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Molecular characterization of an adaptive response to alkylating agents in the opportunistic pathogen *Aspergillus fumigatus*

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ABSTRACT

An adaptive response to alkylating agents based upon the conformational change of a methylphosphotriester (MPT) DNA repair protein to a transcriptional activator has been demonstrated in a number of bacterial species, but this mechanism appears largely absent from eukaryotes. Here, we demonstrate that the human pathogen *Aspergillus fumigatus* elicits an adaptive response to sub-lethal doses of the mono-functional alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). We have identified genes that encode MPT and O⁶-alkylguanine DNA alkyltransferase (AGT) DNA repair proteins; deletions of either of these genes abolish the adaptive response and sensitize the organism to MNNG. *In vitro* DNA repair assays confirm the ability of MPT and AGT to repair methylphosphotriester and O⁶-methylguanine lesions respectively. In eukaryotes, the MPT protein is confined to a select group of fungal species, some of which are major mammalian and plant pathogens. The evolutionary origin of the adaptive response is bacterial and rooted within the Firmicutes phylum. Inter-kingdom horizontal gene transfer between Firmicutes and Ascomycete ancestors introduced the adaptive response into the Fungal kingdom. Our data constitute the first detailed characterization of the molecular mechanism of the adaptive response in a lower eukaryote and has applications for development of novel fungal therapeutics targeting this DNA repair system.

INTRODUCTION

Aspergillus fumigatus (*A. fumigatus*) is responsible for a range of devastating diseases in immunocompromised individuals, and while the extent of disease depends largely on the immune status of the host, mortality rates are extremely high ranging between 50 and 85% (1). Currently, several strategies are available for the treatment of *Aspergillus*-related diseases. These include conventional methods such as the use of antifungal drugs which usually target either the fungal cell wall or cell membrane (2). More recently, other avenues for antifungal development have been explored such as inhibition of fungal natural product and secondary metabolite biosynthesis (3). Despite significant advances in antifungal strategies, many important challenges still remain, in particular the emergence of resistance to most classes of antifungal drugs (4). The availability of the complete genome sequence for *A. fumigatus* creates an opportunity to investigate previously uncharacterized pathways, which may be fungal-specific, providing potential novel drug targets for antifungal development (5). Our particular focus was the identification and characterization of pathogen-specific DNA repair pathways and their potential as new fungal targets for therapeutic intervention, particularly in the context of cancer patients undergoing alkylating chemotherapeutic treatment, who are amongst some of the most at risk individuals for invasive aspergillosis disease.

Mono-functional alkylating agents, such as *N*-methyl-*N*-nitrosourea (MNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and the metabolically active form of dimethylnitrosamine are efficient mutagens and carcinogens (6). Methylating agents can react with nucleophilic sites on DNA molecules (7) and while the majority of evidence indicates that the principal mutagenic and

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toxic lesion that results is *O*⁶-methylguanine (*O*⁶meG) a dozen other sites in DNA bases and the oxygen atoms in the phosphodiester linkage are also targets, the latter results in the formation of phosphotriesters.

Escherichia coli and some other prokaryotes respond to DNA alkylation damage by the induction of an adaptive response which increases resistance to the mutagenic and toxic effects of subsequent doses of alkylating agents (8). The *E. coli ada* gene is the key player in the adaptive response, and this has been extensively studied over the last 30 years (9,10). The Ada protein functions both as a direct DNA repair protein and as a positive regulator of the adaptive response. The C-terminal region of the Ada protein removes methyl groups from *O*⁶meG and also *O*⁴-methylthymine onto cysteine residue 321 (Cys-321) in a stoichiometric and auto-inactivating process that reduces the toxic and mutagenic effects of these lesions (6,11). The transcriptional regulator function is mediated by the N-terminal domain of the Ada protein (12) in which cysteine residue 38 (Cys-38) stoichiometrically removes methyl groups from one of the methylphosphotriesters generated in DNA by MNNG. This converts the Ada protein into an active transcriptional regulator (13) for genes involved in the alkylation response pathway, namely *alkB*, *alkA* and *aidB* and also *ada* itself (14,15). Furthermore, *E. coli* possesses a constitutively expressed *O*⁶-alkylguanine DNA alkyltransferase, encoded by the *ogt* gene, which exhibits significant sequence similarity to the C-terminus of Ada but which is distinct from the adaptive response (16).

Since the early investigations in *E. coli*, an adaptive response to alkylating agents has been found to be widespread throughout bacterial species (17). However, no adaptive response exists in the lower eukaryote, *Saccharomyces cerevisiae* (18,19). A gene encoding *O*⁶-alkylguanine DNA alkyltransferase (AGT) activity has been cloned from *S. cerevisiae* by functional complementation of an *E. coli ada/ogt* double mutant, and this gene has been designated *MGT1* (20). Deletion of *MGT1* sensitizes *S. cerevisiae* to sacrificing and mutagenesis following exposure to alkylating agents. In agreement with the lack of an adaptive response in *S. cerevisiae*, *MGT1* transcript levels were not increased in wild-type *S. cerevisiae* in response to alkylation treatment (21).

However, the biological effects of alkylating agents are largely unexplored in filamentous fungi. An improved growth rate in the presence of MNNG was observed for *Aspergillus nidulans*, following exposure to a sub-lethal dose (22) and DNA methyltransferase activity was also observed in extracts of this fungus (23,24). This activity has been shown to be highly inducible by MNNG treatment of *A. nidulans*, and effective in repairing *O*⁶meG and methylphosphotriesters, hallmarks of an adaptive response. To date, *A. nidulans* is the only eukaryotic organism demonstrated to possess an adaptive response to alkylating agents that may be equivalent to that observed in bacteria. Although up-regulation of repair processes have been reported in mammalian cells and tissues, no such alkyl phosphotriester-mediated mechanism has been reported, indeed there is no evidence for

an alkyl phosphotriester alkyltransferase in higher eukaryotes.

We hypothesized that such DNA alkylation-damage mediated adaptive responses may be more widespread in the Fungal kingdom and may therefore constitute potential novel therapeutic targets against fungal pathogens. We therefore assessed whether an adaptive response to alkylating agents exists in the widespread and highly pathogenic fungus *A. fumigatus*. We identified open reading frames (ORFs) in this organism that were predicted to encode for an AGT and a methylphosphotriester methyltransferase (MPT). We show that *A. fumigatus* does possess an adaptive response to alkylating agents, and that deletion of the genes encoding the AGT and the MPT ablates this response in the fungus, concomitantly increasing sensitivity to MNNG. We have characterized the adaptive response at the transcriptional level and demonstrated that the AGT and MPT proteins can repair *O*⁶meG and methylphosphotriester (methylPT) lesions, respectively. Functional conservation of AGT activity is confirmed by the ability of *AfAGT* to fully complement the MNNG sensitivity of a *S. cerevisiae mgt1* deletion strain. Furthermore, using phylogenetics we identify the potential existence of an adaptive response to alkylating agents in a number of Ascomycete fungi, and we show that the bacterial adaptive response is rooted in the ancient Firmicutes phylum.

These data demonstrate, for the first time, that there is an adaptive response to alkylating agents in the human pathogen *A. fumigatus*. The lack of a corresponding system in mammalian organisms, together with the rise in invasive *Aspergillus* infections, and short-falls in current treatment, make this response an attractive possibility as a novel drug target, warranting further investigation.

MATERIALS AND METHODS

Strains, growth conditions and general DNA manipulation

The *A. fumigatus* Af293 strain was used in this study. Fungal strains were grown in *Aspergillus* minimal media (AMM), containing 1% (w/v) glucose as carbon-source, 5mM ammonium tartrate as nitrogen-source and trace elements according to Pontecorvo *et al.* (25). Fungal culturing was performed as previously described (26). *E. coli* DH5 α was used for general plasmid DNA propagation and cultured in LB-AMP (containing 100 μ g/ml) for plasmid maintenance. *A. fumigatus* genomic DNA was purified using a ZR Fungal/Bacterial DNA Kit (Zymoresearch). *S. cerevisiae* strain used was BY4741 *Amgt1* derivative (Euroscarf).

Sequence data and database searches

E. coli protein sequences were obtained at NCBI (27). BLAST searching was performed against *Aspergillus* genomes and *A. fumigatus* genomic sequence data was obtained from The Central *Aspergillus* Data Repository (28). Amino acid sequences from all completely sequenced prokaryotic genomes were obtained from the NCBI ftp site (release 165). Our fungal data set consisted of 103

fungus genomes. Where available, data was obtained from the NCBI fungal genome FTP site (<ftp://ftp.ncbi.nih.gov/genomes/Fungi>). The remaining data was downloaded from the relevant sequencing centres [see Medina *et al.* (29) for more information]. We also included 35 representative non-fungal Eukaryotes in our database (Supplementary Table S1). These were obtained from the KEGG FTP site (<http://www.kegg.jp/kegg/download/>). All sequence data was merged to produce a local database. We also merged a subset of the genome data to yield a separate, smaller representative database (Supplementary Table S1).

Initially, *Bacillus subtilis* (strain 168) *adaA* (GenBank accession P19219) and *adaB* (GenBank accession P19220) proteins were used as query sequences against the representative database. Taking one protein at a time, gene families were located using the BlastP algorithm (30) with a cut-off expectation (E) value of 10^{-10} . Due to its patchy phyletic distribution a second database search of *adaA* against the complete database was performed. Homologous sequences were retrieved and manually checked to ensure orthology.

