A Gene from *Aspergillus nidulans* with Similarity to *URE2* of *Saccharomyces cerevisiae* Encodes a Glutathione S-Transferase Which Contributes to Heavy Metal and Xenobiotic Resistance

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*Aspergillus nidulans* is a saprophytic ascomycete that utilizes a wide variety of nitrogen sources. We identified a sequence from *A. nidulans* similar to the glutathione S-transferase-like nitrogen regulatory domain of *Saccharomyces cerevisiae* Ure2. Cloning and sequencing of the gene, designated *gstA*, revealed it to be more similar to *URE2* than the *S. cerevisiae* glutathione S-transferases. However, creation and analysis of a *gstA* deletion mutant revealed that the gene does not participate in nitrogen metabolite repression. Instead, it encodes a functional theta class glutathione S-transferase that is involved in resistance to a variety of xenobiotics and metals and confers susceptibility to the systemic fungicide carboxin. Northern analysis showed that *gstA* transcription is strongly activated upon exposure to 1-chloro-2,4-dinitrobenzene and weakly activated by oxidative stress or growth on galactose as a carbon source. These results suggest that nitrogen metabolite repression in *A. nidulans* does not involve a homolog of the *S. cerevisiae* *URE2* gene and that the global nitrogen regulatory system differs significantly in these two fungi.

The ascomycete *Aspergillus nidulans* is particularly robust, with resistance to many xenobiotics, high tolerance to changes in temperature and pH, and the ability to utilize a wide range of carbon and nitrogen sources. An important feature of these responses is the ability to tightly regulate the expression of specific subsets of genes under different environmental conditions. For example, synthesis of molecules which are effective only at acidic or at alkaline pH is restricted to environments of the appropriate acidity through the action of the zinc finger-containing transcriptional regulator PacC (28, 29). A similar restriction is observed for genes required for the utilization of a wide variety of nitrogen sources. The presence of the preferred nitrogen source ammonium or l-glutamine prevents the expression of many catabolic enzymes required for the utilization of more complex nitrogen sources (for a review, see reference 50). In *A. nidulans* this regulation, termed nitrogen metabolite repression, is mediated via the positively acting GATA zinc finger transcription factor AreA (7, 41). In response to nitrogen limitation, AreA activates expression through the consensus sequence HGATAR in the promoters of genes encoding proteins required for the catabolism of secondary nitrogen sources (75). Loss-of-function *areA* mutants are thus unable to grow on nitrogen sources other than ammonium (7, 36, 37, 41).

Regulation of AreA function occurs at multiple levels. The expression of *areA* is controlled by autogenous regulation of transcription and reduced transcript stability during nitrogen sufficiency through an element in the 3′ untranslated region of the transcript (43, 55, 58). AreA activity is negatively affected by NmrA, a homolog of the *Neurospora crassa* NMR1 protein which interacts with the zinc finger and carboxyl-terminal residues of the AreA homolog NIT2 (4, 24, 77). Deletion of the C-terminal amino acids of AreA partially relieves nitrogen metabolite repression, while almost complete relief of repression is obtained when the mRNA stability-affecting element in the 3′ untranslated region (UTR) of the *areA* transcript is mutated (58). A low level of nitrogen metabolite repression is still observed in this double mutant, which suggests that a third mechanism for regulating AreA activity may exist.

Relief of nitrogen catabolite repression in the budding yeast *Saccharomyces cerevisiae* is dependent on the GATA factors Gln3 and Nil1/Gat1 (16, 53, 54, 66). These factors show little homology to AreA at the C-terminal end, the region with which NmrA is proposed to interact. Consistent with this finding, the *S. cerevisiae* genome does not encode a recognizable homolog of NmrA. Instead, the activation of genes involved in secondary nitrogen source catabolism by Gln3 and Nil1 is modulated by interaction with the Ure2 protein. Protein phosphorylation via the Tor signal transduction pathway allows Ure2 to retain Gln3 and Nil1 in the cytoplasm during nitrogen sufficiency (9, 10, 13). Nuclear exclusion of these positively acting GATA factors allows the expression of nitrogen-metabolic genes to be tightly controlled. There is no genetic or molecular evidence at present for the existence of a *URE2* homolog in *A. nidulans*.

