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Identification, cloning, and functional expression of three glutathione transferase genes from *Aspergillus fumigatus*

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Abstract

Analysis of the genome of the human pathogen, *Aspergillus fumigatus*, revealed the presence of several putative glutathione transferase (GST) open reading frames. Three *A. fumigatus* GST genes, termed *gstA*, *B*, and *C*, were cloned and recombinant proteins expressed in *Escherichia coli*. Functional analysis of recombinant gstA–C confirms that the enzymes exhibit GST activity and glutathione peroxidase activity. RT-PCR confirmed low basal expression of *gstA* and *gstC* which was markedly up-regulated (at least 4×–10×) in the presence of either H_2O_2 or 1-chloro-2,4-dinitrobenzene (CDNB). *GstB* expression was only observed in the presence of CDNB. These results demonstrate for the first time the existence of three functional GSTs in *A. fumigatus* and strongly suggest a role for these enzymes in the response of the organism to both oxidative stress and xenobiotic presence.

Keywords: GST; MALDI-TOF; Xenobiotic resistance; Oxidative stress; Fungal

1. Introduction

Aspergillus fumigatus is a human pathogenic fungus capable of inducing a range of disease states in patients with pre-existing lung damage or immunosuppression following organ transplantation (Daly and Kavanagh, 2001). Three forms of aspergillosis are recognised clinically: saprophytic, allergic, and invasive, with the latter form having a mortality rate of >90% in some patient groups (Denning, 1998). Conventional therapy relies upon the use of amphotericin B and, more recently, on novel azole derivatives and the echinocandin class of anti-fungal agents, but mortality rates remain high. *A. fumigatus* displays the ability to withstand attack by macrophages and neutrophils and develop in a potentially hostile environment. Toxin-mediated inhibition of oxidative burst in alveolar macrophages and polymorphonuclear leukocytes by conidia and hyphae is well characterised (Bertout et al., 2002; Mitchell et al., 1997; Muryama et al., 1996). In addition, the physical size of developing hyphae prevent phagocytosis by alveolar macrophages and there is emerging evidence that *A. fumigatus* may be able to tolerate entry of xenobiotics as a result of amphotericin B treatment creating apertures in the fungal cell membrane (Ellis, 2002).

Glutathione transferases (GST; EC 2.5.1.18) are dimeric phase II detoxification enzymes with the ability to conjugate a broad range of potentially harmful xenobiotics to glutathione (GSH), thereby rendering them more susceptible to removal from the cell. GSTs have also been shown to exhibit GSH-dependent peroxidase activity and thus may be involved in resistance to oxidative stress. Cytosolic GSTs have been identified in almost all organisms, with mammalian GSTs the most clearly characterised. These enzymes have been implicated in pesticide resistance in plants and insects (Sheehan et al., 2001), and

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some GST polymorphisms are thought to alter cancer susceptibility in mammals (Hayes and Pulford, 1995). GSTs are divided into several classes based upon substrate specificity, sequence similarity (particularly in the N-terminal region which is involved in GSH binding), immunological cross-reactivity and, where available, structure similarity. GST classes include α, μ, π, θ, σ, ζ, ω, and κ classes, with insect specific (δ and ϵ), plant specific (φ and τ) and bacterial (β) classes also described (Sheehan et al., 2001). In addition, it is likely that many more classes have been already characterised exist in the broad ranging GST category; for example, new protozoan and fungal GST classes have been proposed (Cha et al., 2001; Takada et al., 2004).

Until recently, relatively little was known about the presence and role of GST in fungi, however it is now clear that GST isoforms exist in a number of fungal species including *Schizosaccharomyces pombe*, *Aspergillus nidulans*, *Saccharomyces cerevisiae*, *Issatchenkia orientalis*, *Yarrowia lipolytica*, *Cunninghamella elegans*, *Mucor circinelloides*, and *Phanerochaete chrysosporium* (Cha et al., 2001; Choi et al., 1998, 2002; Dowd et al., 1997; Dowd and Sheehan, 1999; Foley and Sheehan, 1998; Fraser et al., 2002; Kim et al., 2001; Tamaki et al., 1999; Shin et al., 2002; Veal et al., 2002). Fungal GSTs exhibit differential expression patterns, with some isoforms shown to be expressed inducibly in the presence of xenobiotics or oxidative stress. For example, of two GSTs identified in *I. orientalis*, only one was constitutively expressed, and both were induced in the presence of *o*-dinitrobenzene (*o*-DNB) (Choi et al., 1998). Three GSTs in *S. pombe* were induced by oxidative stress, and mutants lacking $gst1^+$ and $gst2^+$ or $gst3^+$ were more sensitive to the presence of the anti-fungal drug fluconazole, thereby indicating a role for GST in mediating anti-fungal drug tolerance (Cho et al., 2002; Kim et al., 2001; Shin et al., 2001; Veal et al., 2002).