Phylogenetic methods

Gene families were aligned using MUSCLE (v3.6) (31), with the default settings. Phylogenetic relationships were inferred using the maximum likelihood criterion. Appropriate protein models of substitution were selected using ModelGenerator (32). One hundred bootstrap replicates were then carried out with the appropriate protein model using the software program PHYML (33) and summarized using the majority-rule consensus method.

RNA isolation and RT-PCR amplification

Fungal RNA was isolated and purified from *Aspergillus* hyphae crushed under liquid nitrogen using the RNeasyTM plant mini kit (Qiagen). Prior to cDNA synthesis, RNA was treated with DNase I (Invitrogen). RNA quality was determined by visualization of intact 16S and 26S rRNA subunits by ethidium bromide staining following agarose gel electrophoresis. cDNA synthesis from RNA (500 ng) was performed using first strand transcription cDNA synthesis kit (Roche) with oligo (dT) primers. The gene encoding *calmodulin* (*calm*) which is constitutively expressed in *A. fumigatus* served as a control in RT-PCR experiments (34).

Investigation of presence of adaptive response

Aspergillus fumigatus conidia (10^4) were used to inoculate MEA agar plates with or without MNNG (0.5 µg/ml). Following overnight growth, 0.5 cm² plugs were taken from these plates and transferred to fresh MEA plates supplemented with increasing concentrations of MNNG (0–4 µg). Plates were incubated for a further 72 h, after which the radial growth of the colonies was measured and statistical analysis was performed by two-way ANOVA. To investigate transcriptional activation of genes in response to MNNG, *A. fumigatus* cultures ($n = 9$, 100 ml each) were incubated at 37°C. MNNG (0.5 µg/ml) was added after 16 h to four of the cultures

(referred to as induced). One culture was harvested as an uninduced reference at $T = 0$ h. Uninduced and induced cultures were then harvested at 30 min, 1 h, 2 h and 3 h post-induction. RT-PCR was then performed for genes of interest as described above.

Generation of *A. fumigatus* AFUA_5G06350 and AFUA_2G02090 mutant strains

Aspergillus fumigatus transformation was carried out according to Tilburn *et al.* (35). For generation of *Afmpt* and *Afagt* mutant strains, the bipartite marker technique was used (36), with modifications. Briefly, *A. fumigatus* Af293 was transformed with two DNA constructs, each containing an incomplete fragment of a pyrithiamine resistance gene (*ptrA*) (37,38), fused to 1.2 kb of 5'- and 3'-gene flanking regions. The marker fragments shared a 557 bp overlap within *ptrA*, serving as a potential recombination site during transformation. Two rounds of PCR were used to generate each fragment. The same strategy was used for the deletion of *Afmpt* and *Afagt*, and primer pairs used for each deletion are outlined in Supplementary Table S1. First, each flanking region was amplified from *A. fumigatus* Af293 genomic DNA using primer 1 and primer 2 for flanking region A, and primer 3 and primer 4 for flanking region B. Subsequent to gel-purification, the fragments were digested with specific restriction enzymes (MfeI and HindIII for *Afmpt* and XmaI and KpnI for *Afagt*) to facilitate ligation to *ptrA*. The *ptrA* selection marker was released from plasmid pSK275 (a kind gift from Professor Sven Krappmann) by digestion with the enzymes indicated above. For transformation, two overlapping constructs were amplified from the ligation products using primer 5 and *ptrA*-F for fragment C (2.6 kb) and primer 6 and *ptrA*-R for fragment D (2.2/2.3 kb). Subsequently, *A. fumigatus* was transformed with the overlapping fragments C and D. The strategies used in this study resulted in the complete deletion of either the *Afmpt* or *Afagt* coding region. Transformants were selected initially on the basis of pyrithiamine resistance. Positive deletion strains were screened by Southern blot analysis and DIG-hybridization probes were generated for detection of mutant alleles. Corresponding abolition of gene expression in deletion strains was confirmed by RT-PCR.

Engineering of *A. fumigatus* genes into *Saccharomyces cerevisiae*

To introduce *Afmpt* and *Afagt* into *S. cerevisiae*, plasmids were constructed as follows: the coding regions of *Afmpt* and *Afagt* were amplified by PCR using primers *Afmpt*-PC210 F and *Afmpt*-PC210 R or *Afagt*-PC210 F and *Afagt*-PC210 R, respectively (Supplementary Table S2). These primers pairs were designed to incorporate 5' NdeI and 3' SphI restriction sites onto both *A. fumigatus* genes for subsequent directional cloning into the pC210 plasmid (39). pC210 contains the *SSA1* coding region under control of the *SSA2* promoter that was cloned into a pRS315 (*LEU2*) backbone. Following digestion of pC210 with NdeI and SphI to remove the *SSA1* coding sequence, similarly digested *A. fumigatus* gene products

were ligated into pC210 to form pC-*Afmpt* and pC-*Afagt*. The integrity of these plasmids was confirmed by sequencing. Rich and minimal yeast medium was as described (40).

Phenotypic assays

Aspergillus fumigatus wild-type and mutant strains were grown on either AMM agar or MEA agar plates for one week at 37°C. Conidia were harvested aseptically in PBST [0.1% (v/v) Tween-80] and filtered through sterile miracloth to remove mycelial matter. Conidia were counted using a haemocytometer. Conidia (10^4) were spotted onto test plates. Plates were incubated at 37°C and colony diameter was measured periodically. Statistical analysis was carried out using two-way ANOVA.

Expression and purification of *Afmpt*

The *Afmpt* gene was obtained by PCR using a suspension of a single colony of *Afmpt*-transformed *S. cerevisiae* (see above) as a template. PCR was carried out using Phusion Polymerase (NEB) according to supplier recommendations with the primers AF forward (5' Phos ATGCATG TGGTCGATGATTCAAATG) and AF reverse (5' Phos CTATTGTAAGTTCGTCTGGCCAAAAG). Cycling conditions comprised an initial cycle of 30 s at 96°C, then 25 cycles of 30 s of 96°C, 30 s at 60°C and 1 min at 72°C. pMALc2 (NEB) was digested with *Xmn*I, purified and ligation to *Xmn*I-digested *Xmn*I at a 1:1 molar ratio. The ligation reaction was incubated at room temperature overnight and then transformed to competent *E. coli* DH5 α . Plasmids were isolated from resulting colonies using a miniprep robot (Qiagen 8000) and sequenced on an ABI3130 16 capillary system. Sequence alignment revealed one incorrect base (137) and this was corrected by site-directed PCR mutagenesis using the primer pair 5'Phos CGTCTACGCCGTCCTAACG and 5'Phos AATGTATTGGCCGTTGCATCG.

For protein over-expression and purification, a 40 μ l aliquot of colony suspension was inoculated into 10 ml of LB-amp and shaken overnight at 37°C. An aliquot (5 ml) of this was then added to 200 ml of LB-amp containing glucose (0.2%) and incubated at 37°C to an OD₅₉₅ of approximately 0.6. IPTG (Bioline) was then added to a final concentration of 0.4 mM to induce expression and the cells were harvested after further 3 h incubation by centrifuging for 20 min at 6000 rpm, washing the pellet with TE pH8 then centrifuging again for 10 min at 2800 rpm. Cell-free extracts were prepared by sonication (Sonicator Ultrasonic Processor XL; Heat Systems) and centrifugation at 15000 rpm for 10 min at 4°C. The supernatant was applied to an Amylose (NEB) column (0.8 $\times$ 2.0 cm) that had been equilibrated with 8 column volumes of binding buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA). The column was then washed with binding buffer and 2 ml fractions were collected until the protein concentration detected using an ND1000 spectrophotometer was less than 0.1 mg/ml. Aliquots (2 ml) of binding buffer containing 10 mM maltose were then added to elute the protein. The fraction containing the

highest concentration of protein (0.85 mg/ml) was stored in ice and used in subsequent experiments.

Determination of methyltransferase activity

To prepare substrate DNA, 32.6 mg of extensively deproteinized calf thymus DNA (Sigma) was dissolved in 3 ml of 40 mM ammonium acetate buffer. [³H]MNU (ARC; 80 Ci/mmol; 5 mCi in 5 ml ethanol:HOAc 99:1) was transferred to a 50 ml conical centrifuge tube and reduced to ~1 ml in a stream of air. The DNA solution was added to this and both DNA and MNU containers rinsed serially four times with 1 ml ammonium acetate buffer and mixed thoroughly. After 4.5 h at room temperature, the DNA was precipitated by addition of 1 ml 3 M NaAc and 24 ml ethanol and collected by centrifugation. The supernatant was carefully removed and stored for disposal and the DNA was washed extensively with 70% ethanol, re-dissolved in TE, re-precipitated in ethanol and washed as above, and finally dissolved in TE at ~3 mg/ml. Methyltransferase assays generally used 0.75 μ g of this DNA which, by assay with recombinant MGMT and recombinant *E. coli* MPT and scintillation counting was calculated to contain ~10 fmoles both of the substrate methylphosphotriester stereoisomer and of O⁶-meG. At a counting efficiency of 25%, this equated to 44.4 cpm per fmole of methyl group.

In the methyltransferase assays, increasing volumes of cell-free extracts, recombinant proteins or extract-recombinant protein mixtures were incubated with 0.75 μ g of substrate in a total volume of 300 μ l made up with buffer I containing 1 mg/ml BSA and incubated for various times at room temperature or 37°C. In the lesion/inhibitor assays, increasing volumes of extracts were incubated with protein limiting amounts of recombinant *AfMPT* (~7 fmoles) or recombinant MGMT (~6 fmoles) for 1 h at room temperature, then substrate DNA added and incubation continued for 1 h. After incubation, assay samples were processed as described previously (41) and radioactivity quantified by scintillation counting.