Cloning of the *URE2* gene revealed it to be constitutively expressed and to encode a small protein with an asparagin-rich N terminus (residues 1 to 65) which allows the protein to form the non-Mendelian genetic element [URE3] (18, 25, 73). [URE3] is an inactive, prion form of Ure2, with strains carrying this self-propagating amyloid form displaying phenotypes equivalent to those of *ure2* loss-of-function mutants, i.e., a loss of nitrogen catabolite repression of various nitrogen-regulated activities. The remainder of the protein is required for nitrogen catabolite repression via Gln3, shows sequence similarity to the members of the glutathione S-transferase (GST) superfamily,
and binds glutathione despite an absence of detectable GST activity (12, 14, 18, 51). GSTs (EC 2.5.1.18) are mainly cyto- 
solic, multifunctional detoxification enzymes that mediate the 
conjugation of the reduced form of the tripeptide glutathione 
to many exogenous and endogenous hydrophobic electrophiles. 
These substrates are typically aliphatic, aromatic, or 
heterocyclic and are difficult for the cell to detoxify. The liga-
tion of a glutathione moiety to such cytotoxic agents is believed 
to confer a common structural determinant and increase their 
water solubility, allowing their glutathione conjugate forms to 
be actively transported to the vacuole (46).

Although analysis of GSTs in higher eukaryotes has been 
extensive, fungal GSTs have not been as well studied. Early 
work revealed that the activities of fungal GSTs varied consid-
erably between species, with low speci-
ficity between species, with low speci-
fic activities typically 
preparing ammonium tartrate.

<table>
<thead>
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<tr>
<td>MH9989........</td>
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* Gene symbols are as described by Clutterbuck (15).

**Molecular techniques.** Standard methods were as described by Sambrook et al. (48). Restriction enzymes (Promega) were used with the supplied buffers. DNA 
fragments were purified from agarose gels using the Agarose Gel DNA Extrac-
tion Kit (Boehringer Mannheim). RNA was isolated from *A. nidulans* cultures 
using the RNA Red Fast Prep kit (BIO 101) and electrophoresed on a form-
amide-containing 1.2% agarose denaturing gel, followed by blotting to an H 
Bond N+ nylon membrane (Amersham) with 40 mM NaOH. PCR was per-
formed using primers ure2a (CCTTACCTACCTCCTGAGC) (C) and ure2b (CC 
TCCCAAGCTTAAAGATGC).

**Construction of gatA plasmids.** An 8-kb XhoI fragment from bacterial artificial 
chromosome (BAC) 16E08 was subcloned into pbLueScript SK(+) (pJAF4858) 
and further subcloned as a 2.8-kb NotI/XhoI fragment (pJAF4989). The gatA 
deletion plasmid pJAF5084 was created by inserting a 2.3-kb Small/BglII riboB 
fragment from pJLI (56) into pJAF4989 digested with BglII and Small, disrup-
ting exon 3. The 4.5-kb SauI/XhoI gatA::vibo**2** fragment was inserted into 
pJAF4888 digested with SauI to give pJAF5086, increasing the flanking genomic 
sequence.

**Nucleotide sequence accession number.** The gatA sequence has been deposited in 
GenBank under accession number AF425746.

## RESULTS

**Cloning of an A. nidulans gene with homology to S. cerevisiae URE2.** A search of the University of Oklahoma *A. nidulans* expressed sequence tag (EST) database (www.genome.ou.edu/ fungal.html) with the *S. cerevisiae* Ure2 sequence revealed multiple 
ESTs (f1a11a1.r1, z4 h02a1.f1, and z4 h02a1.r1) with identity to the yeast protein. The PCR primers ure2a and ure2b were designed based upon these sequences and used to amplify a 365-bp fragment from *A. nidulans* genomic DNA. The fragment was used to probe an *A. nidulans* genomic BAC library high-density filter (kindly provided by Ralph Dean, 
Department of Plant Pathology and Physiology, Clemson Uni-
versity, Clemson, S.C.), resulting in multiple positive clones. Based upon genomic Southern blots, a hybridizing 8-kb XhoI 
fragment was subcloned from one of the hybridizing clones 
(BAC clone 16E08) into pbLueScript SK(+) to give pJAF4858, 
which was in turn subcloned as a 2.8-kb NotI/XhoI fragment, 
creating pJAF4989. Probing of genomic Southern blots at low 
stringency failed to show cross-hybridization with any related 
*URE2*-like sequences (not shown).

