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The AzgA Purine Transporter of *Aspergillus nidulans*

CHARACTERIZATION OF A PROTEIN BELONGING TO A NEW PHYLOGENETIC CLUSTER*

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The *azgA* gene of *Aspergillus nidulans* encodes a hypoxanthine-adenine-guanine transporter. It has been cloned by a novel transposon methodology. The null phenotype of *azgA* was defined by a number of mutations, including a large deletion. In mycelia, the *azgA* gene is, like other genes of purine catabolism, induced by uric acid and repressed by ammonium. Its transcription depends on the pathway-specific UaY zinc binuclear cluster protein and the broad domain AreA GATA factor. AzgA is not closely related to any other characterized membrane protein, but many close homologues of unknown function are present in fungi, plants, and prokaryotes but not metazoa. Two of three data bases and the phylogeny presented in this article places proteins of this family in a cluster clearly separated (but perhaps phylogenetically related) from the NAT family that includes other eukaryotic and prokaryotic nucleobase transporters. Thus AzgA is the first characterized member of this family or subfamily of membrane proteins.

Three purine transporters exist in *Aspergillus nidulans* (1–3). UapA is specific for uric acid and xanthine. UapC is able to transport all purines albeit with different K_m values and capacities. UapC is a xanthine-uric acid transporter with a low capacity activity for hypoxanthine and other purines. These two proteins are homologues and belong to the nucleobase-ascorbate transporter (NAT)[1] family, conserved in fungi, prokaryotes, plants, and mammals (2, 3). Members of this family transport purines, pyrimidines, and surprisingly in mammals, ascorbic acid (4). The determinants of the substrate specificity of this family of proteins have been studied in detail (5, 6). This family is referred also as the nucleobase/cation symporter 2 family (accession number TIGR00801, Ref. 7). The mammalian ascorbate transporter is a Na^+ symporter (8). Four proteins of this family, the fungal transporter UapA, the plant transporter LPE1,[2] and the bacterial transporters YicE and YgfO[3] are most probably H^+ symporters. Transport mediated by these proteins is abolished by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone for UapA and LPE1,[2] or by the ATPase inhibitor quercitin for YicE and YgfO.[3] The results with UapA are in line with the fact that transporters in fungi, with only two exceptions are proton symporters (4, 9–11). Transporters dependent on different ion exchange mechanisms are known to coexist within some phylogenetic family (12).

A third transporter, only known from genetic evidence, is defined by the *azgA* gene. It is specific for hypoxanthine, guanine, and adenine and it transports the toxic analogues 8-azaguanine and purine (1, 3, 13). Inhibition experiments with carbonyl cyanide *m*-chlorophenylhydrazone (see above) suggest that as most fungal transporters this also is an H^+ symporter.[2] Here we identify the *azgA* gene by transposon tagging and we establish that AzgA defines a new group of membrane proteins, which may be distantly related to the NAT family. Homologues of unknown function are found in plants, fungi, bacteria, and Archaea.

In *A. nidulans*, genes coding for proteins involved in purine utilization, including transporters, are under a dual control, induction by uric acid and nitrogen metabolite repression (2, 3, 14). Induction by uric acid is mediated by the zinc binuclear factor UaY (15, 16). Nitrogen metabolite repression acts by preventing the action of the GATA factor AreA (2, 3, 14, 17). We show here that in mycelia, *azgA* is regulated in parallel with *uapA* and *uapC*. We also report the first application of a heterologous transposition tool developed in our laboratory (18).

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—Media and growth conditions of *A. nidulans* have been described (15, 19). Nitrogen sources were at the following concentrations: 5 mM urea; 5 mM ammonium L (+) tartrate; adenine, guanine, hypoxanthine, and uric acid, 0.1 g/liter (around 700 μM).

Strains—The *Escherichia coli* strain used was JM109 (20). *A. nidulans* strains used were CS2498 (*pabaA1*) as the wild type. CS2757 (*yA2*, *pantoB100*, *riboB2*, *niaD353*, carries integrated in the genome plasmids pCM3 (carrying *niaD*::*impala*::*yA*) and pPL5 (carrying *riboB*) (18) were used for transposon tagging of the *azgA* gene. The *azgA*-tagged strain is CS3107. *niaD353* is a deletion in the *niaD* gene. CS2580 (*azgA4*, *uapA24*, *uapC201/401*, *argB2*, *pantoB100*, *yA2*) was used as recipient strain in transformation experiments. *azgA4*, *uapA24*, and *uapC201/401* are loss-of-function mutations in the cognate genes. CS2748 (*yA2*, *pabaA1*, *prnB337*) was crossed with CS3107 to check the co-segregation

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[1] The abbreviations used are: NAT, nucleobase-ascorbate transporter; RACE, rapid amplification of cDNA ends.

[2] S. Amillis and G. Diallinas, unpublished results.

[3] S. Frillingos, personal communication.