RESULTS

Identification of candidate *A. fumigatus* adaptive response genes

The *E. coli* Ada protein sequence (NCBI Accession No. *E. coli* NP_416717) was used as a query sequence to interrogate the *A. fumigatus* genome at the CADRE database (28). A BLASTX search retrieved two significant hits for *E. coli* Ada with the locus identifiers AFUA_5G06350 and AFUA_2G02090. Automatic annotation at CADRE predicted that these genes encode a DNA repair and transcription factor, and a methylated-DNA-protein-cysteine methyltransferase, respectively. The *E. coli* Ada protein sequence was then aligned with the *A. fumigatus* protein sequences using the SIM Alignment Tool at the Swiss Institute of Bioinformatics (<http://www.expasy.ch/tools/sim-prot.html>). The N-terminal domain of Ada preferentially aligned with the putative DNA repair and transcription factor (AFUA_5G06350), while the C-terminal

region of Ada aligned with the *A. fumigatus* putative methylated-DNA-protein cysteine methyltransferase (AFUA_2G02090), making it likely that AFUA_5G06350 and AFUA_2G0290 perform the MPT and AGT functions, analogous to the N- and C-terminal domains of *E. coli* Ada (6,12). Alignments also revealed that critical residues for methyl acceptance in *E. coli* Ada are also present in *A. fumigatus* proteins; AfMPT (Cys-38) and AfAGT (Cys-321), respectively. Protein alignments are shown in Supplementary Figure S1.

Examination of the upstream regions (~300 bp) of the AFUA_5G06350 and AFUA_2G02090 ORFs identified conserved Ada-A and Ada-B boxes, which may represent potential binding sites for the transcriptional activator controlling the adaptive response in *A. fumigatus* (Supplementary Figure S2). Further BLAST searching using bacterial sequences of AlkB and AlkA proteins revealed that there were also homologues in *A. fumigatus* encoded by the genes AFUA_6G07990 and AFUA_4G46800, respectively. Examination of the promoter regions of these ORFs did not identify Ada-A or Ada-B conserved sequences (data not shown). Based on all of these observations, the *A. fumigatus* genes are now referred to as AFUA_5G06350 (*Afmpt*), AFUA_2G02090 (*Afagt*), AFUA_4G06800 (*AfalkA*) and AFUA_6G07990 (*AfalkB*).

Identification of an adaptive response to alkylating agents in *A. fumigatus*

The presence of an adaptive response was investigated phenotypically by measuring fungal growth inhibition in the presence of MNNG following overnight incubation (adaptation) on a sub-lethal dose of MNNG (0.5 µg/ml). At all MNNG concentrations tested, from 1 µg/ml to 4 µg/ml, an approximate 1.5- to 3-fold increase in fungal growth was observed following overnight adaptation compared to the unadapted control ($P < 0.001$; Figure 1A).

Expression of the candidate adaptive response genes, *Afmpt*, *Afagt*, *AfalkA* and *AfalkB* was investigated following exposure to MNNG (0.5 µg/ml) for 30, 60, 120 and 180 min. RT-PCR of cDNA synthesized from extracted RNA indicated that *Afmpt* and *Afagt* were up-regulated at 30 and 60 min, respectively, following MNNG addition and elevated gene expression was maintained for at least 3 h (Figure 1B), while neither *AfalkB* nor *AfalkA* gene expression appeared to be altered (Supplementary Figure S3).

Disruption of *Afmpt* and *Afagt*

Aspergillus fumigatus mpt and *agt* deletion strains (Δ *Afmpt* and Δ *Afagt*) were generated using the bipartite strategy

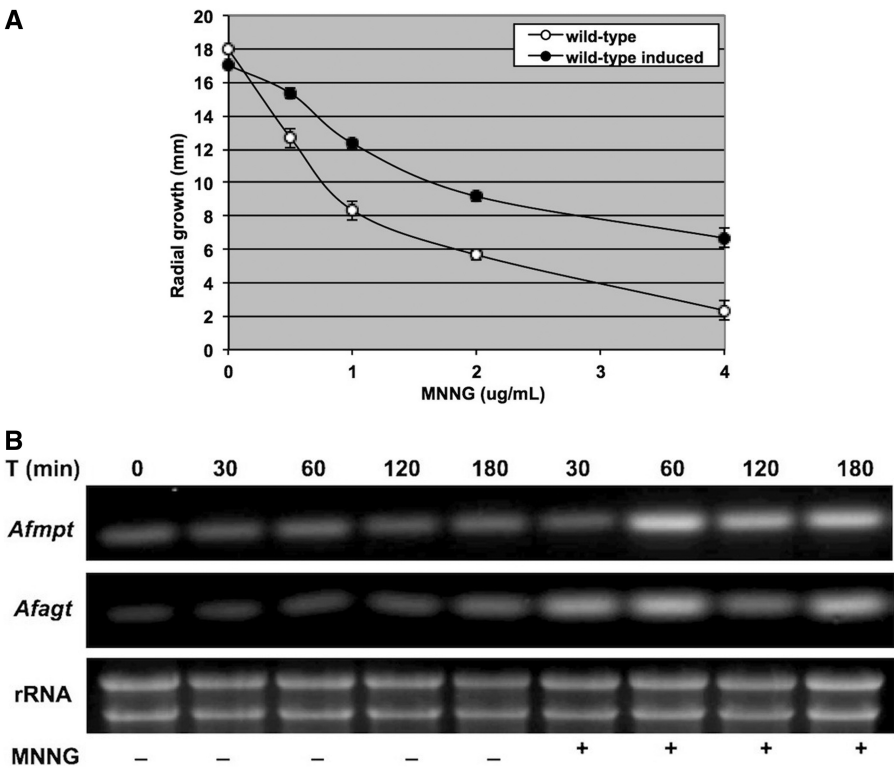


Figure 1. *Aspergillus fumigatus* exhibits an adaptive response to alkylating agents. (A) Adaptive response to the alkylating agent MNNG in *A. fumigatus*. Overnight exposure of *A. fumigatus* conidia to MNNG (0.5 µg/ml; adapted), allowed subsequent growth on elevated concentrations of MNNG (0.5–4 µg/ml, $P < 0.001$) compared to an unadapted control. The radial growths of colonies were measured after 72 h. The mean $\pm$ standard error of three experiments is shown. Key: *** $P < 0.001$. (B) Induction of *A. fumigatus mpt* and *agt* upon exposure to MNNG. Expression of *Afmpt* or *Afagt* in the absence (–) or presence (+) of MNNG over 3 h exposure to MNNG. Lane 1: Time = 0, Lanes 2–5: Uninduced $T = 30, 60, 120$ and 180 min, Lanes 6–9: Induced $T = 30, 60, 120$ and 180 min. *calm* expression remains unchanged. RNA integrity was determined by the presence of intact 26S and 18S ribosomal subunits (rRNA).

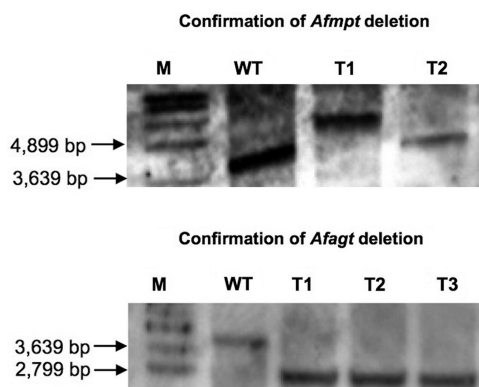


Figure 2. Confirmation of deletion of *Afmpt* and *Afagt* in strain Af293. Identification of $\Delta Afmpt$ and $\Delta Afagt$ by Southern blot analysis. Expected hybridization patterns were observed as described in Supplementary Figure S4. M: Molecular weight marker (Roche VII). WT: Af293 gDNA. $\Delta Afmpt$ candidates; 10, 14: gDNA from two pyrithiamine resistant *Afmpt* transformants, T1 and T2. $\Delta Afagt$ candidates; 9, 11, 24: gDNA from three pyrithiamine resistant *Afagt* transformants, T1, T2 and T3.

(36). Southern blot analysis of genomic DNA from pyrithiamine-resistant transformants confirmed the disruption of *Afmpt* and *Afagt* (Figure 2). Genomic DNA from *Afmpt* transformants was restriction digested with *AfeI*. Expected hybridisation patterns were: wild-type (3951 bp) and $\Delta Afmpt$ (5172 bp). Genomic DNA from $\Delta Afagt$ transformants was restricted with *MfeI*. Expected hybridisation patterns were: wild-type (3967 bp) and $\Delta Afagt$ (2617 bp). Schematic representations of Southern blots are shown in Supplementary Figure S4. Complete replacement of either *Afmpt* or *Afagt* with a pyrithiamine resistance cassette (*ptrA*) was confirmed by Southern blot analysis (Figure 2).

$\Delta Afmpt$ and $\Delta Afagt$ are unable to adapt to MNNG

Phenotypic adaptation experiments indicated that the adaptive response was completely lost upon deletion of *Afmpt* (Figure 3A). $\Delta Afmpt$ showed identical growth rates for all concentrations of MNNG tested regardless of whether this strain was pre-treated or not with an inducing dose of MNNG, and these growth rates were significantly lower than growth of the wild-type strain ($P < 0.001$; Figure 3A). In fact, the $\Delta Afmpt$ strain was unable to grow at the highest MNNG concentration tested (4 $\mu\text{g/ml}$; Figure 3A). Furthermore, phenotypic analysis revealed that $\Delta Afmpt$ and $\Delta Afagt$ were more sensitive to MNNG than wild-type at all concentrations tested when both strains were uninduced ($P < 0.001$; Figure 3B). However, neither of the mutants exhibited increased sensitivity to methyl methanesulphonate (MMS; data not shown).

