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-N0-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 O6-alkylguanine DNA alkyltransferase (AGT) DNA repair proteins; deletions of either of these genes abolish the adaptive response and sensitize the organism to MNNG. 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.

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-N0-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 ($O^6$meG) 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 $O^6$meG 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 $O^6$meG 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 $O^6$meG and methylphosphotriester (methylPT) lesions, respectively. Functional conservation of AGT activity is confirmed by the ability of *Af*AGT 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, 5 mM ammonium tartrate as nitrogen-source and trace elements according to Pontecorvo et al. (25). Fungal culturing was performed as previously described (26). *E. coli* DH5α was used for general plasmid DNA propagation and cultured in LB-AMP (containing 100 μg/ml) for plasmid maintenance. *A. fumigatus* genomic DNA was purified using a ZR Fungal/Bacterial DNA Kit (ZymoResearch). *S. cerevisiae* strain used was BY4741 *Amp1* 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
fungal 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 RNeasy™ 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 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 (10^7) 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 round 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 primer pairs were designed to incorporate 5’ NdeI and 3’ SphI restriction sites onto both Afmpt and Afagt selection markers. The plasmid containing the Afmpt or Afagt marker was then used to transform the S. cerevisiae strain used in this study (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 the S. cerevisiae transformation plasmid, resulting in the construction of the Afmpt or Afagt-harboring S. cerevisiae plasmids.
were ligated into pC210 to form pC-Afmp 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 Afmp**

The *Afmp* gene was obtained by PCR using a suspension of a single colony of *Afmp*-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 TGGTGTAGTCCAATGT) and AF reverse (5' Phos CTTTGTACTTGCTTGCCAAAAG). 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 XmnI, purified and ligated to XmnI-digested XmnI at a 1:1 molar ratio. The ligation reaction was incubated at room temperature overnight and then transformed to competent *E. coli* DH5α. 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 CTTTGTACTTGCTTGCCAAAAG and 5'Phos AA TGTATGCGCGTGGCATCG.

For protein over-expression and purification, a 40 µl 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 OD₆₅₀ 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 x 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 ammediol buffer. [3H]MNU (ARC; 80 Ci/m mole; 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 ammediol 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 µg of this DNA which, by assay with recombinant MGMT and recombinant *E. coli* MPT and scintillation counting was calculated to contain ~10 fmole both of the substrate methylphosphotriester stereoisomer and of O⁵-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 µg of substrate in a total volume of 300 µl 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 *A/MPT* (~7 fmole) or recombinant MGMT (~6 fmole) 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 \textit{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 \textit{E. coli} Ada (6,12). Alignments also revealed that critical residues for methyl acceptance in \textit{E. coli} Ada are also present in \textit{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 \textit{A. fumigatus} (Supplementary Figure S2). Further BLAST searching using bacterial sequences of AlkB and AlkA proteins revealed that there were also homologues in \textit{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 \textit{A. fumigatus} genes are now referred to as AFUA\_5G06350 (\textit{Afmp\textit{t}}), AFUA\_2G02090 (\textit{Afagt}), AFUA\_4G06800 (\textit{AfalkA}) and AFUA\_6G07990 (\textit{AfalkB}).

Identification of an adaptive response to alkylating agents in \textit{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 \(\mu\)g/ml). At all MNNG concentrations tested, from 1 \(\mu\)g/ml to 4 \(\mu\)g/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, \textit{Afmp\textit{t}}, \textit{Afagt}, \textit{AfalkA} and \textit{AfalkB} was investigated following exposure to MNNG (0.5 \(\mu\)g/ml) for 30, 60, 120 and 180 min. RT-PCR of cDNA synthesized from extracted RNA indicated that \textit{Afmp\textit{t}} and \textit{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 \textit{AfalkB} nor \textit{AfalkA} gene expression appeared to be altered (Supplementary Figure S3).

Disruption of \textit{Afmp\textit{t}} and \textit{Afagt}

\textit{Aspergillus fumigatus} \textit{mp\textit{t}} and \textit{agt} deletion strains (\textit{\DeltaAfmp\textit{t}} and \textit{\DeltaAfagt}) were generated using the bipartite strategy...
(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 ΔAfmpt (5172 bp). Genomic DNA from ΔAfgt transformants was restricted with MfeI. Expected hybridisation patterns were: wild-type (3967 bp) and ΔAfgt (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).