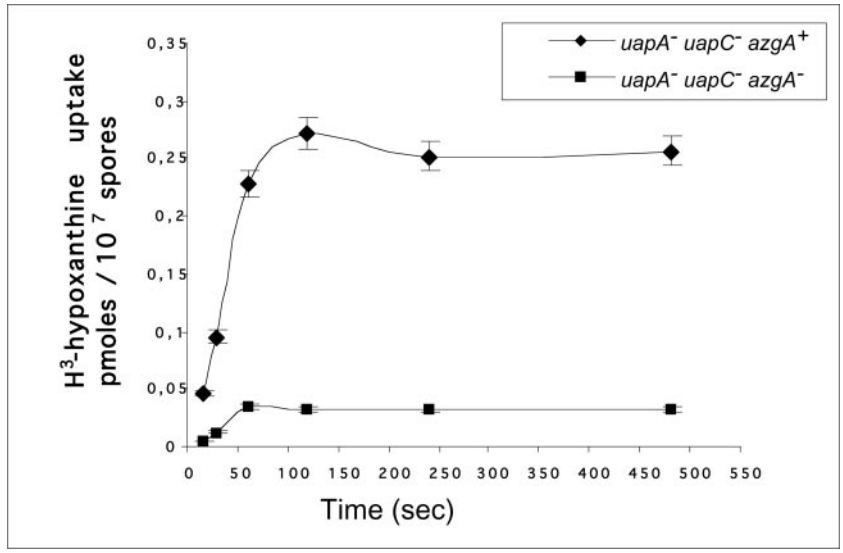


FIG. 1. **Uptake of hypoxanthine in *azgA*$^+$ *uapA*$^-$ *uapC*$^-$ and *azgA*$^-$ *uapA*$^-$ *uapC*$^-$ isogenic strains.** Uptake of hypoxanthine is expressed in picomoles/10^7 cells per min. The final concentration and specific activity of labeled hypoxanthine was 100 μM and 20 Ci/nmol, respectively. Experimental details and genotypes of strains are described under "Experimental Procedures." Results represent mean values of three independent experiments.

of the induced mutation (*azgA108*) with the transposon marker (*yA*$^+$). *prnB337* is a deletion in the proline transporter gene (21). CS2579 (*azgA4*, *uapA24*, *uapC201/401, argB2, yA2*) and CS1316 (*azgA4*, *uapA200*, *pabaA1*) were used to check the linkage between the transposon-induced mutation (*azgA108*) and the *azgA4* mutation. CS3108 (*azgA108*, *uapA24*, *uapC201/401*, *pantoB100*) was used to select transposon excision events. CS2473 (*hxB20*, *xanA1*, *pantoB100*, *yA2*), CS0836 (*uaY2*, *pantoB100*, *yA2*, *fpaD43*), CS1356 (*uaY*^{c}*462*, *pantoB100*), CS1318 (*areA600*, *pabaA1*, *biA1*, *sb43*), and CS1094 (*areA102*, *pyroA4*, *fwA1*) were used in Northern experiments. Relevant mutations are described in the text, auxotrophies in Ref. 22.

UV Mutagenesis—Spores from *A. nidulans* strain CS3103 (*biA1*, *chaA1*, *nicA2*, *pabaA1*) were exposed to 254 nm UV resulting in a survival rate of 8%. Selection of *azgA*$^-$ mutants was on 700 μM 8-azaguanine medium.

Transposition Mutagenesis—To ensure that all *azgA*$^-$ mutants obtained in the transposon-carrying strains originate from independent events, colonies arising from single spore isolates were pre-grown on hypoxanthine as nitrogen source. Colonies were inoculated into medium containing 700 μM 8-azaguanine on 5 mM urea as sole nitrogen source. One resistant sector was isolated per colony.

DNA Manipulations—Plasmid isolation from *E. coli* strains and DNA manipulations were as described in Ref. 23. Total genomic DNA isolation from *A. nidulans* strains has been described (24, 25).

RNA Manipulations—RNA was isolated from *A. nidulans* with RNAPLUS™ following the supplier's instructions (Q-BIOgene) and separated on glyoxal-agarose gels as described in Ref. 23. The hybridization technique is described in Ref. 26. [^{32}P]dCTP-labeled DNA molecules used as gene-specific probes were prepared using the random hexanucleotide-primer kit following the supplier's instructions (Amersham Biosciences).

A 2.4-kb fragment including the whole *azgA* open reading frame (nucleotide 666 to 3013) was amplified by PCR and cloned in pGEM™ easy vector. The EcoRI fragment of the resulting plasmid was used to detect the *azgA* messenger. A 2.7-kb XbaI fragment from pAN503 (2) was used to detect the *uapA* messenger. A 2.3-kb XbaI fragment from pAN903 (3) was used to detect the *uapC* messenger. A 2.5-kb BamHI-KpnI fragment of plasmid pSF5 (27) was used to detect the actin messenger as a control of mRNA loading. The intensities of RNA bands were quantified with a 400A PhosphorImager (Amersham Biosciences). Data were analyzed with ImageQuant.

Inverse PCR Protocol—Inverse PCR (28) was carried out with the Expand long template PCR system kit (Roche Diagnostics) using oligonucleotides 5′-GCGGCCACGTCGTTTTAATCG-3′ and 5′-AACCTGTCAACGCAAATCGC-3′. The resulting product was subjected to a second PCR using nested oligonucleotides 5′-ACCGTAATGAAGGCGTAAGT-3′ and 5′-ACGCCCCGCAGACCTTCTTC-3′. The resulting product was cloned in pGEM™-T easy vector (Promega).

5′ and 3′ RACE Analysis—The start and the end of transcription of the *azgA* gene was determined using the 5′/3′ RACE kit (Roche Diagnostics). In the 5′ RACE experiment, the cDNA was obtained using the specific oligonucleotides 5′-ACCGAGTAAAGTCAAACCG-3′, 5′-GTTGCGCATGAAACGGATAT-3′ and 5′-AGGTCTTCGGCTGGACAG-3′. In the 3′ RACE experiment, the initial cDNA was obtained using the specific oligonucleotide 5′-TCTCCGCCTGTTACAGCATT-3′. Two rounds of nested PCR reactions were then carried out using oligonucleotides 5′-CGGTCTGACATCATGTATGA-3′ and 5′-CCGGTATCTGCTTCTTCAT-3′, respectively, to increase specificity. The resulting product was cloned in the pGEM™-T easy vector (Promega).