Afmpt is essential for the induction of *Afagt* following exposure to MNNG

To investigate if the loss of the adaptive response in $\Delta Afmpt$ coincided with a loss of *Afagt* gene induction, we undertook expression analysis and showed that induction of *Afagt* by MNNG addition was lost in the *Afmpt*

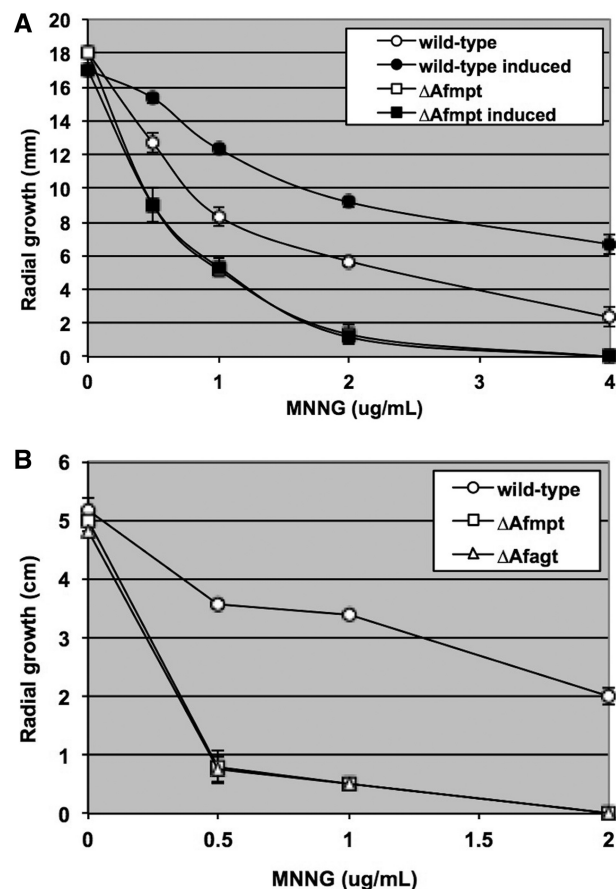


Figure 3. Deletion of *Afmpt* abolishes the adaptive response and sensitizes *A. fumigatus* to MNNG. (A) Deletion of *Afmpt* leads to loss of the adaptive response. Following treatment with a sub-lethal dose of MNNG (0.5 $\mu\text{g/ml}$), radial growth (mm) of $\Delta Afmpt$ in increasing concentrations of MNNG (0–4 $\mu\text{g/ml}$) showed significantly reduced growth compared to wild-type at all concentrations of MNNG ($P < 0.001$). Growth was monitored at 72 h. (B) Unadapted $\Delta Afmpt$ and $\Delta Afagt$ are extremely sensitive to MNNG. $\Delta Afmpt$ and $\Delta Afagt$ display significantly reduced radial growth compared to wild-type on media containing all concentrations of MNNG tested (0–4 $\mu\text{g/ml}$; $P < 0.001$). Growth was monitored at 72 h. Data in A and B displays the mean of three independent experiments and the standard error of the mean. Key *** $P < 0.001$.

deletion strain (Figure 4A). Thus, in contrast to the pattern of *Afagt* expression observed in wild-type cultures exposed to MNNG (Figure 1B), the housekeeping gene *calm* and *Afagt* displayed constitutive transcript levels when cultures were uninduced or induced (Figure 4A). In addition, *Afmpt* gene expression in $\Delta Afagt$ (Figure 4B) showed a similar pattern to that of *A. fumigatus* wild-type (Figure 1B). No changes in gene expression of *AfAlkA* or *AfAlkB* were observed in any of the strains tested with or without addition of MNNG (data not shown).

Afagt can fully complement the alkylation sensitivity of a *Saccharomyces cerevisiae* *mg1* deletion strain

A *S. cerevisiae* *mg1* deletion strain (Euroscarf) was engineered to constitutively express *Afagt* and the resistance of the resulting transformant to MNNG was determined by

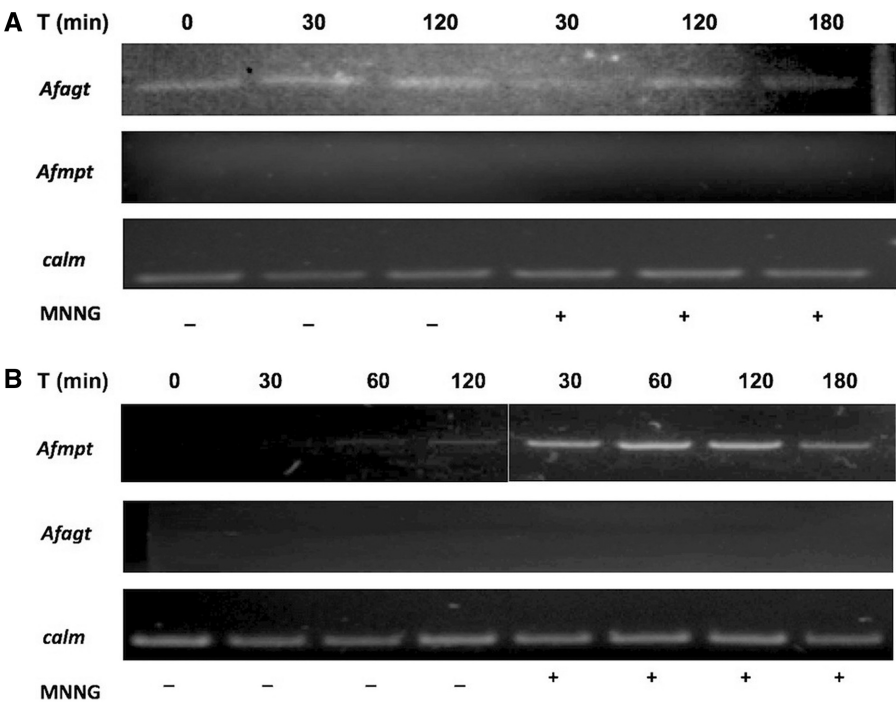


Figure 4. Deletion of *Afmpt* leads to loss of *Afagt* induction by MNNG. (A) RT-PCR analysis demonstrates that *Afagt* mRNA expression is not up-regulated in Δ *Afmpt* following exposure to MNNG, indicating that *Afmpt* transcriptionally regulates the induction of *Afagt*. Lane 1: zero time, Lanes 2 and 3: uninduced (–) 30 min and 120 min. Lanes 4–6: induced (+) 30, 120 and 180 min. (B) RT-PCR analysis demonstrates that *Afmpt* mRNA expression is up-regulated in Δ *Afagt* following exposure to MNNG, indicating that *Afmpt* expression is not dependant on *Afagt*. Lane 1: zero time, Lanes 2 and 3: uninduced (–) 30 and 120 min. Lanes 4–6: induced (+) 30, 120 and 180 min. *calm* expression remains constant over all conditions.

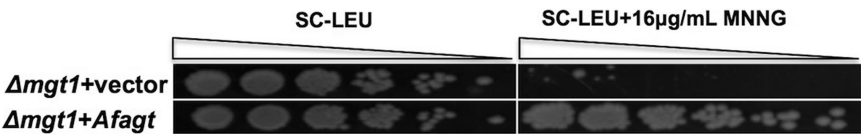


Figure 5. Complementation of *S. cerevisiae* MGT1 deletion by *A. fumigatus* *Afagt*. *S. cerevisiae* expressing *Afagt* exhibited increased growth compared to empty vector control following exposure to medium containing MNNG.

comparison to an appropriate control strain. Expression of *Afagt* in *S. cerevisiae* *mgt1* was able to restore growth on medium containing high levels of MNNG (Figure 5). Protection was observed even at concentrations up to 64 µg/ml MNNG, showing that *in vivo* *AfAGT* is indeed a functional orthologue of the Mgt1 protein.

Up-regulation of methyltransferase activities by MNNG

We determined methyltransferase activity by quantifying the transfer of [³H]-methyl groups from [³H]-*N*-methyl-*N*-nitrosourea-methylated calf thymus DNA to protein: methyl transfer from *O*⁶[³H]meG and [³H]methylphosphotriesters allowed determination of AGT and MPT activity, respectively (see ‘Materials and Methods’ section and Supplementary Figure S5). In initial studies, we first investigated if the putative *AfAGT* and *AfMPT* activities were quantifiable using this assay procedure. Activity was easily detected in cell-free extracts of the *S. cerevisiae* *mgt1* deletion that had been transformed with an expression vector harbouring *Afagt* but activity in

extracts of *Afmpt* transformants was close to that of extracts of vector-transfected controls (Figure 6A). To confirm that the assay was capable of detecting the methylphosphotriester (methylPT) demethylation function of *AfMPT*, we ligated its ORF into pMALC2, expressed the fusion protein in *E. coli* and purified using amylose affinity chromatography. The purified *AfMPT* fusion protein (*rAfMPT*) transferred radioactivity from substrate DNA to an extent that was closely similar to that observed for the amino-terminal region of the Ada MPT protein, that had been expressed and purified under identical conditions (Figure 6A). Furthermore, when *rAfMPT* was mixed with purified recombinant human MGMT, approximately twice as much radioactivity was transferred to protein, but mixtures of *rAfMPT* with *rE. coli* MPT were not additive (Supplementary Figure S5) indicating that like the *E. coli* protein, *rAfMPT* acted only on one of the methylPT isomers. We also found that pre-incubation of the *rAfMPT* at 37°C led to a rapid loss of activity (*t* = 1/2 ~ 5 min) whereas at room