ΔAfmpt and ΔAfgt are unable to adapt to MNNG

Phenotypic adaptation experiments indicated that the adaptive response was completely lost upon deletion of Afmpt (Figure 3A). ΔAfmpt 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 ΔAfmpt strain was unable to grow at the highest MNNG concentration tested (4 µg/ml; Figure 3A). Furthermore, phenotypic analysis revealed that ΔAfmpt and ΔAfgt 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 ΔAfmpt 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 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 ΔAfgt (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).

Afgt can fully complement the alkyltransferase activity of a Saccharomyces cerevisiae mgt1 deletion strain

A S. cerevisiae mgt1 deletion strain (Euroscarf) was engineered to constitutively express Afagt and the resistance of the resulting transformant to MNNG was determined by

![Figure 2. Confirmation of deletion of Afmpt and Afagt in strain A293.](image)

![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 µg/ml), radial growth (mm) of ΔAfmpt in increasing concentrations of MNNG (0–4 µg/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 ΔAfmpt and ΔAfgt are extremely sensitive to MNNG. ΔAfmpt and ΔAfgt display significantly reduced radial growth compared to wild-type on media containing all concentrations of MNNG tested (0–4 µg/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 or the mean. Key ***P < 0.001.)](image)
comparison to an appropriate control strain. Expression of \textit{Afagt} in \textit{S. cerevisiae} \textit{mgt1} was able to restore growth on medium containing high levels of MNNG (Figure 5). Protection was observed even at concentrations up to 64 \text{mg/ml} MNNG, showing that \textit{in vivo} \textit{Af} AGT is indeed a functional orthologue of the \textit{Mgt1} protein.

### Up-regulation of methyltransferase activities by MNNG

We determined methyltransferase activity by quantifying the transfer of \[^{[3]H}\]-methyl groups from \[^{[3]H}\]-N-methyl-N-nitrosourea-methylated calf thymus DNA to protein: methyl transfer from \(O[^{[3]H}]\text{meG}\) and \[^{[3]H}\]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 \textit{AfAGT} and \textit{AfMPT} activities were quantifiable using this assay procedure. Activity was easily detected in cell-free extracts of the \textit{S. cerevisiae} \textit{mgt1} deletion that had been transformed with an expression vector harbouring \textit{Afagt} but activity in extracts of \textit{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 \textit{AfMPT}, we ligated its ORF into pMALC2, expressed the fusion protein in \textit{E. coli} and purified using amylose affinity chromatography. The purified \textit{AfMPT} fusion protein (r\textit{AfMPT}) 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 r\textit{AfMPT} was mixed with purified recombinant human MGMT, approximately twice as much radioactivity was transferred to protein, but mixtures of r\textit{AfMPT} with r \textit{E. coli} MPT were not additive (Supplementary Figure S5) indicating that like the \textit{E. coli} protein, r\textit{AfMPT} acted only on one of the methylPT isomers. We also found that pre-incubation of the r\textit{AfMPT} at 37°C led to a rapid loss of activity \((t = 1/2 \sim 5\text{min})\) whereas at room
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 MPT) 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 A/MPT 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 O6-meG repair function, A/AGT. MNNG treatment thus substantially up-regulates the expression of A/AGT. 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

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**Figure 6.** Up-regulation of methyltransferase activity by MNNG. (A) Methyltransferase activity in extracts of S. cerevisiae transformed with A/MPT (open square) or A/AGT (open triangle)-encoding DNA or empty vector (open circle) or of recombinant MGMT (filled diamond), A/MPT (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)], A/AgT (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 A/MPT (rA/MPT) 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 ΔA/AgT strains). All activities were at substrate limiting levels. Values are means ± 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), A/AgT (open and filled triangles) or ΔAfMPT (open and filled squares) strains on the methyltransferase activity of recombinant A/MPT (7.1 fmoles) under protein-limiting conditions. See ‘Materials and Methods’ section for experimental details.
induced level of activity was calculated to equate to 2.7 × 10^3 molecules of AfAGT per cell. Based on the assay's lower limit of quantitation of activity, the uninduced wild-type strain contained less than ~30 molecules of AfAGT 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 AFMPT (~7 fmoles). None of the control extracts had a measurable effect on the levels of rAfMPT 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 AfMpt cell-free extract markedly inhibited methyl transfer to rAfMPT, indicating that high levels of unrepaired methylPT were, as anticipated, present in the DNA within the extracts. The inactivation of recombinant AFMPT by extracts of 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 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 AFMPT 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 rAfMPT had an additive effect on methyl transfer as a result of the combined effects of up-regulated AFAGT and the added rAFMPT (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 AFMPT. These results provide additional evidence for MNNG-mediated up-regulation of AFMPT, but also explain why we were unable to detect any increase in AFMPT activity, in contrast to that for AFAGT.