Sequence Determination—Sequences were determined using the "Big Dye terminator cycle sequencing ready reaction with AmpliTaq DNA polymerase" kit on an ABI310 sequencing apparatus (PerkinElmer Life Sciences). The *azgA* gene was sequenced on both strands using two independent clones. When necessary a third clone was sequenced.

Transformation Techniques—Transformation of *E. coli* was performed by electroporation with a MicroPulser™ (Bio-Rad). Transformation of *A. nidulans* was according to Ref. 29.

Purine Transport Assays—[^{3}H]Hypoxanthine uptake was assayed in conidiospores at 37 °C (3, 30). Initial velocities were corrected by subtracting background uptake values, measured in the triple *azgA*$^-$*uapA*$^-$*uapC*$^-$ mutant. Competition assays were performed in the simultaneous presence of [^{3}H]hypoxanthine (1 μM) and 300 μM non-radiolabeled putative competitors. Competition by L-ascorbic acid was performed at 300 μM and 5 mM. The apparent Michaelis constant (K_m) and maximal velocity (V) values for [^{3}H]hypoxanthine were determined from double reciprocal plots of the initial uptake rate plotted against substrate concentrations (1–13 μM). The concentration of adenine resulting in 50% inhibition of [^{3}H]hypoxanthine uptake (K_{50}) was obtained at constant [^{3}H]hypoxanthine concentration (1 μM) and adenine concentrations from 0 to 200 μM. All transport assays were carried out in at least three different experiments. In each experiment three measures were taken for each point and standard errors were calculated. Radiolabeled [G-^{3}H]hypoxanthine (specific activity 13 Ci/mmol) was from PerkinElmer Life Sciences.

RESULTS

In Vivo Characterization of the AzgA Transporter—The early mutants in the AzgA transporter were obtained as spontaneous sectors resistant to the toxic analogue 8-azaguanine. These strains were cross-resistant to purine and showed severely reduced growth on adenine, hypoxanthine, and guanine as nitrogen sources (1). Fig. 1 shows the time course of hypoxanthine uptake for an *azgA*$^+$ and an *azgA*$^-$ strain. Hypoxanthine uptake showed hyperbolic kinetics in relation to substrate concentration (not shown). The apparent K_m and V for hypoxanthine were calculated to be 1.4 ± 0.5 μM and 1.2 pmol/min 10^7 conidiospores, respectively. Although the K_m characterizes this transporter, V depends on the absolute quantity of transporter inserted into the membrane. The latter depends on growth conditions but is also under developmental control.[4] Thus this parameter is contingent to the exact conditions of the experi-

[4] S. Amillis, G. Cecchetto, V. Sophianopoulou, C. Scazzocchio, and G. Diallinas, unpublished results.

TABLE I
Competition of [^{3}H]hypoxanthine uptake by excess unlabeled purine-related compounds or ascorbic acid in an azgA$^+$ uapA$^-$ uapC$^-$ strain

Uptake assays were performed as described under "Experimental Procedures." Values shown express per cent of [^{3}H]hypoxanthine (1 μM) uptake in the presence of excess (300 μM) unlabeled competitors. The uptake value obtained in the absence of competitor was taken as 100%. Results represent averages of three independent experiments.

Unlabeled competitor	[^{3}H]hypoxanthine uptake rate
	%
None	100 ± 3
Hypoxanthine	7 ± 0.5
Xanthine	119 ± 9
Uric acid	113 ± 8
Adenine	9 ± 1
Guanine	40 ± 3
Thymine	120 ± 9
Cytosine	71 ± 4
Uracil	103 ± 7
Adenosine	94 ± 5
Guanosine	46 ± 3
Inosine	66 ± 3
Thymidine	78 ± 5
Cytidine	106 ± 8
Uridine	79 ± 3
2-Thioxanthine	74 ± 3
6-Thioguanine	45 ± 2
8-Azaguanine	104 ± 12
8-Azaxanthine	35 ± 2
5-Fluorouracil	71 ± 4
5-Fluorouridine	82 ± 5
5-Aminouracil	71 ± 6
Caffeine	65 ± 4
Allopurinol	98 ± 7
Ascorbic acid	72 ± 5
Ascorbic acid (5 mM)	30 ± 3

ment (see "Experimental Procedures"). The high toxicity of 8-azaguanine allowed investigation of the specificity of the AzgA transporter by growth competition experiments. Guanine, hypoxanthine, adenine, 2-aminopurine, and 2–6-diaminopurine protect completely from 8-azaguanine toxicity. Guanosine protects moderately (13). Adenine competes for hypoxanthine uptake with a K_i of 3.0 ± 0.6 μM. Table I shows competition of hypoxanthine uptake by a number of metabolites. These data agree very well with the plate competition tests. A surprising feature is that 8-azaguanine does not compete with hypoxanthine uptake. Thus the *in vivo* inhibition of growth by 8-azaguanine was re-investigated. The analogue inhibits growth on solid media at concentrations as low as 7 μM and all *azgA*$^-$ mutants tested are resistant to concentrations as high as the solubility limit. At the standard concentration of 700 μM 8-azaguanine, 7 μM hypoxanthine affords clear protection, which confirms that the AzgA protein has a much higher affinity for hypoxanthine than for 8-azaguanine (not shown). Allopurinol, an isomer of hypoxanthine, does not compete with hypoxanthine uptake, and this agrees with experiments that suggested that this analogue is taken in by the UapA and UapC transporters.[5] The human ascorbic acid transporter is a member of the NAT family (8). UapA and the related plant LPE1 transporter bind ascorbic acid at concentrations higher than 5 mM (31, 32). Competition of hypoxanthine uptake by L-ascorbic acid is shown in Table I. 300 μM L-ascorbic acid did not compete with hypoxanthine uptake but 5 mM L-ascorbic acid reduced hypoxanthine uptake to 35%.

