faecalis* [47], an unidentified open reading frame from *Mycobacterium tuberculosis* (p97180), an unidentified open reading frame from *M. leprae* (g466843) and XYLD from *P. fluorescens* subsp. *cellulosa* [42]. Residues identified as important in catalysis are marked with an asterisk. In both panels regions containing identical residues or conservative replacements are boxed. The numbers denote the positions of the residues within their respective full-length sequences.

fibrisolvens E14 [41,48]. Inspection of the aligned sequences indicated that the homology spanned regions within EstA that contained residues putatively involved in catalysis. Analysis of

the regions identified in each sequence led to the identification of four blocks of conserved sequence which contained residues that have been previously demonstrated to be components of the

Figure 6 Purification of EstA'

EstA' was purified as described in the Materials and methods section. Samples are as follows: lane 1, cell-free extract from *E. coli* BL21 (DE3):plysS; lane 2, cell-free extract from *E. coli* BL21(DE3):plysS harbouring pIF1; lane 3, EstA' purified as a thioredoxin fusion protein by nickel affinity chromatography and released from the resin column by cleavage with thrombin; lane 4, EstA' further purified by anion-exchange chromatography. The positions of molecular-mass standards (kDa) are indicated.

Table 1 Substrate specificity of EstA'

Assays were performed using 33 μ M substrate. In the case of acetylated xylan the acetate groups were at 33 μ M. n.d., no activity detected.

Substrate	Specific activity (units/mg)
Methyl caffeate	51.5
Methyl <i>p</i> -coumarate	50.4
Methyl ferulate	1.26
Methyl sinapate	n.d.*
Acetylated xylan	n.d.*
4-Nitrophenyl acetate	55.7
1-Naphthyl acetate	121
FAXX	51
Ara ₂ F	3.0

catalytic triad or the oxyanion hole [46,49–52] (Figure 5). This last feature is responsible for stabilizing the catalytic intermediates produced during catalysis, including the transiently produced tetrahedral intermediate of the carbonyl carbon. Blocks

1, 2, 3 and 4 contain the putative oxyanion hole, nucleophilic serine, catalytic triad aspartate and histidine residues respectively. No other significant homologies were detected between EstA and the PHA depolymerases.

High-level expression, purification and characterization of EstA

To confirm that the C-terminal half of EstA contains the esterase catalytic domain, the region of the *estA* gene between nt 784 and 1611 was amplified using PCR and ligated into pET32a to produce pIF1. *E. coli* BL21 (DE3):plysS containing pIF1 expressed an inducible thioredoxin fusion protein with a molecular mass of 50000 Da which hydrolysed methyl ferulate and 4-nitrophenyl acetate. Fusion protein expressed during culture and induction at 37 °C was largely included and was found to be insoluble in the cell envelope fraction after sonication of *E. coli* cells; however, culture of the recombinant *E. coli* strain to mid-logarithmic phase at 30 °C, followed by induction with IPTG for 2 h at the same temperature, resulted in the production of a soluble thioredoxin–esterase fusion protein. Purification of the hybrid protein by nickel affinity chromatography, followed by cleavage with thrombin and further purification by anion-exchange chromatography, resulted in a protein with molecular mass of 37000 Da, corresponding to the catalytic domain of EstA (EstA') (Figure 6).

The range of substrates attacked by purified EstA' is depicted in Table 1; no activity was detected against cellulosic or other hemicellulosic substrates. Full-length EstA, contained in cell-free extract from *E. coli* XL1-Blue harbouring pBPA51, displayed activity against the same substrates as the truncated form of EstA comprising the catalytic domain alone (EstA'). At pH 6.0 and 37 °C, EstA', in the presence or absence of 10 units of *Trichoderma viride* xylanase, released only trace amounts of acetic acid from chemically acetylated birchwood xylan (20 mg/ml) during prolonged incubation (up to 16 h); under the same conditions, AxeA from *A. niger* released close to 100 % of the theoretical yield of acetic acid. With the exception of methyl sinapate, the methyl esters of hydroxycinnamic acids were cleaved by EstA'; specific activity against methyl caffeate and methyl *p*-coumarate was similar, but activity against methyl ferulate was lower. EstA' released ferulic acid from FAXX and Ara₂F, confirming its capacity to cleave both 1 → 5 and 1 → 2 ester linkages between ferulic acid and arabinose. The K_m for FAXX was in the low micromolar range, but that for Ara₂F was 20 times higher (Table 2); catalytic efficiency was 65 times higher for FAXX than for Ara₂F, suggesting that the 1 → 5 ester linkage is cleaved preferentially.