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°-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. AFMPT and AFAGT, respectively. Protein sequence alignment of AFMPT indicated a high level of sequence conservation with the N-terminal transcriptional activator domain of E. coli Ada, strongly indicating that AFMPT could act as a transcriptional activator for the adaptive response in A. fumigatus. Alignment of AFAGT
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 *AfAGT* is likely to be a functional homologue of the C-terminal domain of Ada, possessing the $O^6$meG and $O^6$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 *AfMPT* and *AfAGT* 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 $O^6$-alkyguanine DNA alkyltransferase (*Afagt*). In this article, we show that this gene is clearly part of an adaptive response to alkylating agents, and hence we consider that *Afagt* 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*, *Afagt*, *AfalkB* and *AfalkA* following exposure to MNNG. RT-PCR indicated that the expression levels of *AfMpt* and *Afagt* were induced upon exposure to MNNG, suggesting that *AfMpt* and *Afagt* are important in the adaptive response in *A. fumigatus*. Induction of *Afagt* mRNA was observed after 30 min exposure to MNNG and persisted for at least 3 h, indicating that the induced expression of *Afagt* 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 *Afagt* 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 pyrimidine resistance cassette as a selection marker, with modifications (36–38). When tested for an adaptive response, *AfMpt* failed to adapt to MNNG following induction with a non-lethal dose when compared to wild-type (*P* < 0.001). In fact, *AfMpt* displayed almost identical growth rate on MNNG from 0 µg/ml to 4 µg/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 *Afagt* lead to significantly increased sensitivity to MNNG (0.5–2 µg/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 µg/ml). No differences were observed between wild-type, *AfMpt* or *Afagt* regarding sensitivity to MMS, indicating that *AfMpt* or *Afagt* 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 *AfMpt* and *Afagt* strains to MMS is not surprising. Overall, these phenotypic analyses suggest that the roles of *AfMpt* and *Afagt* are specific in the response to and protection against specific alkyllyation lesions caused by MNNG, of which the main biologically relevant lesion is $O^6$meG.

RT-PCR analysis showed no *Afagt* or *AfMpt* expression in *ΔAfagt* or *ΔAfMpt*, respectively, under non-inducing conditions, thus validating the gene disruption strategy used in this study. While *Afagt* expression was evident in *ΔAfMpt*, there was no difference in the level of expression between uninduced and induced conditions. This indicates that *Afagt* expression was not up-regulated by MNNG in the *ΔAfMpt* mutant strain and hence that *AfMpt* is essential for elevated transcription of *Afagt* by MNNG in *A. fumigatus*. This confirms the role of *AfMpt* as the functional equivalent of the *E. coli* Ada transcriptional activator in *A. fumigatus*. Thus, the expression level of *AfMpt* was markedly increased in the *ΔAfagt* strain under inducing conditions indicates that *AfMpt* expression in response to MNNG is not dependent on the presence of *Afagt* but induces it 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 *Afagt* is deleted. However, the vastly increased sensitivity to MNNG in *ΔAfagt* strongly argues that *Afagt* is essential for the repair of toxic lesions caused by MNNG.

The *Afagt* 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, *ΔAfagt* and *ΔAfimpt* 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 *ΔAfagt* and *ΔAfimpt* strains. That DNA in the *ΔAfagt* and wild-type extracts contained much lower levels of methylPT than the *ΔAfimpt* extract clearly demonstrated that *Af*MPT expression had been induced by MNNG in the wild-type and *ΔAfimpt* 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 by the levels of damage introduced by MNNG.

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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