The identification of a functional theta class GST (gene: *gstA*) in *A. nidulans* has further elucidated the role of GST in fungal metabolism. *GstA* appears to be up-regulated by the presence of either 1-chloro-2,4-dinitrobenzene (CDNB)[2] or H_2O_2 in the culture medium and may also play a role in mediating heavy metal resistance in *A. nidulans* (Fraser et al., 2002).

Given the significance of *A. fumigatus* as a human pathogen and the limited success of anti-fungal agents to treat aspergillosis, particularly in immunocompromised patients, it is surprising that the putative presence and role of GST has merited little attention. In addition, the potential role of fungal GST in allowing *A. fumigatus* to withstand neutrophil attack may represent a key element in the cell's ability to survive in the host and colonise pulmonary tissue. Here we describe the identification, cloning, heterologous expression and characterisation of three GST genes from *A. fumigatus*. We also investigate the response of GST gene expression following exposure of *A. fumigatus* to both CDNB and H_2O_2.

[2] *Abbreviations used:* CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; GST, glutathione transferase; GSH, glutathione; MALDI-TOF, matrix assisted laser desorption ionisation-time of flight; MEM, minimal essential medium Eagle; CALM, calmodulin.

2. Experimental

2.1. Genomic DNA isolation

Aspergillus fumigatus ATCC 26933 (obtained from the American Type Culture Collection, Maryland, USA) was used in this study. *Aspergillus* cultures were grown in 5%(v/v) fetal calf serum in minimal essential medium Eagle (MEM) (Sigma–Aldrich, Dorset, UK) for 2 days at 37 °C. Genomic DNA was isolated as described by Nicholson et al. (2001). Briefly, ca. 4 g *A. fumigatus* mycelia were crushed in liquid N_2 and suspended in 10 ml extraction buffer (10 mM Tris–HCl, 10 mM EDTA, 0.5% (w/v) SDS pH 8.0). Phenol:chloroform:isoamyl alcohol (25:24:1, 10 ml) was added to the mycelial suspension and mixed gently for 30 min. Phases were separated by centrifugation at 5000*g* at 4 °C. The aqueous layer was removed a fresh tube and phenol extraction repeated until the interface was clear. The final aqueous layer was treated with chloroform:isoamyl alcohol (24:1) and phases separated as before. The remaining aqueous layer was treated with ribonuclease A (20 μl; 10 mg ml^{-1}) at 37 °C for 30 min, followed by phenol extraction, then chloroform extraction. The DNA was precipitated from the aqueous layer with 2 volumes of 100% ethanol and 1/10 volume of LiCl (4 M) at −20 °C overnight. DNA was recovered by centrifugation at 13,000*g* for 10 min. The pellet was washed with 70%(v/v) ethanol, air-dried, and resuspended in 1 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.2. DNA sequence and bioinformatic analysis

All DNA sequence analysis was performed using a Perkin-Elmer ABI Prism 310 genetic analyser, commercially by MWG Biotech (Milton Keynes, UK) or Lark Technologies (Essex, UK) and sequence similarities were determined using the BLAST algorithm (www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). Sequence alignments and neighbor-joined phylogenetic trees were generated using ClustalW (Thompson et al., 1994; http://www.ebi.ac.uk/clustalw). A bootstrapping value of 1000 was used, with bootstrapping percentages noted at tree branch points. Trees were visualised in Treeview (Page, 1996; http://taxonomy.zoology.gla.ac.uk/rod/rod.html). Preliminary sequence data was also obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of *A. fumigatus* genome is near completion with support from the Wellcome Trust and NIH.

2.3. PCR amplification

All PCR reagents were obtained from Sigma–Aldrich. PCR was performed using AccuTaq polymerase with 1–10 ng genomic DNA as template and 1.0 μM each of forward and reverse primer (Table 1) in a total volume of 50 μl. PCR conditions were as follows: 95 °C denaturation for 5 min; (94 °C denaturation for 30 s, 55 °C annealing for 90 s, 72 °C extension for 60 s) × 35 cycles; 68 °C extension for 7 min. Optimal cDNA amplification was found to require 45 cycles of PCR. PCR-amplified DNA was electrophoresed on 1% (w/v) agarose containing 0.5 μg ml^{-1} of ethidium bromide for 30 min at 100 V. Visualisation of amplicons was performed using an 'Eagle-Eye II' digital still video system (Stratagene, CA, USA).