Genetic Characterization of azgA—Scazzocchio (13) had characterized 11 *azgA* non-complementing alleles. One allele, *azgA4* was still available. This was located in linkage group V, in agreement with the location of the lost allele *azgA1*. *azgA4*,

[5] C. Scazzochio and H. N. Arst, Jr., unpublished results.

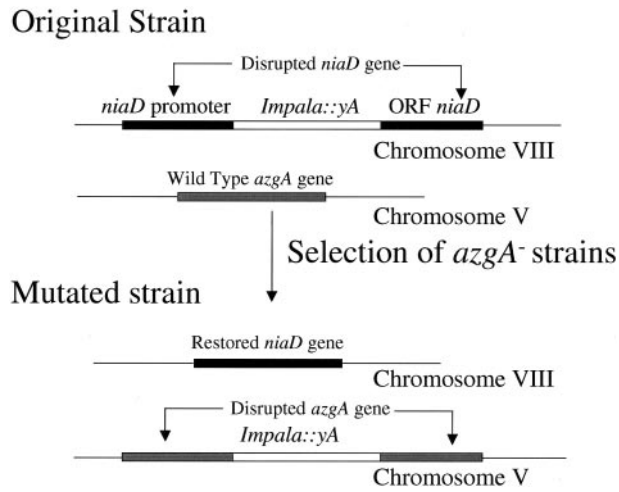


FIG. 2. **Selection of *azgA*-tagged mutants.** This figure schematizes the transposon tagging of the *azgA* gene. The original strain was inoculated onto minimal medium containing urea as nitrogen source and 8-azaguanine (see "Experimental Procedures"). The 8-azaguanine-resistant mutant sectors obtained were tested for their ability to grow on nitrate. The original strain is *niaD*$^-$ (unable to grow on nitrate) and *azgA*$^+$ (8-azaguanine sensitive). The expected phenotype of a strain carrying a tranposon insertion in the *azgA* gene is *niaD*$^+$ (able to grow on nitrate) and *azgA*$^-$ (8-azaguanine resistant).

chosen as the representative allele was mapped more precisely in chromosome V and was loosely linked to the *lysB*, *nudC*, and *nicA* genes. The nearest marker is *lysB* (23.5 ± 2.4 centimorgan). 11 new putative alleles (*azgA208* to *azgA219*) were selected as 8-azaguanine-resistant colonies after UV mutagenesis. A new allele (*azgA201*) was isolated as an 8-azaguanine spontaneous resistant sector. All these 8-azaguanine-resistant strains have impaired growth on hypoxanthine as nitrogen source. Crosses and complementation tests showed *azgA4*, *azgA201*, *azgA212*, and *azgA217* to be allelic.

Transpositional Cloning of the azgA Gene—Transformation with gene libraries or pools of cosmids from chromosome V failed to yield *azgA*$^+$ transformants. To attempt transposon tagging of *azgA* we used strain CS2757 (see "Experimental Procedures," Ref. 18). The CS2757 strain is deleted for the resident *niaD* gene, encoding nitrate reductase, and carries a single copy of a *niaD* transgene interrupted in its 5′-untranslated leader with a modified *impala* transposon from *Fusarium oxysporum*. It also carries the *yA2* mutation, resulting in yellow conidiospores and the *yA*$^+$ gene inserted in the transposable element. *yA*$^+$ is normally expressed and thus this strain is unable to grow on nitrate as nitrogen source but has green conidiospores. On excision of *impala* the *niaD* leader will be restored and the strain will recover the ability to utilize nitrate. The characteristics of the strain and the strategy used are shown in Fig. 2. We selected 248 8-azaguanine-resistant mutants in the CS2757 strain. Of these, only one had become *niaD*$^+$. This phenotype shows that the transposon has been excised from the *niaD* promoter, but not that it has inserted concomitantly in the *azgA* gene. This was shown as follows. The resulting strain CS3107 was crossed with a *yA*$^-$ strain (CS2748), and the progeny checked for conidiospore color, 8-azaguanine resistance, and ability to utilize hypoxanthine as nitrogen source. In 300 progeny analyzed, all strains resistant to 8-azaguanine and defective in hypoxanthine utilization (*azgA*$^-$) were *yA*$^+$, all *azgA*$^+$ strains were *yA*$^-$. Thus the transposon is genetically tightly linked to (and presumably the cause of) the *azgA*$^-$ phenotype. Complementation and recombination (4000 progeny tested) tests show that the transposition insertion and *azgA4* are alleles. The new allele will be called *azgA108*. Thus we have tagged *azgA* with the *impala*-modified