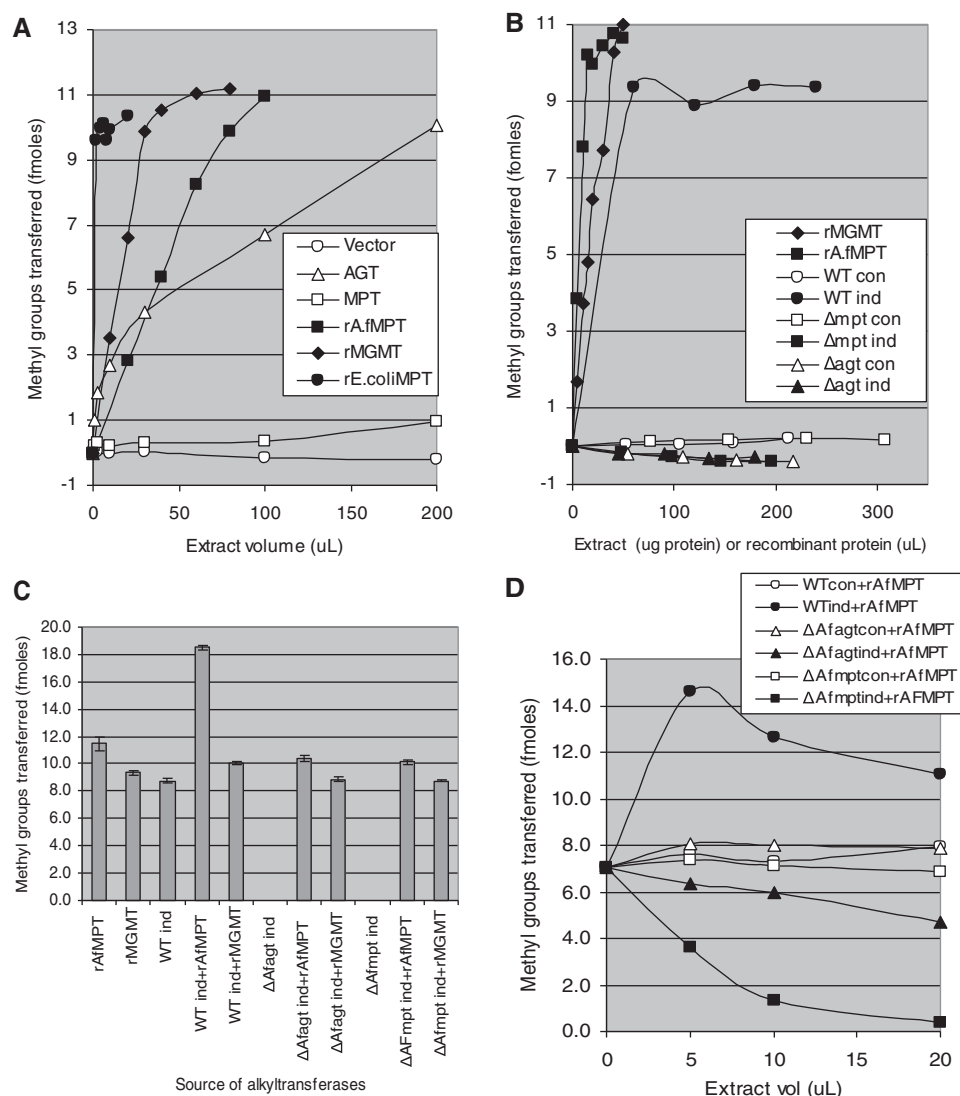


Figure 6. Up-regulation of methyltransferase activity by MNNG. (A) Methyltransferase activity in extracts of *S. cerevisiae* transformed with *AfMPT* (open square) or *AfAGT* (open triangle)-encoding DNA or empty vector (open circle) or of recombinant MGMT (filled diamond), *AfMPT* (filled square) or *E. coli* MPT (filled circle). (B) Methyltransferase activity in extracts of *A. fumigatus* wild-type [control (uninduced), open circle; induced (filled circle)]; *ΔAfagt* (control, open triangle; induced (filled triangle) or *ΔAfMPT* (control, open square; induced (filled square)) and of recombinant MGMT (filled diamond) and *E. coli* MPT (filled circle). (C) Methyltransferase activity of recombinant AfMPT (rAfMPT) or human MGMT (rMGMT) and the effect of co-incubation of these with crude extracts of MNNG-induced (ind) wild-type *A. fumigatus* (Note: no activity was detected in extracts of *ΔAfMPT* or *ΔAfagt* strains). All activities were at substrate limiting levels. Values are means $\pm$ SD of triplicate determinations. (D) Effect of increasing amounts of cell-free extracts of control (open symbols) or MNNG-induced (closed symbols) wild-type (open and filled circles), *ΔAfagt* (open and filled triangles) or *ΔAfMPT* (open and filled squares) strains on the methyltransferase activity of recombinant *AfMPT* (7.1 fmol) under protein-limiting conditions. See 'Materials and Methods' section for experimental details.

temperature there was very little loss of activity over 40 min (Supplementary Figure S5). At room temperature, methyl transfer to rAfMPT (but not to the *E. coli* protein) was complete in approximately 20 min (Supplementary Figure S5). Room temperature incubation was therefore used for assessing transferase activity in crude extracts of the *A. fumigatus* strains.

No [^3H]methyltransferase activity was detectable in cell-free extracts of any of the uninduced *A. fumigatus* strains under the assay conditions used. However, activity was easily detected in extracts of the MNNG-induced wild-type strain, but not in those of the deletants

(Figure 6B). To investigate the nature of the induced activity, under substrate-limiting conditions, we added recombinant *AfMPT* or human MGMT to the extracts prior to addition of substrate DNA. This resulted in higher levels of transferase activity only when rAfMPT was added to the induced wild-type extract (Figure 6C), demonstrating that the activity seen was exclusively the O^6 -meG repair function, *AfAGT*. MNNG treatment thus substantially up-regulates the expression of *AfAGT*. The extent of this induction cannot be accurately calculated because activity was not quantifiable in the pre-MNNG extract under the conditions used, but the

induced level of activity was calculated to equate to 2.7×10^3 molecules of *Af*AGT per cell. Based on the assay's lower limit of quantitation of activity, the uninduced wild-type strain contained less than ~ 30 molecules of *Af*AGT per cell, suggesting a ~ 100 -fold induction of expression at the protein level.

Our inability to detect MPT activity in the cell-free extracts of induced wild-type *A. fumigatus* might indicate that the levels of its expression are extremely low, as might be the case if its function is principally as a signal transduction factor. One of the possible consequences of this putative low level activity could be increased levels of persistent methylPT DNA damage from the MNNG used to induce expression. To examine this, we added incrementally increasing amounts of the cell-free extracts of control or induced wild-type or deletant *A. fumigatus* to protein-limiting amounts of recombinant *Af*MPT (~ 7 fmoles). None of the control extracts had a measurable effect on the levels of *rAf*MPT activity indicating that there was little or no endogenous methylation damage in the DNA in the cell-free extracts (Figure 6D). In contrast, the MNNG-induced $\Delta Afmpt$ cell-free extract markedly inhibited methyl transfer to *rAf*MPT, indicating that high levels of unrepaired methylPT were, as anticipated, present in the DNA within the extracts. The inactivation of recombinant *Af*MPT by extracts of $\Delta Afagt$ was considerably less, indicating that substantial, but nevertheless incomplete, repair of methylPT had taken place in this strain. It is reasonable to conclude that, in the $\Delta Afagt$ strain, the intact *Afmpt* gene had been up-regulated following MNNG treatment, but to an extent that was insufficient to eliminate all the methylPT from DNA. This probably explains our inability to detect *Af*MPT functional activity in the induced extracts. We also confirmed our earlier conclusion that *Afmpt* up-regulation is not dependent upon the presence of a functional *Afagt* gene. While the addition of small amounts of induced WT extract to *rAf*MPT had an additive effect on methyl transfer as a result of the combined effects of up-regulated *Af*AGT and the added *rAf*MPT (Figure 6C and D), addition of larger volumes reduced methyl transfer (Figure 6D): again this suggests that the repair of methylPT was incomplete despite the up-regulation of *Af*MPT. These results provide additional evidence for MNNG-mediated up-regulation of *Af*MPT, but also explain why we were unable to detect any increase in *Af*MPT activity, in contrast to that for *Af*AGT.

The eukaryotic adaptive response to alkylating agents is confined to the Fungal kingdom and is of bacterial origin

Using comparative genomics, we assessed the extent of the adaptive response in Eukaryotes. We hypothesize that in order for an organism to potentially possess the adaptive response to alkylating agents, analogous to what we have observed in *A. fumigatus*, it must contain orthologues of MPT and AGT.

Automated BLAST database searches followed by manual inspection of homologues showed that the AGT gene is ubiquitous in the tree of life (Supplementary Table S1). Where it is absent we expect that other DNA

repair proteins such as alkyltransferase-like proteins may be present. However, our analysis indicates that MPT is largely absent from Eukaryotes and where it is present, it is confined to the Fungal kingdom (Supplementary Table S1). Closer inspection infers that the potential adaptive response is actually restricted to members of the Ascomycota lineage (some of which are major human or plant pathogens; Figure 7) as they contain a copy of both MPT and AGT, similar to what is observed in *A. fumigatus*.