2.4. Cloning and expression of gstA, B, and C

The *gstA* sequence was amplified from cDNA and the *gstB*/*gstC* sequences were amplified from *A. fumigatus* DNA, using primers incorporating terminal *Eco*RI and *Pst*I sites to facilitate downstream cloning (Table 1). PCR products were cloned into the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. *gstA*, *gstB*, and *gstC* were subsequently cloned into the pProEX-Hta expression vector (Invitrogen), which facilitates $(His)_6$ affinity tag introduction, utilising the engineered restriction sites. Ligations were performed using Quickstick ligase (Bioline, London, UK) according to the manufacturer's instructions. pPXA*gstA*, pPXA*gstB*, and pPXA*gstC*, the resultant expression vectors containing *gstA*, *gstB*, and *gstC*, respectively, were individually transformed into *E. coli* strain DH5α by electroporation according to Dower et al. (1988). Expression of all three recombinant GST proteins was induced by the addition of 0.6 mM isopropyl β-D-thiogalactoside (IPTG) and monitored by SDS–PAGE and Western blot analysis. For enzyme purification, induced cells were lysed by incubation with lysozyme (90 μg ml^{-1}) and sodium deoxycholate (0.04% (w/v)), in the presence of protease inhibitors (1 μg ml^{-1} leupeptin and pepstatin, respectively, and 1 mM PMSF). Cell debris was removed by centrifugation at 10,000*g* for 10 min and N-terminal $(His)_6$-tagged recombinant proteins were purified from the supernatant by Ni–NTA chromatography (Qiagen, West Sussex, UK) by elution with 250 mM imidazole in 50 mM sodium phosphate/300 mM NaCl. Purified GST proteins were dialysed (twice; once overnight, and once for 4 h) against phosphate-buffered saline (PBS) containing 0.02% (w/v) sodium azide for storage at 4 °C. Protein concentrations were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

2.5. MALDI-TOF MS

Mass spectrometry was carried out using an Ettan MALDI-TOF mass spectrometer (Amersham Biosciences (Europe) GmbH, Freiburg, Germany). Protein samples for peptide mass determination were either (i) separated by SDS–PAGE and digested with trypsin or (ii) obtained following in-solution enzymatic digestion and deposited (1 μl) with 1 μl α-cyano-4-hydroxycinnaminic acid (4-HCCA; 5 mg/200 μl 50%(v/v) acetonitrile in aqueous trifluoroacetic acid) onto mass spectrometry slides and allowed to dry prior to delayed extraction, reflectron TOF analysis at 20 kV.

2.6. GST activity assays

Glutathione transferase activity was determined using methods based on those described (Habdous et al., 2002; Habig and Jakoby, 1981) whereby the change in absorbance at 340 nm ($\Delta A_{340\,nm}$) was recorded, and enzyme activity calculated as micromoles CDNB utilised/mg GST/min. Activity with 1,2-dichloro-4-nitrobenzene (DCNB) was performed at 345 nm in the same way as for CDNB, with some exceptions; 100 mM phosphate buffer (pH 7.5), 100 mM DCNB in 100% ethanol, and 50 mM GSH in phosphate

Table 1
Nucleotide sequence of oligonucleotide primers used to amplify *Afugst* genes *A–C* from *A. fumigatus* genomic DNA and cDNA, respectively

Gene	Primers	Sequence (5′–3′)
gstA	*gstA*-F	GAGAGAATTCATGGCAAATAGACCTGATATTACACTG
	gstA-R	GAGACTGCAGATTAATGCTTCGCCTATTCG
gstB	*gstB*-F	GAGAGAATTCATGTCTTTGAAGCCTATCGTC
	gstB-R	GAGACTGCAGTTACTTTTCCTGTGCGGC
gstC	*gstC*-F	GAGAGAATTCATGCCGGACATCCAACCCATC
	gstC-R	GAGACTGCAGTCAGGTCGAGGGGAAGATGTC
Calmodulin	LCALM	CCGAGTACAAGGAAGCTTTCTC
Calmodulin	RCALM	GAATCATCTCGTCGACTTCGTCGTCAGT

Nucleotide sequence of control calmodulin primers (Romero et al., 2003) are also given. Oligonucleotide primers were designed based on sequence data obtained from the *A. fumigatus* genome sequencing effort (http://www.tigr.org). All *Afugst* forward primers (F1–F3) contained a 5′ *Eco*RI restriction site, and reverse primers contained 3′ *Pst*I sites, to facilitate directional cloning into pProEx-Hta.

buffer were used. Activity with ethacrynic acid was recorded at 270 nm, using 100 mM phosphate buffer, pH 6.5, 20 mM ethacrynic acid in 100% ethanol, and 2.5 mM GSH. Glutathione peroxidase activity was determined as described (Veal et al., 2002). Briefly, test samples (100 μl) were mixed with 880 μl of assay buffer (50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U ml^{-1} glutathione reductase and 1 mM GSH) and incubated at 30 °C for exactly 5 min, after which the mixture was transferred to a cuvette and 20 μl of 69 mM cumene hydroperoxide added. The depletion of NADPH was measured over 3 min at 340 nm.