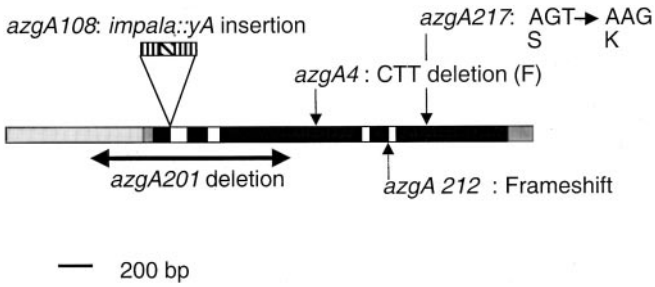


FIG. 3. **The *azgA* gene.** The distribution of introns, and the position of a number of $azgA^-$ mutations is shown. *Light gray*, promoter region. *Dark gray*, 5′- and 3′-untranslated regions of the *azgA* mRNA. *Black*, translated regions. *White*, introns. The 5′ start point is at −58 bp from the ATG. Polyadenylation occurs at +144 bp after the stop codon. Intron positions are in nucleotides from the ATG: intron 1, 91–191; intron 2, 296–370; intron 3, 1185–1234; intron 4, 1340–1385.

transposon. The precise location of the transposon in the *azgA* gene is described below and shown in Fig. 3.

Molecular Characterization of the azgA Gene—We cloned the *azgA* gene by inverse (circular) PCR (28) using SphI-digested DNA from strain CS3107 and primers able to hybridize the *impala*::*yA* transposon sequences. Two nested series of primers allowed us to amplify a 3-kb DNA fragment containing the right and left boundaries of the known *impala*::*yA* sequence. This fragment was sequenced and shown to contain a sequence highly similar to the amino terminus of a *Schizosaccharomyces pombe* membrane protein of unknown function. We could thus assume that we have cloned the 5′ of the *azgA* gene. The missing 3′ sequences were obtained as a cDNA by a 3′ RACE experiment using RNA from the wild type strain and primers able to hybridize with the previously determined sequence. This cDNA included the 3′ terminus of the RNA. Then, the corresponding genomic DNA was amplified by PCR and its sequence determined. We then sequenced a 3013-bp fragment containing a 2012-bp open reading frame interrupted by 4 putative introns. The start point of transcription and the exact location of introns 1 and 2 were determined by the 5′ RACE experiment using RNA from the wild type strain grown on adenine containing medium (see "Experimental Procedures"). We amplified by reverse transcriptase-PCR an internal cDNA fragment overlapping both the 5′ and 3′ RACE fragments. Sequencing this fragment allowed us to map the boundaries of introns 3 and 4. The exact position of the 5′ and 3′ mRNA termini and of the introns is shown in Fig. 3. The insertion of *impala* had occurred at the TA sequence at the 5′ splice site of intron 1 as shown by the inverse PCR sequence (see above).

A 2.6-kb fragment overlapping the insertion point was amplified and used as a probe in Southern blots against DNA extracted from wild type, *azgA4* and *azgA108* strains. The wild type and *azgA4* strains have identical restriction patterns, whereas the *azgA108* strain pattern shows the presence of the transposon (data not shown). Thus the cloned fragment corresponds to the primary transposition event in the CS3107 mutated strain. The *azgA* sequence data have been submitted to the EMBL data base under accession number AJ575188.

The whole 3013-bp fragment was amplified from total genomic DNA of the wild type strain and cloned in the pGEM™-T easy vector. The resulting plasmid is able to complement an *azgA4* mutation in transformation experiments (results not shown).

We find *azgA* in the recently released *A. nidulans* genomic sequence[6] in contig 1.156, Scaffold 12, which anchors this supercontig to chromosome V. No other homologues of *azgA* were found.

Transposon Excision from the azgA Gene—The *azgA108* allele results from an *impala* insertion exactly in the 5′ extremity of the first intron of the *azgA* gene. To obtain $azgA^+$ revertants it is necessary for an excision event to generate sequences compatible with intron splicing. A *azgA108 uapC201/401* double mutant (CS3108) was constructed (see "Experimental Procedures" for details). This is necessary as a single $azgA^-$ mutation shows residual growth on hypoxanthine, which enters via the UapC transporter (1, 3). From this strain, we have obtained 6 revertants as strains able to grow on hypoxanthine as sole nitrogen source. To determine the footprints left by the excision of the transposon we amplified and sequenced for each revertant a 598-bp fragment overlapping the insertion. In four cases, the sequence was identical to the wild type one, whereas the two other strains show a TACATA footprint, comprising a TA duplication (characteristic of the Tc1-mariner family, of which *impala* is a member). This duplication does not affect intron splicing, as the wild type intron/exon junction (read as customary in the opposite, coding strand) GATGT is replaced by GTAT**GTAT**GT (duplicated sequence, including the TA in bold) that maintains a 5′ intron consensus splicing sequence.

AzgA Defines a New Family or Subfamily of Membrane Proteins—No AzgA homologues of known function were identified. Many homologues of unknown function were found in data bases of fungi, plants, bacteria, and Archaea. AzgA is different from every previously described family of purine or pyrimidine transporters. The most similar proteins are found in *Neurospora crassa* and *S. pombe*, followed by two proteins of *A. thaliana*, and a second one of *N. crassa* (see Fig. 4A). No homologues are found in *Saccharomyces cerevisiae*, *Candida albicans,* or *Aspergillus fumigatus*. Similar proteins were found in a large variety of prokaryotes such as the Archaea *Pyrococchus abyssi*, the cyanobacterium *Trichodesmium erythaeum* and *Staphylococcus spp.* or *Pseudomonas spp*. A related human gene was also identified as a cDNA (GenBank™ accession number AAK25745), corresponding to two EST sequences from heart tissue (accession number R15594 and T12436). This sequence was not found in either the human or mouse genomes, it is most similar to bacterial sequences and it is most probably a contaminant.