The origin of the MPT gene in the Ascomycota lineage is intriguing. There are two competing possibilities. First, the MPT gene may have arisen in the last common ancestor (LCA) of the Ascomycete species represented in our analysis. The Ascomycete MPT was then retained through multiple speciation events, and at some point acquired by bacterial species *via* horizontal gene transfer (HGT). Alternatively, the MPT gene may be of bacterial origin and was acquired through HGT from a bacterial source by the Ascomycete LCA and subsequently retained during speciation. We tested these hypotheses by reconstructing a robust maximum likelihood phylogenetic gene-tree based on clearly identifiable MPT orthologues from the Bacterial and Fungal kingdoms (Figure 8). Our phylogeny infers that both bacterial and fungal MPTs form independent, strongly supported (82% bootstrap support) monophyletic clades (Figure 8). Interestingly, the vast majority of bacterial species represented in our analysis belong to the Firmicutes phylum. The Firmicutes are an ancient bacterial lineage that shared a common ancestor approximately 2.5 billion years ago (42), whereas the Ascomycetes are relative newcomers, having diverged from their Dikarya ancestor approximately 450–968 million years ago (43–45). Due to its distribution in a wide variety of Firmicutes species, the most parsimonious explanation would suggest an ancient HGT event of MPT from a Firmicutes ancestor into the LCA of the Ascomycetes represented in this analysis. The alternative (unparsimonious) explanation requires multiple independent HGTs of the Ascomycete MPT gene into different Firmicutes species.

DISCUSSION

The *E. coli* Ada protein confers resistance to the biological effects of alkylating agents through its dual functions as both a transcriptional activator of the adaptive response genes (*ada*, *aidB*, *alkA* and *alkB*), and as a direct repair protein for the principal toxic and mutagenic lesion, *O*⁶-alkylguanine, generated in DNA by alkylating agents (14,15). Bioinformatic and protein alignment analysis revealed *ada* orthologues in *A. fumigatus*, referred to here as *Afmpt* and *Afagt*, which encode individual proteins that resemble the N and C terminal domains of *E. coli* Ada, i.e. *Af*MPT and *Af*AGT, respectively. Protein sequence alignment of *Af*MPT indicated a high level of sequence conservation with the N-terminal transcriptional activator domain of *E. coli* Ada, strongly indicating that *Af*MPT could act as a transcriptional activator for the adaptive response in *A. fumigatus*. Alignment of *Af*AGT

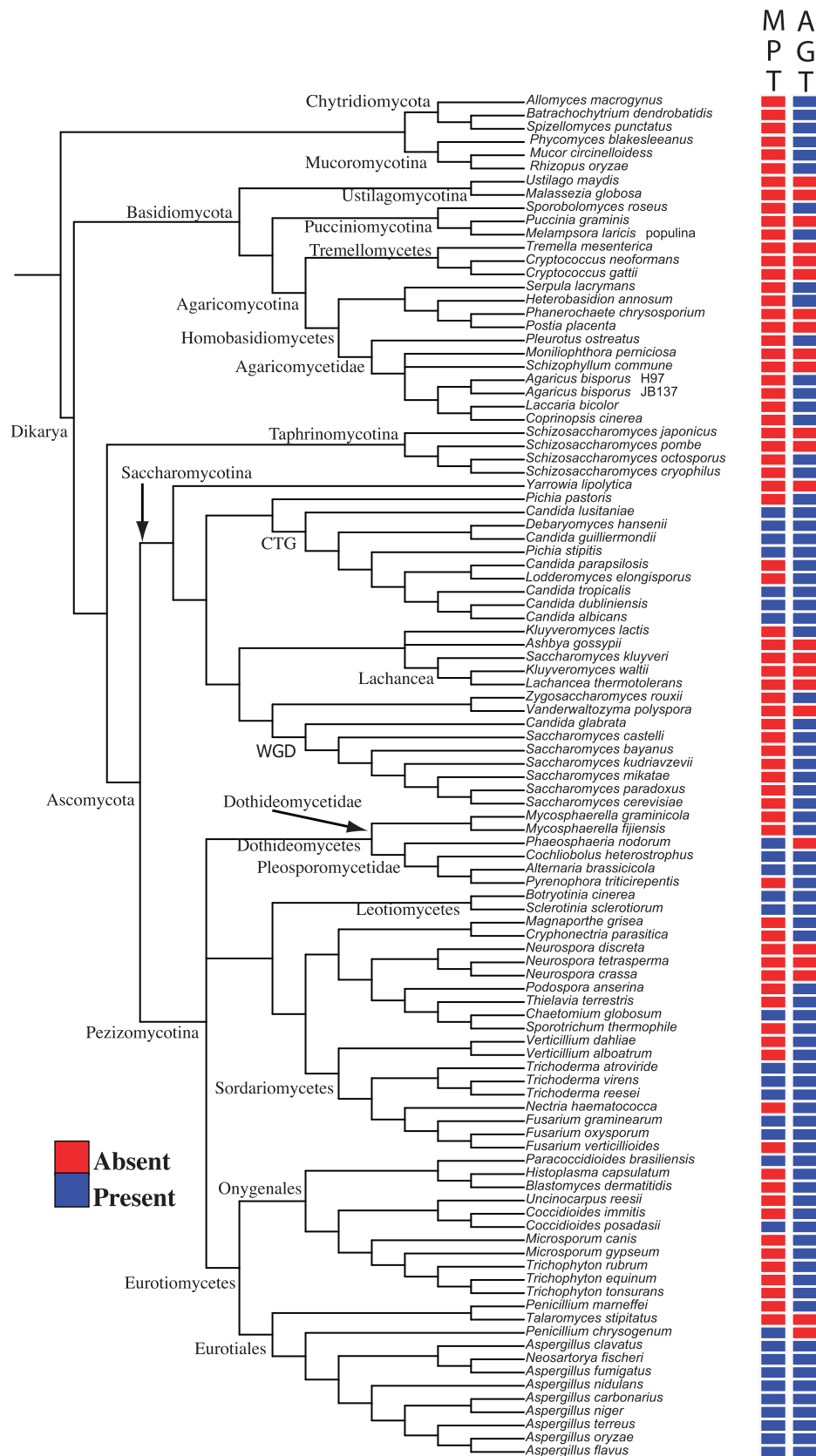


Figure 7. MPT protein maximum likelihood phylogeny. MPT orthologues were sourced from a local database that contained 35 Eukaryote species in addition to all available Fungal and Bacterial genome data (see 'Materials and Methods' section). Bootstrap scores are displayed above branches. Fungal MPT proteins are found as a strongly supported monophyletic clade (82% bootstrap support). All fungal orthologues are belonging to the Ascomycetes. Bacterial MPT orthologues also form a monophyletic clade; all bacterial orthologues belong to the Firmicutes phylum, the two exceptions are *Desulfovibrio desulfuricans* and *Bradyrhizobium japonicum* (highlighted) which are both proteobacteria.



Figure 8. Fungal species phylogeny. Species phylogeny modified from Medina *et al.* (2011). The presence or absence of MPT and AGT in each species is signified by red (absent) and blue (present) rectangles. AGT is distributed throughout the fungal tree of life. MPT is restricted to the Ascomycota lineage.

with *E. coli* Ada indicates that *Af*AGT is likely to be a functional homologue of the C-terminal domain of Ada, possessing the O^6 meG and O^4 meT transferase activities. This situation is similar to the organization of the adaptive response operon in *B. subtilis*, whereby the dual function of Ada is split into two distinct proteins, AdaA and AdaB (46). Indeed, based on Ada's structure, both *Af*MPT and *Af*AGT appeared to possess the critical cysteine residues for acceptance of the methyl groups. It should also be noted that *A. fumigatus* only contains one ORF encoding an O^6 -alkylguanine DNA alkyltransferase (*Aafgt*). In this article, we show that this gene is clearly part of an adaptive response to alkylating agents, and hence we consider that *Afagf* should be considered as a true Ada C-terminal domain orthologue rather than orthologue of the *E. coli* Ogt, which also acts on O^6 meG, but is not up-regulated by MNNG exposure (47). Although it has been demonstrated that mammalian O^6 -methylguanine DNA methyltransferase can be induced in some cells to provide protection against subsequent exposure to methylating agents, an adaptive response similar to that in bacterial cells is clearly absent from mammalian systems (48).

In this article, we have clearly shown the existence of the adaptive response in *A. fumigatus*. Previously, an adaptive response to MNNG has been reported in *A. nidulans* (22), whereby incubation of cell free extracts with radiolabelled substrate DNA resulted in the detection of four proteins in gel electrophoresis. These were designated ANAT1-4 (for *Aspergillus nidulans* AlkylTransferase), three being predicted as AGTs and one as a MPT (24). Interrogating the *A. nidulans* genome identifies only two ORFs and these clearly encode an AGT and a MPT (G. W. Jones, unpublished observation). It therefore seems that two of the original ANAT proteins identified by Baker *et al.* (24) as possessing AGT activity are processed or modified versions of a single AGT in this organism. Thus, the number of repair proteins involved in the adaptive response to alkylating agents appears to be conserved between *A. fumigatus* and *A. nidulans*, as may be the overall control of the response.

The adaptive response was further investigated by examining the mRNA expression levels of *Afmpt*, *Afagf*, *AfalkB* and *AfalkA* following exposure to MNNG. RT-PCR indicated that the expression levels of *Afmpt* and *Afagf* were induced upon exposure to MNNG, suggesting that *Afmpt* and *Afagf* are important in the adaptive response in *A. fumigatus*. Induction of *Afagf* mRNA was observed after 30 min exposure to MNNG and persisted for at least 3 h, indicating that the induced expression of *Afagf* is initiated rapidly following exposure to MNNG and that this response is persistent for several hours. *Afmpt* expression was elevated at 1 h following addition of MNNG and this was also persistent throughout the 3 h period investigated in this study. No induction was observed for *AfalkB* or *AfalkA* mRNA upon MNNG exposure. This lack of induction may reflect the absence of potential Ada box-like transcriptional binding sites within the upstream promoter regions (~300 bp) of either *AfalkA* or *AfalkB*.