2.7. Induction of GST expression in *A. fumigatus* and analysis by RT-PCR

Aspergillus fumigatus ATCC 26933 was cultured, with agitation, in 500 ml MEM + 5% (v/v) fetal calf serum at 37 °C for 47 h, before addition of either CDNB (final concentration: 200 μM) or H_2O_2 (final concentration: 5 mM). Aliquots (50 ml) were removed both prior to induction and at 1, 2, and 3 h post-induction. *A. fumigatus* mycelia were collected by filtration, rapidly frozen in liquid nitrogen, and stored at −80 °C prior to RNA extraction. RNA was extracted from *A. fumigatus* mycelia using the RNeasy plant mini kit (Qiagen). Quantification of RNA was performed using Total Lab software (NonLinear Dynamics) to ensure equal amounts of RNA were used subsequently, and ca. 1 μg RNA was used for cDNA synthesis. RNA was treated with DNase I (Sigma–Aldrich) prior to cDNA synthesis to remove DNA contamination. cDNA synthesis from mRNA was performed using the SuperScript kit (Invitrogen) using oligo(dT) primers. Subsequent PCR of GST cDNA was performed as described above. Control PCRs were performed with primers LCALM and RCALM (Table 1) which amplify 348 and 617 bp regions from *A. fumigatus* cDNA and genomic DNA, respectively (Romero et al., 2003). Densitiometric quantification of PCR products was performed using Genetools software (Syngene).

3. Nomenclature

In accordance with recommendations from the *A. fumigatus* sequencing group (http://www.man.ac.uk), the glutathione transferase genes disclosed here are termed *gstA*, *gstB*, and *gstC* and the corresponding proteins identified as gstA, gstB, and gstC. In addition, as recommended, the corresponding *S. pombe* orthologs are identified as superscripts as follows: $gstA^{gst3}$, $gstB^{gst1}$, and $gstC^{gst2}$. Finally, as recommended, the three-letter prefix '*Afu*', for *A. fumigatus* gene identification, has only been used when necessary.

4. Results and discussion

4.1. Cloning and sequence analysis of *A. fumigatus* GST open reading frames

Similarity searching of the *A. fumigatus* genome database with *A. nidulans* gstA (Genbank Accession No. AAM48104; Fraser et al., 2002) revealed the presence of an *A. fumigatus* GST (*AfugstA*) which, following amplification with primers *gstA*-F/R yielded a PCR product of 909 bp when a template of *A. fumigatus* genomic DNA was used, and 762 bp when cDNA was employed; sequence examination revealed two introns of 95 and 52 bp proximal to the 5′ end. The *A. fumigatus* genome database (http://www.tigr.org) was also interrogated with *S. pombe* protein sequences corresponding to GSTI (Genbank Accession No. AAK77864; Cho et al., 2002) and GSTII (GenBank Accession No. AAF21054; Kim et al., 2001), and a number of GST-like open reading frames were revealed which exhibited approximately 30% sequence similarity to the query sequences. PCR primers *gstB*-F/R and *gstC*-F/R (Table 1) were designed based on these *A. fumigatus* GST sequences and used to amplify open reading frames of 663 and 675 bp, respectively, from *A. fumigatus* genomic DNA. *GstA*, *B*, and *C* open reading frames corresponding to protein sequences shown in Fig. 1 were cloned into pProEx-Hta for recombinant expression.

Cloned *gstA*, *B*, and *C* sequences were compared with the *A. fumigatus* genome database; observed differences were minimal, resulting in translated amino acid sequences which exhibited 100% sequence identity to the database for gstA and gstB. GstC exhibited a single amino acid difference, which was a conservative change from V (http://www.tigr.org) to I (gstC sequence) at position 33. *GstC* was independently cloned and sequenced several times, indicating a true polymorphism rather than a sequence error. From the amino acid sequence alignment of gstA to C shown in Fig. 1, it is apparent that isolated regions of identity exist between all three proteins throughout their entire sequence, with gstA apparently consisting of a 30 amino acid C-terminal extension relative to gstB and C. However, more

Fig. 1. Amino acid sequence alignment of and gstA (254 aa), gstB (221 aa) and gstC (225 aa). Identical residues are highlighted in black boxes while those residues only common to two sequences are shaded grey. Gaps (–) are introduced for optimal alignment. It appears that gstA contains a 30 amino acid C-terminal extension relative to both gstB and C.

criteria are required to confidently assign the GSTs to this class, such as immunological cross-reactivity. As relatively few fungal GSTs have been characterised, it is difficult to determine whether these enzymes are homologues of those found in other organisms or are fungal specific. Cha et al. (2001), identified a GST from *Cunninghamella elegans* which bore little resemblance to known classes, and a new fungal class was proposed; it is possible that several classes are yet to be discovered.