A number of data bases classify transporters into discrete families. The Pfam data base (33) classifies AzgA and some of its homologues (*i.e.* that of *S. pombe*) as members of the NAT family that includes UapA and UapC (4). The BLOCK data base finds domains conserved between the NAT family and the AzgA homologues (34). The TransportDB[7] includes transporters from a number of completely sequenced genomes, including 109 bacteria, 16 Archaea, and 6 eukaryotes, including *S. pombe*, *N. crassa*, and *A. thaliana* but not *A. nidulans*. No eukaryotic UapA-like or AzgA-like protein are included in the data base but their bacterial homologues (all included by TransportDB in one nucleobase/cation symporter 2 family) are split into two distinct clusters giving a topology very similar to that shown in Fig. 5. The COG data base (88 complete genomes) groups the AzgA-like proteins separately from all others. The NCBI search for conserved domains gives probability values for this and alternative groupings. The highest probability (COG2252, $6e^{-97}$) correspond the COG group of "unknown function" that includes the (and only) AzgA-like proteins. The second (COG0659, $2e^{-11}$) groups the AzgA-like proteins together with the sulfate transporters and the third

[6] genome.wi.mit.edu/annotation/fungi/aspergillus, March 2003.

[7] 66.93.129.133/transporter/wb/index.html.