Comparison with other reported cinnamoyl ester hydrolases revealed that the catalytic efficiency of *P. equi* EstA' against

Table 2 Comparative activities of *P. equi* EstA' and other esterases against plant cell wall substrates

Enzyme and source	Catalytic parameters					
	FAXX as substrate			Ara ₂ F as substrate		
	K_m (μ M)	V_{max} (units/mg)	V_{max}/K_m	K_m (μ M)	V_{max} (units/mg)	V_{max}/K_m
<i>Piromyces equi</i> EstA'	4.39	56.3	12.8	75.4	14.7	0.20
<i>Aspergillus niger</i> FAE III (ref. [39])	19	114	6.0	No activity detected		
<i>Aspergillus niger</i> CinnAE (ref. [40])	410	14.1	0.034	290	59.1	0.20
<i>Pseudomonas fluorescens</i> subsp. <i>cellulosa</i> XYLD (ref. [42])	560	2.9	0.007	Not determined		

Table 3 Release of ferulic acid from destarched wheat bran [containing 0.55% (w/w) ferulic acid] by *P. equi* EstA'

EstA' (m-units)	Xylanase* (m-units)	Percentage of total ferulic acid released during 3 h at 37 °C
14	—	0.6
140	—	3.0
14	2	63.5

* *Trichoderma viride* xylanase (Megazyme) was electrophoretically homogeneous and devoid of esterase activity.

Figure 7 Temperature (A) and pH (B) dependence of EstA' activity

Assays were carried out as detailed in the Materials and methods section using FAXX as substrate at a concentration of 33 μ M.

FAXX was comparable with that of *A. niger* FAEIII, but was 400-fold and 1000-fold greater than that of *A. niger* cinnamic acid esterase and *P. fluorescens* subsp. *cellulosa* XYLD respectively (Table 2). *P. equi* EstA' and *A. niger* cinnamic acid esterase were equally efficient at cleaving Ara₂F, whereas *A. niger*

Figure 8 Production of EstA by *P. equi*

P. equi was cultured for 48 h in medium containing cellobiose (lane 1) or wheat bran (lane 2) as the source of fermentable substrates. The multiprotein cellulose-binding cellulase-hemicellulase complex, produced during growth on cellobiose [24], and total proteins attached to residual wheat bran were fractionated by SDS/PAGE, electroblotted onto Immobilon-P (Amersham) and probed with anti-EstA' antiserum. The positions of molecular-mass standards (kDa) are shown on the left.

FAEIII was inactive against this substrate. To assess the capacity of *P. equi* EstA to release hydroxycinnamic acids from native plant cell walls, the purified enzyme was incubated with destarched wheat bran at 37 °C and pH 6.0 in the presence and absence of *T. viride* xylanase. EstA' (14 m-units) alone released 0.61 % of the total esterified ferulic acid from 10 mg of destarched wheat bran during 3 h at 37 °C (Table 3); a 10-fold increase in EstA' loading increased the yield of ferulic acid to 3.0 % of maximum. However, in the presence of *T. viride* xylanase (2 units), EstA' (14 m-units) released 63.5 % of total esterified ferulic acid during 3 h at 37 °C. No ferulic dehydromers were detected in the products. The temperature dependence of EstA activity was measured by incubating EstA' with FAXX for 3 min at temperatures ranging from 30 °C to 70 °C. Activity was maximal between 50 and 60 °C (Figure 7A). EstA' cleaved FAXX over a broad pH range: activity was maximal at pH 6.7 and remained at 75 % of its peak value over the pH range 5.8–7.7 (Figure 7B).

Inhibition by AEBSF

AEBSF, an irreversible inhibitor of serine proteases, inhibited the activity of EstA' in a time- and concentration-dependent fashion. At 1 mM AEBSF, EstA' activity, measured with methyl ferulate as substrate, diminished to 5 % of its initial value in 5 h; at 10 mM AEBSF, complete inhibition occurred within 5 h.