Fig. 2 shows the relationship between GSTs and many of the fungal GST sequences characterised to date. It can be seen gstB and C are most closely related to the gstA enzymes from *A. fumigatus* and *A. nidulans*, respectively. While *S. pombe* GST1 and 2 also exhibit significant relatedness to the GST proteins from *A. fumigatus*, those from *I. orientalis* and *Cu. elegans* are more distantly related. When the three *A. fumigatus* GST protein sequences were used in a general BLAST search at http://www.ncbi.nlm.nih.gov/entrez/, similarities to putative GST-like proteins from several fungal species, which have not been fully characterised, were revealed. GstA exhibited the highest similarities, indicating that this GST may have developed prior to speciation. Novel sequences identified using gstA included a putative GST from the artichoke pathogen *Botryotinia fuckeliana* (GenBank Accession No. AAG43132, 69% identity) and putative proteins from the rice blast fungus *Magnaporthe grisea* (GenBank Accession No. EAA55090, 58% identity), *Neurospora crassa* (GenBank Accession No. CAD36970, 58% identity), and the cereal pathogen *Gibberella zeae* (GenBank Accession No. EAA71824, 49% identity). GstB and C also showed similarity to the *B. fuckeliana* putative GST, at the level of 40% identity. While gstB also exhibits similarity to the *N. crassa* protein mentioned previously (GenBank Accession No. CAD36970, 44% identity) and gstC shares similarity with the *G. zeae* protein (39% identity), there is little crossover between similar proteins identified by searching with gstA and with the other two *A. fumigatus* GSTs. GstB and C, however, share similarity with several of the same proteins, although these are less similar than the gstA-like sequences. In addition, both gstB and gstC displayed some similarity with the URE2 group of nitrogen metabolism proteins that have been identified from several *Saccharomyces* species (Fig. 2), but which have not been shown to exhibit GST activity with CDNB (Rai et al., 2003); this similarity was not evident with gstA. URE2 from *S. cerevisiae* was shown to be involved in defense against heavy metal ions and oxidative stress (Rai et al., 2003). Recently, a putative GST sequence was identified from the *A. fumigatus* genome as part of a co-regulated gene cluster, which is postulated as responsible for production of gliotoxin (Gardiner et al., 2004). While this GST has not been characterised and is not the same as any of the GSTs described here, it suggests a metabolic role for GSTs in *A. fumigatus*, as has been postulated in other organisms (Hayes and Pulford, 1995), and also suggests there may be further, as yet undiscovered, GSTs in this fungus.

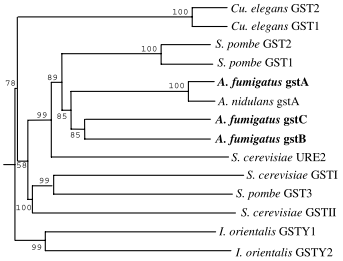

Fig. 2. Phylogenetic analysis of the three *A. fumigatus* GST proteins compared to 11 other characterised fungal GSTs. Sequences were aligned and a neighbor-joined tree generated using ClustalW, with bootstrapping of 1000. Percentage bootstrapping values are shown at branch points. Sequence GenBank Accession numbers are as follows: *Cunninghamella elegans* GST2, AAL02369; *Cu. elegans* GST1, AAL02368; *S. pombe* GST2, AAF21054; *S. pombe* GST1, AAK77864; *A. nidulans* GSTA, AAM48104; *S. cerevisiae* URE2, A39609; *S. cerevisiae* GSTI, P40582; *S. pombe* GST3, AAK59430; *S. cerevisiae* GSTII, Q12390; *I. orientalis* GSTY1, BAA77459; and *I. orientalis* GSTY2, S16178.