Subsequently, the roles of the *Afmpt* and *Afagf* encoded proteins within *A. fumigatus* were investigated further using a gene disruption approach. Both genes were individually deleted in *A. fumigatus* Af293 using the bipartite strategy and a pyrithiamine resistance cassette as a selection marker, with modifications (36–38). When tested for an adaptive response, Δ *Afmpt* failed to adapt to MNNG following induction with a non-lethal dose when compared to wild-type ($P < 0.001$). In fact, Δ *Afmpt* displayed almost identical growth rate on MNNG from 0 μ g/ml to 4 μ g/ml whether or not it was induced with MNNG, indicating complete loss of the adaptive response when *Afmpt* was absent, and confirming that adaptation to MNNG is mediated by this protein within *A. fumigatus*.

Deletion of either *Afmpt* or *Afagf* lead to significantly increased sensitivity to MNNG (0.5–2 μ g/ml) when compared to wild-type ($P < 0.001$) and no growth was observed for either strain for the highest concentration of MNNG tested (4 μ g/ml). No differences were observed between wild-type, Δ *Afmpt* or Δ *Afagf* regarding sensitivity to MMS, indicating that *Af*MPT or *Af*AGT are most likely not involved in protection against or repair of the lethal lesions caused by this alkylating agent. Given that the primary toxic lesions produced by MMS treatment would be repaired by the *AfalkA* and *AfalkB* gene products, and that these genes are not part of the adaptive response in *A. fumigatus*, the lack of sensitivity of the Δ *Afmpt* and Δ *Afagf* strains to MMS is not surprising. Overall, these phenotypic analyses suggest that the roles of *Afmpt* and *Afagf* are specific in the response to and protection against specific alkylation lesions caused by MNNG, of which the main biologically relevant lesion is O^6 meG.

RT-PCR analysis showed no *Afagf* or *Afmpt* expression in Δ *Afagf* or Δ *Afmpt*, respectively, under non-inducing conditions, thus validating the gene disruption strategy used in this study. While *Afagf* expression was evident in Δ *Afmpt*, there was no difference in the level of expression between uninduced and induced conditions. This indicates that *Afagf* expression was not up-regulated by MNNG in the Δ *Afmpt* mutant strain and hence that *Afmpt* is essential for elevated transcription of *Afagf* by MNNG in *A. fumigatus*. This confirms the role of *Afmpt* as the fungal equivalent of the *E. coli* Ada transcriptional activator in *A. fumigatus*. Thus, the expression level of *Afmpt* was markedly increased in the Δ *Afagf* strain under inducing conditions indicates that *Afmpt* expression in response to MNNG is not dependent on the presence of *Afagf* but induces its own expression. The presence of the AdaA and AdaB boxes in the promoter, coupled with the biochemical data discussed below, certainly support this hypothesis. At this point, it cannot be excluded that *Afmpt* could be affecting the transcription of other gene targets when *Afagf* is deleted. However, the vastly increased sensitivity to MNNG in Δ *Afagf* strongly argues that *Afagf* is essential for the repair of toxic lesions caused by MNNG.

The *Afagf* and *Afmpt* ORFs were confirmed to encode active methyltransferase proteins using a functional *in vitro* assay of extracts of *S. cerevisiae* transformed

with *Af*AGT or purified recombinant *Af*MPT expressed as an MBP-fusion protein in *E. coli*. Mixing experiments indicated that *Af*MPT demethylates the same methylPT stereoisomer as does the C-terminal domain of the Ada protein. The *Af*MPT was found to be much less stable than the *E. coli* equivalent, and to transfer methyl groups rapidly at room temperature, but no further characterization was undertaken in this study. The levels of methyltransferase activities in cell-free extracts of wild-type, $\Delta Afagt$ and $\Delta Afmpt$ strains were lower than the lower limit of quantification (LOQ) of the assay, suggesting the constitutive expression of about 30 or less molecules per cell. Adaptive MNNG treatment up-regulated expression of *Af*AGT activity at least 100-fold in wild-type but *Af*MPT activity remained lower than the LOQ, as did both functions in the MNNG-treated $\Delta Afagt$ and $\Delta Afmpt$ strains. That DNA in the $\Delta Afagt$ and wild-type extracts contained much lower levels of methylPT than the $\Delta Afmpt$ extract clearly demonstrated that *Af*MPT expression had been induced by MNNG in the wild-type and $\Delta Afagt$ strains. It is reasonable to conclude that *Af*MPT is required for the up-regulation of both itself and *Af*AGT but that the level of induction of *Af*MPT is much lower than that of *Af*AGT, so that it is completely inactivated by the levels of damage introduced by MNNG.

Since Baker *et al.* (24) reported the existence of both AGT and MPT activities in *A. nidulans*, no further work has been reported demonstrating the presence of a MPT in any eukaryotic organism. We reasoned that if MPTs are indeed absent from most eukaryotes but can be shown to exist in pathogenic fungi, there is potential for these DNA repair proteins to be novel therapeutic targets. We clearly show that the presence of MPTs in the Eukarya is confined to the Fungi (Supplementary Table S1). This absence from non-fungal eukaryotes may be a direct reflection of reliance on other highly efficient ways of dealing with alkylation damage. The presence of MGMT in higher eukaryotes will provide protection from lesions such as O^6 -meG, while other biologically relevant lesions may be efficiently dealt with through the action of the mammalian AlkB dioxygenase orthologues, of which humans have two [for review see Sedgwick (49)]. Within the Fungi the presence of the MPT, and hence presumably an adaptive response, is restricted to a number of Ascomycetes (Figure 7). Identifying an adaptive response and presence of a MPT in *A. fumigatus*, as well as predicting the same in pathogenic *Fusarium* and *Candida* species, further highlights this adaptive pathway as a possible novel therapeutic target. Perhaps the presence of the adaptive response in these pathogens has been evolutionary conserved in part as a defence mechanism from host-attack during infection. The apparent absence of MPT and AGT from some fungi mostly reflects the presence of other proteins capable of dealing with alkylated DNA lesions, namely the alkyltransferase-like proteins (ATLs) (16). While it has long been assumed that the adaptive response to alkylating agents is of bacterial origin, this has not been formerly proven. Our comparative genomic and phylogenetic analysis clearly demonstrates that the MPT (and hence the adaptive response based upon the MPT) is bacterial in origin and

arose in the Firmicutes phylum (Figure 8). The Firmicutes species analysed all possess individual copies of MPT and AGT and we predict that the Ada-type proteins have arisen through fusion of separate MPT and AGT rather than *vice-versa*. The phylogenetic MPT gene-tree (Figure 8) clearly splits the bacterial and fungal MPTs. The main conclusion from this tree is that the MPT arose in the Firmicutes phylum and was transferred *via* inter-kingdom HGT to the LCA of the Ascomycete species analysed in this study. These data represent the most comprehensive assessment to date of the evolutionary origin of the adaptive response.

In conclusion, this study reports the first demonstration of an adaptive response to alkylating agents in the opportunistic pathogen *A. fumigatus* and the genetic and phenotypic characterization of the key components. Our work also clearly defines the evolutionary history of the adaptive response. The lack of an equivalent adaptive response pathway in mammalian cells makes this pathway an interesting area for further characterization, as it could prove to be a novel therapeutic drug target in cases of aspergillosis where immunocompromised individuals are being treated with chemotherapeutic alkylating agents. In addition, as MPT are not known in man, this represents a clear distinction between *A. fumigatus* and human cells, so, for example, the possibility exists to develop a prodrug that is activated by *Af*MPT and should specifically sacrifice *A. fumigatus* cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figures 1–5.

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REFERENCES

1. Abad,A., Victoria Fernandez-Molina,J., Bikandi,J., Ramirez,A., Margareto,J., Sendino,J., Luis Hernando,F., Ponton,J., Garaizar,J. and Rementeria,A. (2010) *Rev Iberoam Micol*, 2010 Revista Iberoamericana de Micología. Espana: Elsevier.
2. Beauvais,A. and Latge,J.P. (2001) Membrane and cell wall targets in *Aspergillus fumigatus*. *Drug Resist. Updat.*, **4**, 38–49.
3. Cisar,J.S. and Tan,D.S. (2008) Small molecule inhibition of microbial natural product biosynthesis: an emerging antibiotic strategy. *Chem. Soc. Rev.*, **37**, 1320–1329.