**Sequence analysis of gatA.** Sequencing of the insert of 
pJAF4989 revealed a single region in the center of the clone 
with sequence similarity to GSTs. With the aid of the available 
ESTs, the gene (designated gatA) was determined to contain 
two introns and to encode a predicted 250-amino-acid product.Comparison (22) to yeast proteins revealed that the encoded protein 
much more closely resembled Ure2 (47% similarity and 35% 
identity) than the two characterized *S. cerevisiae* GSTs Gtt1 
and Gtt2 (35% similarity and 24% identity and 35% similarity 
and 25% identity, respectively), indicating that this gene might be 
a *URE2* ortholog. The predicted GstA sequence lacked an 
asparagine-rich N terminus, which functions as a prion-form-
ing domain in *Ure2* (residues 1 to 94) (30, 68). Database 
searches revealed higher similarity to several GST-like 
sequences, only one of which has been characterized. Identity 
was highest to the protein GST1 from the plant pathogen 
*Botrytis cinerea* (73%) (60), with lower identity to uncharacter-
ized GST-like sequences in the genomes of *N. crassa* (68%) (sequence contig 1.1301, assembly version 1, Neurospora 
Sequencing Project) and *Candida albicans* (48%, sequence contig 6-2410, unfinished fragment of complete *C. albicans* genome).

A second unpublished GST-like sequence from *C. albicans*...
(accession no. AF260777) with a much higher level of similarity to *S. cerevisiae* Ure2 has already been identified as having Ure2-like sequences from *Saccharomyces douglassi* (AF260775) and *Kluveromyces lactis* (AF260776). As with *S. cerevisiae* Ure2, these all contain the long asparagine-rich prion forming N terminus, in addition to a conserved 27-residue insertion in the center of the GST-like nitrogen regulatory domain. Both of these features are lacking in *A. nidulans* GstA and the GstA-like sequences from *B. cinerea*, *N. crassa*, and *C. albicans*. The fact that both GstA and URE2 homologs exist in *C. albicans* suggests that *A. nidulans* GstA is not an ortholog of URE2.

**GstA resembles a theta class GST.** GSTs have been classified based on substrate specificity, antibody cross-reactivity, crystal structure, and amino acid sequence data into five main classes: alpha, mu, pi, sigma, and theta (35, 47, 52, 62). The low level of similarity of GstA to most characterized GSTs suggests that the *A. nidulans* enzyme belongs to the theta class, as this is the most heterogenous class. Comparison to the structurally derived consensus pattern for theta class GSTs confirmed this designation, with only 7 deviations from 35 consensus residues (62). The most significant of these differences is in one of six theta class-specific residues with the conservative change of serine to threonine at position 13. Mutagenesis studies and analysis of crystal structures have shown that this is the catalytic residue required for glutathione ligation in theta class enzymes (11, 74). Significantly, Rossjohn et al. (62) found deviation at this position only in GST-like proteins that have no detectable GST activity (LIGF from *Pseudomonas pacifico* mobilis, SSP1 from *Escherichia coli*, SSP2 from *Haemophilus somnus*, and Ure2 from *S. cerevisiae*). One possibility is that in *A. nidulans* threonine 13 is responsible for activation of glutathione by promoting thiolate formation. Alternatively, tyrosine 9 may participate in the binding of the sulfhydryl moiety of glutathione instead, as occurs in the alpha, mu, and pi classes (39, 47, 49, 57, 67).

**GstA does not play a role in nitrogen metabolism repression.** A *gstA* deletion strain was generated by homologous recombination of a disruption construct (pJAF5086) in which the 148-bp BglII/StuI fragment in exon 3 was replaced with the *riboB* selectable marker, disrupting the gene after codon 91. To allow more sensitive testing for potential nitrogen-regulatory phenotypes, the recipient strain MH9870 carried the *nmrAΔ* (4) and *areA-ΔUTR5* (an *areA* 3’UTR deletion [59]) alleles. pJAF5086 was linearized with XhoI, and transformants were selected for riboflavin prototrophy. Analysis by Southern blotting of 40 Ribo transformants revealed a *gstA* knockout frequency of 35%. One such transformant (MH9985) was selected for further study.

Several studies have used a variety of plate tests to qualitatively determine nitrogen metabolite repression of several activities required for the utilization of poor nitrogen sources. One such approach is the detection of increased sensitivity to toxic nitrogen source analogs in the presence of a repressing nitrogen source (4, 58). A comparison between *gstAΔ* and *gstAΔ* strains was performed using *areA-ΔUTR5*, *nmrAΔ*, and otherwise wild-type backgrounds (strains are described in Table 1). *gstA* deletion strains showed sensitivity to 200 mM potassium chloride (a toxic analog of nitrate) equivalent to that of *gstAΔ* strains in the presence of 5 mM ammonium tartrate. Growth tests on 5 mM thiourea (a toxic analog of urea) with 2.5 mM ammonium tartrate also failed to show any difference in sensitivity (not shown). Inappropriate derepression of extracellular proteases has previously been shown to cause a halo of milk clearing on plates containing 1% skim milk (Diploma) with 10 mM ammonium tartrate (17). This test also failed to show any difference from the *gstAΔ* phenotype in the equivalent *gstAΔ* strains (not shown). These plate assays strongly suggest that *gstA* plays no role in the global regulation of genes involved in the utilization of poor nitrogen sources.