Expression, purification, and activity analysis of recombinant GST proteins

Protein expression plasmids pPXA*gstA*, pPXA*gstB*, and pPXA*gstC*, consisting of the vector pProEx-Hta containing the open reading frames of *gstA* (cDNA), *gstB*, and *gstC* respectively, were transformed into *E. coli* DH5α and expression induced by the addition of

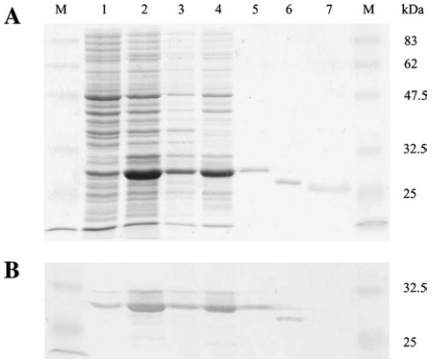

Fig. 3. SDS–PAGE and Western blot analysis of recombinant GST expression in *E. coli*. Duplicate SDS–PAGE gels were loaded as follows: M, protein size marker; lane 1, uninduced *E. coli* harbouring expression plasmid pPXA*gstC*; lane 2, *E. coli* harbouring expression plasmid pPXA*gstC* induced with 0.6 mM IPTG; lane 3, insoluble proteins extracted from induced *E. coli*; lane 4, soluble proteins extracted from induced *E. coli*; lane 5, $(His)_6$-purified recombinant gstC (5 μg); lane 6, $(His)_6$-purified recombinant gstB (5 μg); and lane 7, $(His)_6$-purified recombinant gstA (4 μg). One gel was Coomassie stained (A) and the other was probed with anti-$(His)_6$ murine monoclonal antibody to identify recombinant proteins (B). GstB was purified in the same manner as gstC. Both gstB and gstC were detected with anti-$(His)_6$ monoclonal antibody, but not gstA. However, the identity of this protein was confirmed by mass spectrometry.

0.6 mM IPTG (Fig. 3). All three recombinant proteins were present in cell lysate supernatants, indicating solubility, and recombinant gstB and gstC were purified using the N-terminal $(His)_6$-tag, with a yield of approximately 17 and 18 mg per gram of *E. coli* cells cultured, respectively (Fig. 3). Detection of recombinant gstA using the $(His)_6$ tag was not possible, and large-scale metal chelate affinity chromatography was required to purify sufficient enzyme for activity analysis (Fig. 3; Table 2). Purified recombinant proteins were analysed by MALDI-TOF MS and peptides (following tryptic digestion) were identified corresponding to the theoretical amino acid sequence for all three proteins whereby 4/83 peptides (20% sequence coverage), 7/57 peptides (34% sequence coverage), and 4/46 peptides (15% sequence coverage) were observed for recombinant gstA, B, and C, respectively. SDS–PAGE data confirm molecular masses of 26, 27, and 30 kDa for gstA, B, and C, respectively (Fig. 3). These are consistent with theoretical molecular masses of 28.99 and 28.72 kDa for the $(His)_6$-tagged gstB and gstC proteins, but smaller than the theoretical mass of 32.76 kDa for gstA. Protein molecular mass analysis via FPLC gel filtration chromatography (Superose 6) confirmed the dimeric status of purified gstB (56 kDa) and C (61 kDa), respectively (data not shown). No binding to glutathione–Sepharose affinity columns was observed for any recombinant GST (data not shown). Furthermore, attempts to purify native gstA to C from *A. fumigatus* extracts by GSH affinity chromatography were unsuccessful (data not shown), which has also been noted in the purification of some theta GST enzymes (Hayes and Pulford, 1995). Crystal structures of some theta GSTs have indicated that the GSH-binding site may be sited further inside the protein than in other classes of GST, and that conventional GSH-affinity matrices may be unable to bind the active site (Hayes and Pulford, 1995).

The enzymatic activities of purified, recombinant gstA–C were assessed with several substrates. CDNB, DCNB, and ethacrynic acid are substrates for glutathione transferase activity, whereas cumene hydroperoxide is a substrate for glutathione peroxidase activity (Veal et al., 2002). GstA–C exhibited low level GST and glutathione peroxidase activities, which were detectable due to the high concentrations of recombinant enzyme obtained (Table 2). GstB exhibited a four to six times higher specific activity against CDNB than either gstA or C, respectively. No activity was observed for either enzyme when DCNB or ethacrynic acid were used as substrates. The specific activity observed against cumene hydroperoxide was almost six times higher for gstB then gstA/C (Table 2). Although relatively lower, the actual ratio of relative activity against CDNB was similar to that previously found (Veal et al., 2002) whereby *S. pombe* gst1 showed three times greater activity towards CDNB than gst2. Conversely, the glutathione peroxidase activity of *S. pombe* gst2 was about twice that observed for gst1 albeit at a six times (approx.) lower level to that found for *A. fumigatus* gstB. GST specific activity determinations for *S. pombe* gst1 (Kim et al., 2001) were carried out on cell lysates using impure gst1, thus making exact comparison difficult. However, gst1 activity was detectable against CDNB with no glutathione peroxidase activity evident. *S. pombe* gst2 also possesses glutathione transferase activity, as measured by CDNB conjugation (Cho et al., 2002). The specific activities of the *A. fumigatus* GSTs are low in comparison with other characterised GSTs, however, any loss of activity due to the presence of the $(His)_6$ tag is unlikely as TEV protease removal of the $(His)_6$ tag (from gstC) did not result in enhanced activity (data not shown). Ultimately, activity analysis of the native GSTs is required to fully assess activity.