4. Loeffler, J. and Stevens, D.A. (2003) Antifungal drug resistance. *Clin. Infect. Dis.*, **36**, S31–S41.
5. Nierman, W.C., Pain, A., Anderson, M.J., Wortman, J.R., Kim, H.S., Arroyo, J., Berriman, M., Abe, K., Archer, D.B., Bermejo, C. *et al.* (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature*, **438**, 1151–1156.
6. Demple, B., Sedgwick, B., Robins, P., Totty, N., Waterfield, M.D. and Lindahl, T. (1985) Active-site and complete sequence of the suicidal methyltransferase that counters alkylation mutagenesis. *Proc. Natl Acad. Sci. USA*, **82**, 2688–2692.
7. Wyatt, M.D. and Pittman, D.L. (2006) Methylating agents and DNA repair responses: methylated bases and sources of strand breaks. *Chem. Res. Toxicol.*, **19**, 1580–1594.
8. Samson, L. and Cairns, J. (1977) New pathway for dna-repair in *Escherichia coli*. *Nature*, **267**, 281–283.
9. Sedgwick, B. and Lindahl, T. (2002) Recent progress on the Ada response for inducible repair of DNA alkylation damage. *Oncogene*, **21**, 8886–8894.
10. Kleibl, K. (2002) Molecular mechanisms of adaptive response to alkylating agents in *Escherichia coli* and some remarks on O(6)-methylguanine DNA-methyltransferase in other organisms. *Mutat. Res.*, **512**, 67–84.
11. Olsson, M. and Lindahl, T. (1980) Repair of alkylated DNA in *Escherichia coli*. Methyl group transfer from O6-methylguanine to a protein cysteine residue. *J. Biol. Chem.*, **255**, 10569–10571.
12. Kataoka, H., Hall, J. and Karran, P. (1986) Complementation of sensitivity to alkylating agents in *Escherichia coli* and Chinese hamster ovary cells by expression of a cloned bacterial DNA repair gene. *EMBO J.*, **5**, 3195–3200.
13. Takinowaki, H., Matsuda, Y., Yoshida, T., Kobayashi, Y. and Ohkubo, T. (2004) 1H, 13C and 15N resonance assignments of the N-terminal 16 kDa domain of *Escherichia coli* Ada protein. *J. Biomol. NMR (Netherlands)*, **29**, 447–448.
14. Teo, I., Sedgwick, B., Demple, B., Li, B. and Lindahl, T. (1984) Induction of resistance to alkylating-agents in *Escherichia coli*: the ADA+ gene-product serves both as a regulatory protein and as an enzyme for repair of mutagenic damage. *EMBO J.*, **3**, 2151–2157.
15. Nakabeppu, Y. and Sekiguchi, M. (1986) Regulatory mechanisms for induction of synthesis of repair enzymes in response to alkylating-agents: ADA protein acts as a transcriptional regulator. *Proc. Natl Acad. Sci. USA*, **83**, 6297–6301.
16. Margison, G.P., Butt, A., Pearson, S.J., Wharton, S., Watson, A.J., Marriott, A., Caetano, C.M., Hollins, J.J., Rukzenkova, N., Begum, G. *et al.* (2007) Alkyltransferase-like proteins. *DNA Repair (Amst)*, **6**, 1222–1228.
17. Sedgwick, B. and Vaughan, P. (1991) Widespread adaptive response against environmental methylating agents in microorganisms. *Mutat. Res.*, **250**, 211–221.
18. Maga, J.A. and McEntee, K. (1985) Response of *S. cerevisiae* to N-methyl-N'-nitro-N-nitrosoguanidine: mutagenesis, survival and DDR gene expression. *Mol. Gen. Genet.*, **200**, 313–321.
19. Polakowska, R., Perozzi, G. and Prakash, L. (1986) Alkylation mutagenesis in *Saccharomyces cerevisiae*: lack of evidence for an adaptive response. *Curr. Genet.*, **10**, 647–655.
20. Sassanfar, M. and Samson, L. (1990) Identification and preliminary characterization of an O6-methylguanine DNA repair methyltransferase in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **265**, 20–25.
21. Xiao, W., Derfler, B., Chen, J. and Samson, L. (1991) Primary sequence and biological functions of a *Saccharomyces cerevisiae* O6-methylguanine/O4-methylthymine DNA repair methyltransferase gene. *EMBO J.*, **10**, 2179–2186.
22. Hooley, P., Shawcross, S.G. and Strike, P. (1988) An adaptive response to alkylating-agents in *Aspergillus nidulans*. *Curr. Genet.*, **14**, 445–449.
23. Swirski, R.A., Shawcross, S.G., Faulkner, B.M. and Strike, P. (1988) Repair of alkylation damage in the fungus *Aspergillus nidulans*. *Mutat. Res.*, **193**, 255–268.
24. Baker, S.M., Margison, G.P. and Strike, P. (1992) Inducible alkyltransferase DNA-repair proteins in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res.*, **20**, 645–651.
25. Pontecorvo, G., Roper, J.A., Hemmons, L.M., Macdonald, K.D. and Bufton, A.W.J. (1953) The genetics of *Aspergillus nidulans*. *Adv. Genet. Incorporating Mol. Genet. Med.*, **5**, 141–238.
26. Schrettli, M., Carberry, S., Kavanagh, K., Haas, H., Jones, G.W., O'Brien, J., Nolan, A., Stephens, J., Fenelon, O. and Doyle, S. (2010) Self-protection against gliotoxin-A component of the gliotoxin biosynthetic cluster, GliT, completely protects *Aspergillus fumigatus* against exogenous gliotoxin. *Plos Pathogens*, **6**, 15.
27. Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. and Sayers, E.W. (2009) GenBank. *Nucleic Acids Res.*, **37**, D26–D31.
28. Mabey, J.E., Anderson, M.J., Giles, P.F., Miller, C.J., Attwood, T.K., Paton, N.W., Bornberg-Bauer, E., Robson, G.D., Oliver, S.G. and Denning, D.W. (2004) CADRE: the Central *Aspergillus* Data Repository. *Nucleic Acids Res.*, **32**, D401–D405.
29. Medina, E.M., Jones, G.W. and Fitzpatrick, D.A. (2011) Reconstructing the fungal tree of life using phylogenomics and a preliminary investigation of the distribution of yeast prion-like proteins in the fungal kingdom. *J. Mol. Evol.*, **73**, 116–133.
30. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, **25**, 3389–3402.
31. Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.*, **32**, 1792–1797.
32. Keane, T.M., Creevey, C.J., Pentony, M.M., Naughton, T.J. and McLnerney, J.O. (2006) Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. *BMC Evol. Biol.*, **6**, 29.
33. Guindon, S. and Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.*, **52**, 696–704.
34. Burns, C., Geraghty, R., Neville, C., Murphy, A., Kavanagh, K. and Doyle, S. (2005) Identification, cloning, and functional expression of three glutathione transferase genes from *Aspergillus fumigatus*. *Fungal Genet. Biol.*, **42**, 319–327.
35. Tilburn, J., Scazzocchio, C., Taylor, G., Lockington, R.A., Sissman, J.O.Z. and Davies, R.W. (1983) Transformation in *Aspergillus nidulans*. *Heredity*, **51**, 519–520.
36. Nielsen, M.L., Albertsen, L., Lettier, G., Nielsen, J.B. and Mortensen, U.H. (2006) Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. *Fungal Genet. Biol.*, **43**, 54–64.
37. Kubodera, T., Yamashita, N. and Nishimura, A. (2000) Pyrithiamine resistance gene (ptrA) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. *Biosci. Biotechnol. Biochem.*, **64**, 1416–1421.
38. Kubodera, T., Yamashita, N. and Nishimura, A. (2002) Transformation of *Aspergillus* sp and *Trichoderma reesei* using the Pyrithiamine resistance gene (ptrA) of *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.*, **66**, 404–406.
39. Schwimmer, C. and Masison, D.C. (2002) Antagonistic interactions between yeast PSI+ and URE3 prions and curing of URE3 by Hsp70 protein chaperone Ssa1p but not by Ssa2p. *Mol. Cell. Biol.*, **22**, 3590–3598.
40. Loovers, H.M., Guinan, E. and Jones, G.W. (2007) Importance of the Hsp70 ATPase domain in yeast prion propagation. *Genetics*, **175**, 621–630.
41. Watson, A.J., Sabharwal, A., Thorncroft, M., McGown, G., Kerr, R., Bojanic, S., Soonawalla, Z., King, A., Miller, A., Waller, S. *et al.* (2010) Tumor O(6)-methylguanine-DNA methyltransferase inactivation by oral lomeguatrib. *Clin. Cancer Res.*, **16**, 743–749.
42. Battistuzzi, F.U., Feijao, A. and Hedges, S.B. (2004) A genomic timescale of prokaryote evolution: insights into the origin of methanogenesis, phototrophy, and the colonization of land. *BMC Evol. Biol.*, **4**, 44.
43. Taylor, J.W. and Berbee, M.L. (2006) Dating divergences in the fungal tree of life: review and new analyses. *Mycologia*, **98**, 838–849.
44. Douzery, E.J., Snell, E.A., Baptiste, E., Delsuc, F. and Philippe, H. (2004) The timing of eukaryotic evolution: does a relaxed molecular clock reconcile proteins and fossils? *Proc. Natl Acad. Sci. USA*, **101**, 15386–15391.

45. Hedges, S.B., Blair, J.E., Venturi, M.L. and Shoe, J.L. (2004) A molecular timescale of eukaryote evolution and the rise of complex multicellular life. *BMC Evol. Biol.*, **4**, 2.
46. Morohoshi, F., Hayashi, K. and Munakata, N. (1990) *Bacillus subtilis* ada operon encodes two DNA alkyltransferases. *Nucleic Acids Res.*, **18**, 5473–5480.
47. Potter, P.M., Wilkinson, M.C., Fitton, J., Carr, F.J., Brennand, J., Cooper, D.P. and Margison, G.P. (1987) Characterisation and nucleotide sequence of ogt, the O6-alkylguanine-DNA-alkyltransferase gene of *E. coli*. *Nucleic Acids Res.*, **15**, 9177–9193.
48. Fritz, G., Tano, K., Mitra, S. and Kaina, B. (1991) Inducibility of the DNA repair gene encoding O6-methylguanine-DNA methyltransferase in mammalian cells by DNA-damaging treatments. *Mol. Cell. Biol.*, **11**, 4660–4668.
49. Sedgwick, B. (2004) Repairing DNA-methylation damage. *Nat. Rev. Mol. Cell Biol.*, **5**, 148–157.