The acetamidase enzyme, encoded by the *amds* gene, allows growth on acetamide as a carbon and/or nitrogen source by producing ammonium and acetate. Regulation of *amds* expression is complex, involving multiple induction signals as well as control by carbon and nitrogen metabolites (for a review, see reference 38). By crossing the *gstAΔ* mutant with various *amds:αlacZ* reporter strains (20), we were able to determine whether *gstA* played a role in nitrogen metabolism repression. Comparison of *amds:αlacZ* expression in wild-type (MH3408) and *gstAΔ* (MH9986) strains in cultures grown overnight with either 10 mM ammonium tartrate (a repressing nitrogen source) or 10 mM alanine (a limiting nitrogen source) showed that deletion of *gstA* had no effect (Table 2). Similarly no effect on expression of *amds:αlacZ* was observed following transfer of ammonium-grown mycelium to minimal medium lacking a nitrogen source for 4 h. Furthermore, deletion of *gstA* did not affect *amds:αlacZ* regulation in genetic backgrounds with altered nitrogen metabolite repression (*areA-ΔUTR5* [MH9987]; *nmrAΔ* [MH9988], and *areA-ΔUTR5 nmrAΔ* [MH9989]) (Table 2). Therefore, no role for *gstA* in nitrogen metabolism repression was detected.

**GstA contributes to resistance to multiple xenobiotics and heavy metals.** The absence of a nitrogen-regulatory role for GstA suggested that despite the higher identity to Ure2 than to the *S. cerevisiae* GSTs Gtt1 and Gtt2, this gene might encode a true GST. In agreement with this, a strain carrying the *gstA* allele (MH9986) showed increased sensitivity to the antimicrobial phenylpyruvrole derivative pyroloxin [3-chloro-4-(2’-nitro-3’-chloro-phenyl)pyruvrole], a compound that inhibits the electron transport system in *N. crassa* (6, 27), at 10 μg/ml. The deletion mutant also grew more poorly than the wild type in

<table>
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<th>Genotype</th>
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<tr>
<td>Wild type</td>
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<tr>
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<tr>
<td><em>nmrAΔ</em></td>
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<tr>
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<td>6</td>
</tr>
<tr>
<td><em>areA-ΔUTR5</em> <em>nmrAΔ</em> <em>gstAΔ</em></td>
<td>7.5</td>
</tr>
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* Mycelium was grown in 100 ml of 1% glucose medium with either 10 mM ammonium tartrate or 10 mM alanine at 37°C for 16 h or was nitrogen starved (i.e., grown in 1% glucose-10 mM ammonium tartrate medium for 16 h, washed with 1% glucose-nitrogen-free medium, and incubated for a further 4 h in ANM lacking a nitrogen source). Full genotypes and names of the strains assayed are described in Table 1.

* Values of β-galactosidase activity are given in units per minute per milligram of protein and represent the results of at least two separate experiments.
the presence of sulfanilamide (at 50 μg/ml) or oxidative stress induced by diamide (0.2 μl/ml) compared to the wild-type strain MH1 (Fig. 1A) (40, 72). No difference in sensitivity was apparent when the mutant strain was grown in the presence of the toxic molecule CDNB (5 μg/ml), a common GST substrate (44).

Surprisingly, growth tests in the presence of various heavy metals revealed that the gstAΔ mutant had increased sensitivity relative to the wild type. The mutant strain exhibited poor growth in the presence of 2 mM selenium (as Na2SeO3) and, to a lesser extent, in the presence of 0.1 mM silver (as AgNO3) and 2 mM nickel (as NiSO4) (Fig. 1B). Work with S. cerevisiae has revealed that a glutathione S-conjugate pump contributes to heavy metal resistance (31, 46, 61). These data suggest that the same is true in A. nidulans.

In contrast to the susceptibility to other xenobiots and heavy metals, the gstAΔ strain showed increased resistance to the systemic fungicide carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) at 10 μg/ml, with increased conidiation and denser colonial growth. Mutations in three different loci, i.e., carA (cxbB), carB (cxbC), and carC (cxbA), have previously been reported to confer resistance to carboxin in A. nidulans (32, 71). One of these loci may correspond to gstA.