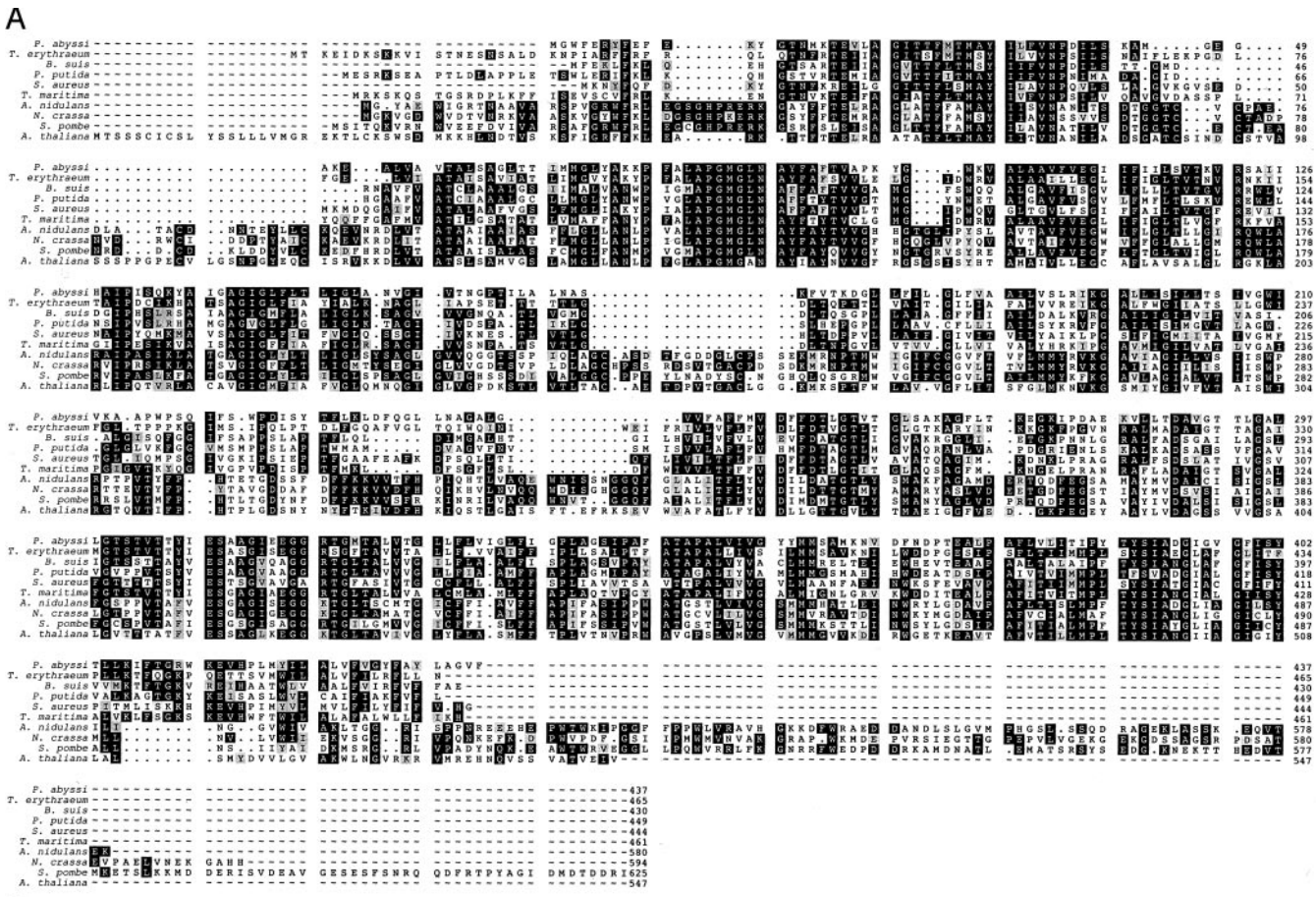


FIG. 4. **Comparison of AzgA with related proteins.** *A*, alignment of the *azgA* amino acid sequence with putative homologous transporters identified using BlastX: *P. abyssi* strain Orsay (accession number CAB49984), *Trichodesmium erythraeum* IMS101 (accession number ZP_00074263), putative xanthine/uracil permease from *Brucella suis* (accession number NP_699845), *Pseudomonas putida* KT2440 (accession number NP_746400), *S. aureus* subsp. *aureus* N315 (accession number BAB43345), *Thermotoga maritima* strain MSB8 (accession number AAD35679), *A. nidulans* (this work), *N. crassa* (deduced *in silico* from the DNA sequence of cosmid B13N4 (MIPS) and EST a6b06np), *S. pombe* (accession number T40742), and *A. thaliana* (accession number BAB09401). The alignment was obtained using the pileup program from GCG package (Wisconsin Package version 10.0, Genetics Computer Group (GCG), Madison, WI); identical amino acids are shown in *black*, similar amino acids in *gray*. There are two putative homologues in both *N. crassa* and *A. thaliana*. Only homologues most similar to *azgA* are shown in this alignment. *B*, hydrophobicity profile of the *azgA* sequence. The figure was generated by the SOSUI program (37). Putative transmembrane segments are *boxed*. *C* and *D*, putative homologous regions. Alignments were created by BLOCKS (*C*) using the sequence of AzgA or its homologue in *N. crassa* as queries (34) or Multalin (D) (58). *UapA*, xanthine/uric acid permease from *A. nidulans* (accession number Q07307); *UapC*, purine permease from *A. nidulans* (accession number P48777); *PYRP*, uracil permease from *B. subtilis* (accession number P39766); *S. pombe* (accession number T40742). The NAT family signature is defined in Ref. 5. *X*, any amino acid; *φ*, hydrophobic amino acid.