Production of EstA by *P. equi*

Isolation of the *estA* cDNA was based on its capacity to specify a protein (EstA) which cross-reacts with an antiserum raised against the *P. equi* multienzyme cellulose-binding cellulase-hemicellulase complex. This suggests that EstA is either a component of the complex or contains an epitope that is conserved in proteins which are part of the complex. We and others [24,29,30] have shown that during culture in medium containing cellulosic substrates, the multienzyme cellulase-hemicellulase complex produced by anaerobic fungi is sequestered onto the surface of residual substrate through its tight binding to cellulose. To establish definitively whether EstA is part of this complex, antiserum raised against the catalytic domain of EstA was used to probe the multienzyme complex

Figure 9 Western blot analysis of *P. equi* (hemi)cellulase complex probed with GST fusion proteins

(A) SDS/10%-PAGE analysis of fractionated proteins stained with Coomassie Blue. (B, C, D) Immobilized proteins preincubated with GST-1DOC, GST-2DOC and GST-3DOC respectively. Blots were developed using the enhanced chemiluminescence kit (NEN-Du Pont). The positions of molecular-mass markers (kDa) are shown on the left. Binding of the GST–dockerin fusion protein to the putative 97 kDa scaffolding protein is depicted by an arrow in (B), (C) and (D).

obtained by affinity purification from cellobiose-grown cultures [24] that remained bound to washed residual substrate during culture of *P. equi* in medium containing wheat bran (0.5%, w/v) as the main carbon source. The results (Figure 8) showed that complex produced during growth of *P. equi* on cellobiose contained two polypeptides of about 55 and 26 kDa which were immunoreactive with anti-EstA' antibodies; complex produced during growth on wheat bran contained a single immunoreactive polypeptide of 55 kDa.

Western blot analysis of *P. equi* cellulase–hemicellulase complex

In order to determine (i) whether one, two and three copies of the 40-residue docking sequence recognize and bind to a ligand contained in the fungal complex and, (ii) to deduce the size of the polypeptide recognized by each, purified GST-1DOC, GST-2DOC and GST-3DOC polypeptides were used to probe proteins contained in the multienzyme complex from *P. equi*. Individual Western blots were each pre-incubated with one of the three GST fusion proteins and bound proteins were subsequently identified. A control blot containing immobilized *P. equi* proteins was preincubated with GST before immunodetection. The results (Figure 9) indicated that each of the GST-DOK fusion proteins recognized a similar-sized polypeptide of about 97 kDa in the cellulase–hemicellulase complex of *P. equi*. No binding of fungal proteins was observed in blots probed with GST alone (results not shown), demonstrating that binding to the putative scaffolding polypeptide of the fungal complex was mediated by the dockerin domain in either one, two or three copies. To establish whether the conserved 40-residue docking domain bound to the scaffolding protein of the *C. thermocellum* cellulosome (CipA), immobilized cellulosome proteins were probed in a manner identical with that described above (results not shown). No interactions between the hybrid proteins and cellulosome components was observed, indicating that the mechanism of interaction between the catalytic and non-catalytic components of bacterial and fungal cellulolytic complexes are likely to be different.

DISCUSSION

Results presented here provide the first description of a cinnamoyl ester hydrolase gene and its encoded protein from an anaerobic

fungus. It has been known for some time that fungi belonging to the genus *Neocallimastix* produce such enzymes and, in addition, acetylxyylan esterases [18–20,41], but this report provides the first evidence that production of cinnamoyl ester hydrolases is not restricted to a single genus.

Analysis of the primary structure of *P. equi* EstA indicates that the enzyme has the same modular architecture as cellulases and hemicellulases from this organism. A distinctive feature of this architecture is the 40-residue domain which is located at the N-terminus of mature EstA and is not required for catalytic activity. This domain is highly conserved in cellulases and hemicellulases from three different species of anaerobic fungus and has been shown to mediate binding of the enzymes to a putative scaffolding polypeptide during formation of a multi-enzyme cellulase complex [26–28,30]. The occurrence of a single copy of the putative docking domain in EstA contrasts with the two or three reiterated copies reported previously in other fungal enzymes and raises the interesting possibility that the number of copies of the domain may influence binding affinity. The results of this study demonstrate that only one copy of the 40-residue dockerin domain is required for efficient binding to the putative 97 kDa scaffolding protein. The results also indicate that the defining characteristic of a catalytic component of the cellulase–hemicellulase complex from anaerobic fungi is the presence of at least one copy of the non-catalytic 40-residue motif.