Table 2
Specific activities of purified recombinant gstA, gstB, and gstC with both glutathione transferase and glutathione peroxidase substrates

Substrate	Specific activity (U mg^{-1}) (±SD)		
	gstA	gstB	gstC
CDNB	0.004 ± 0.0001	0.025 ± 0.0028	0.006 ± 0.0005
DCNB	N.D.	N.D.	N.D
Ethacrynic acid	N.D.	N.D.	N.D
Cumene hydroperoxide	0.019 ± 0.0009	0.145 ± 0.0088	0.025 ± 0.006

GST activity was assayed with CDNB, DCNB and ethacrynic acid, and glutathione peroxidase activity was assayed with cumene hydroperoxide. Units are expressed as micromoles substrate utilised per minute. Specific activities stated are the average of five replicates and were corrected against blank reactions that had been performed in triplicate. N.D., no activity detected.

4.2. Induction of *A. fumigatus* GST expression with CDNB and H_2O_2

GSTs are thought to be involved in the response against external and cellular toxins, and may also aid the cell when challenged by oxidative stress. After initial growth in the absence of either CDNB or H_2O_2, *A. fumigatus* was further cultured in the presence of 200 μM CDNB or 5 mM H_2O_2 and expression levels of *gstA*, *gstB*, and *gstC* determined by RT-PCR (Fig. 4). Moreover, the difference in amplicon size for *gstA* can be seen thereby confirming the removal of intronic sequence. Primers specific for the calmodulin gene (Romero et al., 2003) were used to confirm absence of genomic DNA and RNA equivalence between time-points. Low basal expression of *gstA* and *gstC* was detected, but *gstB* expression was not detectable under basal conditions. Upon induction with CDNB, all three *gst* genes were up-regulated within one hour, with *gstA* showing a 10-fold induction (approximately) and *gstC* showing at least a 4-fold induction at all time-points, and *gstB* exhibiting only weak induction, with a 20% drop in RNA expression at

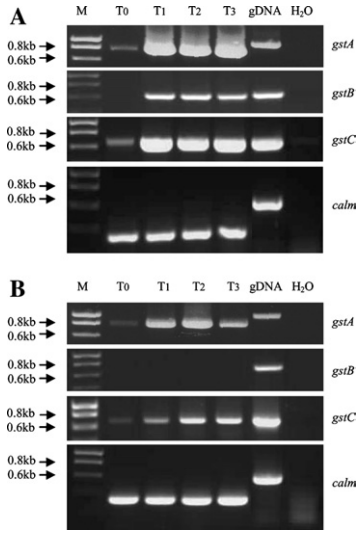

Fig. 4. RT-PCR of *A. fumigatus* cDNA isolated from cultures induced with either CDNB (A) or H_2O_2(B). *A. fumigatus* RNA was isolated prior to induction (T_0), 1 h post-induction (T_1), 2 h post-induction (T_2), and 3 h post-induction (T_3). PCR was performed on cDNA using *gstA*, *B*, and *C* specific primers and primers specific for the calmodulin gene (CALM) (Romero et al., 2003). Optimal cDNA amplification was found to require 45 cycles of PCR. Agarose gels were loaded as follows: M, DNA size marker; T_0; T_1; T_2; and T_3; gDNA, genomic DNA control; and H_2O, no DNA control. Expression of all three *gst* genes was observed when induced with CDNB, and induction of *gstA* and *gstC* only was seen with H_2O_2 induction.

3 h post induction (Fig. 4A). This result is quite interesting as it is somewhat at variance with the above observation that gstB exhibits greater activity towards CDNB than does gstA or gstC, however it is possible that the higher specific activity of gstB may necessitate production of smaller amounts of actual protein to conjugate available CDNB. Previous work, whereby β-galactosidase expression was placed under the control of the upstream regulatory regions associated with gst1 and gst2 of *S. pombe*, has shown that *gst1*$^+$ gene expression was enhanced by mercuric chloride and menadione (generates superoxide radicals), whereas gst2 expression was only significantly induced by the presence of *o*-dinitrobenzene (*o*-DNB) (Cho et al., 2002; Kim et al., 2001; Shin et al., 2002). *o*-DNB also induces GST gene expression in *I. orientalis* (Tamaki et al., 1999).