Transformation of the riboB2 homozygous diploid strain MH6590 allowed the creation of a gstAΔ heterozygous strain (MH9951). Haploidization using the techniques described by Clutterbuck (15) revealed that the gstA gene maps to linkage group III, the same linkage group as carC. Unfortunately a strain carrying this mutation was unavailable for further analysis, so we were unable to determine whether carC and gstA are the same gene.

Regulation of gstA expression. Transcriptional regulation of gstA was consistent with the gene encoding a GST required in the presence of toxic compounds and oxidative stress. Treatment with 40 μg of CDNB per ml for 1 h resulted in very strong activation of gstA transcription, despite the apparent failure of this hydrophobic electrophile to be a GstA substrate (Fig. 2). Overexposure revealed a low level of transcriptional activation in response to oxidative stress caused by 5 mM H2O2, but this was not seen during growth on 10 mM uric acid as a nitrogen source, catabolism of which produces H2O2 (64). Neither osmotic shock (0.5 M NaCl) nor heat shock (45°C for 1 h) led to an increase in expression. Surprisingly, slightly increased transcript levels were observed following growth overnight on galactose as a carbon source. Alteration of the quality of the available nitrogen source by overnight growth on 10 mM alanine rather than 10 mM ammonium tartrate revealed no alteration in expression, indicating that gstA transcription is unaffected by nitrogen metabolite repression (Fig. 2).

**DISCUSSION**

Nitrogen metabolite repression in both A. nidulans and S. cerevisiae involves GATA factors activating the expression of genes required for the catabolism of secondary nitrogen sources. Recent advances in the understanding of this global regulatory response in yeast center on the ability of Ure2 to control the subcellular localization of Gln3 and Nil1/Gat1 and hence their function (9, 10, 13). Unpublished Ure2-like sequences (accession numbers AF260777, AF260775, and AF260776) have al-

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**FIG. 1.** Phenotypes of the gstA deletion strain (gstAΔ) (MH9986) and the wild-type strain (WT) (MH1). Colonies were grown on 1% glucose–ANM supplemented with 10 mM ammonium tartrate for 2 days at 37°C. Xenobiots (A) and heavy metals (B) were added at the indicated concentrations. Metals were added as salts (selenium as Na2SeO3, silver as AgNO3, and nickel as NiSO4).
metals is affected by the activity of yeast cadmium factor 1 (Ycf1p), an ATP-dependent glutathione \textit{S}-conjugate pump with broad substrate specificity (31, 46, 61). In S. cerevisiae, the GSTs are implicated in xenobiotic metabolism, a subject of both pharmacological and agronomic interest. The contribution of GST activity to insecticide detoxification in several insect species has been recognized (33, 34). The role of GSTs in fungicide resistance may also be important, particularly in the cases of emerging fungal pathogens with reduced antimicrobial susceptibility and the development of resistance in existing pathogenic strains (for reviews, see references 8 and 69). GST activity provides one of several mechanisms (including altered targets, decreased drug accumulation, increase of cell damage repair, and increased drug inactivation) that may render cells refractory to cytotoxic agents (76).

\textbf{ACKNOWLEDGMENTS}

J.A.F. was a recipient of an Australian Postgraduate Award. This work was supported by a grant from the Australian Research Council. The predicted \textit{N. crassa} GST sequence was identified via the Neurospora Sequencing Project, Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu). The \textit{C. albicans} predicted GST sequence was generated at the Stanford DNA Sequencing and Technology Center with the support of the NIDR and the Burroughs Wellcome Fund. We thank Ralph A. Dean for providing the \textit{A. nidulans} genomic BAC library.

\textbf{REFERENCES}


**FIG. 2.** Northern blot analysis of \textit{gstA} transcription. RNA was isolated from \textit{A. nidulans} strain MH1. Mycelium was grown in 1% glucose–10 mM ammonium tartrate medium at 37°C for 16 h unless an alternative carbon or nitrogen source is specified. Subsequent treatments were for 1 h. Northern blots were hybridized with probes specific for \textit{gstA} or \textit{A. nidulans} histone H3 as a loading control (26). The nitroson source was used was 10 mM ammonium tartrate (except for the alanine [10 mM] and uric acid [10 mM] cultures), and the carbon source was 1% glucose (except for the 1% galactose culture). Heat shock at 45°C and treatment with H$_2$O$_2$ (5 mM), NaCl (0.5 M), or CDNB (40 
ug/ml) were for 1 h to cultures pregrown for 16 h at 37°C. The CDNB treatment lane was underloaded, as shown by the histone loading control. The last two lanes show shorter exposures (2 days rather than 11 days) of the CDNB and uric acid treatments.