A second interesting feature of EstA is the domain between residues 74 and 262, which contains 12 repeats of a 13-residue motif. The fact that the full sequence of this domain, together with upstream and downstream flanking sequences, could be obtained from a single sequencing gel suggests that it is not a sequencing artefact. A precise role for the domain has not been established; it is, however, not required for catalytic activity, and a likely role would be as a hinge or linker. Typically linkers separating the different domains of modular plant cell wall hydrolases are in the size range 10–50 residues [53,54]. Unusually large linkers containing multiple repeats of a more or less conserved motif have been reported in xylanase B from *N. patriciarum* (455 residues; [36]) and xylanaseA from *Ruminococcus flavefaciens* (374 residues; [55]).

Though comparatively little is known about the primary structures of the catalytic regions of cinnamoyl ester hydrolases, the sequences of several acetylxyylan esterases, including two from the anaerobic fungus *N. patriciarum*, have been reported [41]. Comparison of these sequences has revealed considerable diversity and has led to the recognition of several different esterase families. One such family comprises the esterase catalytic domain of acetylxyylan esterase D (XYLD) from *P. fluorescens* subsp. *cellulosa* [42], acetylxyylan esterase (AxeA) from *A. niger* (g832903), an *M. leprae* protein of unknown function (g466863) and, based on limited sequence data, probably also an acetylxyylan esterase (AXEI) from *Penicillium purpurogenum* [56]. Our results suggest that EstA from *P. equi* is also a member of this small family of esterases but, unlike other members of the family, has little or no activity against acetylxyylan. In common with other serine hydrolases, EstA would be predicted to contain an active-site Ser-His-Asp/Glu triad; inhibition of EstA by the serine-specific protease inhibitor AEBSF confirms that a serine residue is crucially involved in catalysis by the esterase, and distinguishes EstA from *A. niger* Fae III, which is not inhibited by AEBSF at mM concentrations. Inspection of the aligned sequences of the small family of esterases to which EstA apparently belongs revealed a number of potential candidate residues, including S394, D497 and H517 (Figure 5; EstA numbering). The fact that the active-site serine of several lipases occurs within the motif GXSG [56] supports the tentative identification of S394 as the

nucleophilic serine of EstA. The catalytic domain of EstA also displayed homology with several other unrelated serine hydrolases, primarily over regions identified as containing residues important in catalysis, serving to highlight the conservation of catalytic architecture within serine hydrolases whose native substrates and biological function are markedly different.

Results presented here indicate that EstA is apparently produced by *P. equi* during culture in medium containing cellulose and soluble xylan, cellobiose or wheat bran. A pivotal question that this work sought to address is whether cinnamoyl ester hydrolase activity resides in the multienzyme cellulase-hemicellulase complex produced by *P. equi*. Several pieces of evidence suggest that EstA is indeed part of the aggregated enzyme complex. First, the enzyme contains a single copy of the docking domain, which apparently enables cellulases and hemicellulases that are part of the complex to bind to a putative scaffolding polypeptide. Secondly, antibodies raised against the catalytic domain of EstA cross-react with a 55 kDa protein, which is part of the multienzyme complex that binds tightly to residual substrate during culture of *P. equi* in medium containing wheat, and to affinity-purified complex produced during growth on cellobiose; the size of the immunoreactive polypeptide is consistent with that of the translated *estA* open reading frame. Thirdly, cellulose affinity purified complex produced by *P. equi* in medium containing cellobiose has cinnamoyl ester hydrolase activity associated with it. Taken together, these results suggest that EstA is part of the *P. equi* cellulase-hemicellulase complex and an essential component of the fungal repertoire of plant cell wall degrading enzymes. This view is further supported by biochemical data [2] indicating that EstA acts synergistically with xylanase by cleaving the 1 → 5 ester linkages in feruloylated xylo-oligosaccharides like FAXX, which are generated by the action of xylanases on plant cell walls.

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