In the work presented here, exposure of cells to H_2O_2 did not result in *gstB* induction. However, weak induction of *gstC* was detected within 1 h post-induction (approx. 3-fold increase over T_0) which stabilised at 2 and 3 h (approx. 7-fold increase over T_0). In addition, strong expression of *gstA* at 1 h post-induction was evident (at least 5-fold increase) which appeared to further increase at 2 h (approx. 10-fold increase over T_0) before reducing at 3 h post-induction (approx. 4.5-fold with respect to T_0), possibly due to depletion of added H_2O_2 (Fig. 4B). *GstB* and *gstC* were shown to be differentially inducible by H_2O_2, whereby up-regulation of *gstC* expression only was observed. This indicates the possibility of different roles for these proteins within the organism when subjected to environmental stress. Previous work has also indicated that expression of *gst1*, as well as *gst2*, in *S. pombe* is induced by H_2O_2. This observation suggests potentially different mechanisms are involved in the response to oxidative stress for both organisms and that the *A. fumigatus* gstB plays a different role than gst1 in *S. pombe*—an hypothesis supported by greater sequence divergence between *gstB* and *C* in *A. fumigatus* compared to the corresponding genes in *S. pombe* (Fig. 2). It is intriguing that *gstB* expression was not induced in the presence of H_2O_2, yet it exhibited the greatest glutathione peroxidase activity against cumene hydroperoxide. In vivo, it is postulated that GST enzymes may be involved in detoxification of secondary oxidation products produced by initial conversion of reactive oxygen species and H_2O_2 by enzymes such as glutathione peroxidase and superoxide dismutase (Hayes and McLellan, 1999). These secondary oxidation products include lipid and DNA oxidation products, similar to cumene hydroperoxide, and it is possible that *gstB* expression is induced by the secondary oxidation products rather than directly by H_2O_2. Thus, a putative secondary compound required for induction of *gstB* may not have been produced when H_2O_2 was used as the inducer. *A. fumigatus* gstA was identified via homology searching of the *A. fumigatus* genome using *A. nidulans* gstA. In fact, these proteins exhibit a very high degree of sequence similarity (83%) which indicates that they may share a common function in both *Aspergillus spp.* Fraser et al. (2002) have shown that *gstA* encodes a GST which is involved in xenobiotic and metal ion resistance in *A. nidulans*. *A. fumigatus gstA* was strongly up-regulated in the presence of both CDNB and H_2O_2. In addition, *gstA* harbours sequences upstream of the initial ATG with similarity to the xenobiotic (XRE) and antioxidant responsive elements (ARE) involved in regulation of mammalian GSTs (Hayes and Pulford, 1995; Rushmore et al., 1991; Rushmore and Pickett, 1993). Although such responsive elements have not been demonstrated to be functional in the regulation of fungal GSTs to date, XRE and ARE have been identified in the promoter regions of *gst* genes in *S. cerevisiae* (Choi et al., 1998). DNA sequences from the *A. fumigatus* genome (http://www.tigr.org) 2 kb upstream of each *gst* gene were examined for the consensus XRE (TNGCGTG) and ARE (TGACNNNGC) regions. Several XRE and ARE-like regions were observed upstream of all three *gst* genes. Examination of transcriptional regulation via promoter analysis linked to a reporter gene would be necessary to confirm whether these responsive elements were functional and involved in regulation of gene expression in the presence of CDNB and H_2O_2. The expression of all three *gst* genes in response to organism exposure to CDNB and of *gstA* and *C* following exposure of

A. fumigatus to H_2O_2 indicates a high likelihood that GSTs play a role in the response of *A. fumigatus* to both xenobiotic presence and oxidative stress and, as in other fungal species (Veal et al., 2002), may be involved in mediating anti-fungal drug resistance.

5. Summary

Three previously unidentified open reading frames encoding glutathione transferases have been identified in *A. fumigatus*, which are either constitutively expressed under experimental conditions employed (*gstA* and *C*) or inducible in response to xenobiotic (*gstA*, *B*, and *C*) or oxidative stress (*gstA* and *C*). Recombinant proteins corresponding to all three genes have been expressed and enzymatic activity characteristic of glutathione transferase and glutathione peroxidase defined. The availability of this information will facilitate further exploration of the possible role(s) played by GST in xenobiotic tolerance and mediating response to oxidative stress in *A. fumigatus*.

6. Accession numbers

Sequences for the three *Aspergillus fumigatus* glutathione transferases described are deposited in GenBank under Accession Nos. AY770045, AY770043, and AY770044 (*gstA* to *C*) and AY770046 (*gstA* cDNA).

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