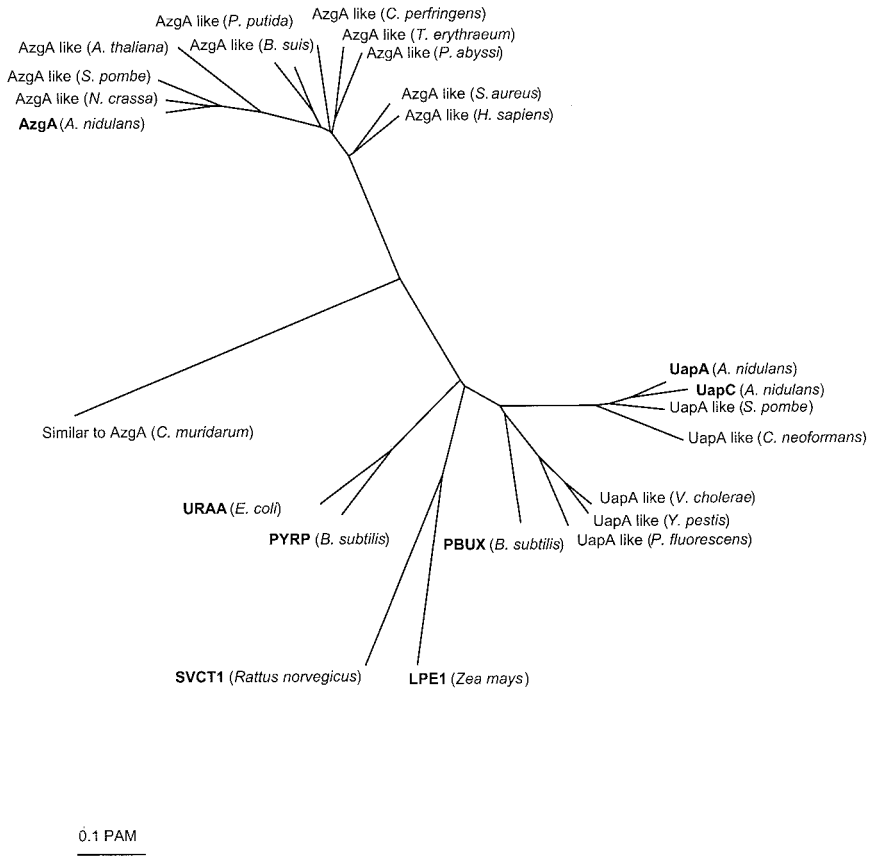


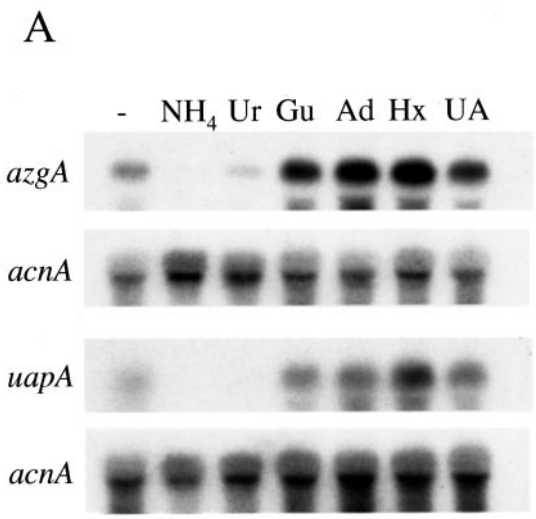
FIG. 5. **Phylogenetic relationship between purines transporters.** Putative or characterized transporters from the NAT family were identified using BlastX and the UapA sequence from *A. nidulans* (accession number Q07307) as a probe: *Pseudomonas fluorescens* (accession number ZP_00088216); *Yersinia pestis* KIM (accession number NP_667450); *Vibrio cholerae* (accession number NP_232339); UAP1 protein from *Cryptococcus neoformans* var. *grubii* (AAN75179); *S. pombe* (accession number NP_593513); *A. nidulans* UapC permease (accession number P48777); SVCT1 human Na^+-dependent vitamin C (L-ascorbic acid) transporter (accession number JC7182); xanthine permease PBUX from *Bacillus subtilis* (accession number P42086); uracil permease PYRP from *B. subtilis* (accession number P39766); uracil permease URAA from *E. coli* (accession number P33780); xanthine and uric acid permease (31) LPE1 from *Zea mays* (accession number T02719). Putative transporters from the AzgA family were identified using BlastX and the AzgA sequence as a probe: *P. abyssi* strain Orsay (accession number CAB49984), *T. erythraeum* IMS101 (accession number ZP_00074263), *P. putida* KT2440 (accession number NP_746400), *S. aureus* subsp. *aureus* N315 (accession number BAB43345); putative xanthine/uracil permease from *B. suis* (accession number NP_699845), *Clostridium perfringens* (accession number NP_562667), AzgA from *A. nidulans* (this work), *N. crassa* (deduced by *in silico* analysis from DNA sequence of cosmid B13N4 (MIPS) and EST a6b06np), *S. pombe* (accession number T40742), *A. thaliana* (accession number BAB09401), P41 *Homo sapiens* (accession number AAK25745). The *Chlamydia muridarum* sequence (accession number NP_296624) was identified using BlastX and the AzgA sequence as a probe. This sequence is classified in the sulfate transporter family. The alignment was created using ClustalW. The tree was created using the neighbor joining method, version 3.6a3 from the PHYLIP package (Phylogeny Inference Package, Joe Felsenstein, Department of Genetics at the University of Washington). In *bold*: proteins of known function.

one (COG2233 $9e^{-5}$) with the proteins of the NAT family. The COG data base displays three different phylogenetic trees corresponding with each classification scheme.

The comparison of UapA, UapC on the one hand and AzgA and its homologues in *S. pombe* and *N. crassa* reveal about 4% identical residues in a ClustalW alignment. Similarity between AzgA and its homologues and NAT proteins is found in a region corresponding to transmembrane segment 1 of UapA and UapC and transmembrane segment 2 of AzgA (Fig. 4*C*). This region is one of the most highly conserved in all proteins of the AzgA family. Mutational evidence suggest that transmemebrane segment 1 is involved in topogenesis of UapA.[8] This domain comprises residues that are conserved in all (Gln^{126} in the UapA sequence) or almost all (His^{127} in the UapA sequence) members of NAT family. Two other conserved regions, typical of the NAT family, were identified in the AzgA sequence (34). The first one (see below, Fig. 4*D*) overlaps a region that is essential for substrate recognition in UapA and UapC (5, 6). Within this region, a typical motif we have described as a signature of the NAT family, (FYS)*X*(QEP)N*X*G$\phi\phi X\phi$T(KRG), where ϕ is a hydrophobic amino acid (4, 5), is not present in AzgA and its homologues. The Asn residue included in the motif is one of the two polar residues that are conserved among all members of the NAT family. In UapA, the Asn residue and the preceding Gln (also absent in AzgA), are determinants of substrate specificity and transport kinetics[9] (6, 32). The second region includes another motif (defined in PROSITE as LIVM)P*X*(PASIF)-V(LIVM)GG*XXXX*(LIVM)(FY)(GSA)*X*(LIVM)*XXX*G, Ref. 35) that is described as typical of proteins of the xanthine-uracil permease family (all included in the NAT superfamily). This motif is present in UapA and UapC but is absent in AzgA and its homologues. 10–12 helical transmembrane segments were predicted for the AzgA family by different programs (36–40). Thus their topology may thus be different from that of members of the NAT family (12 to 14 transmembrane segments according to the program). Fig. 5 shows the phylogenetic rela-

[8] A. Pantazopoulou, M. Koukaki, and G. M. Diallinas, unpublished results.

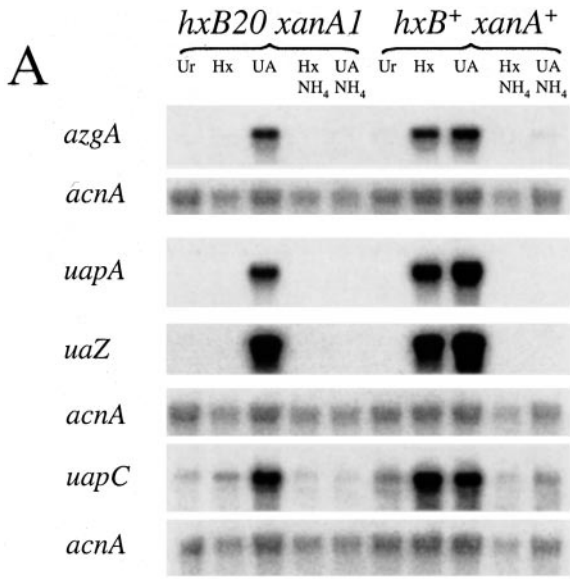
[9] M. Koukaki and G. Diallinas, unpublished results.



B

	-	NH_4	Ur	Gu	Ad	Hx	UA
azgA	28	9	14	129	120	100	72
uapA	52	nd	nd	71	95	100	99

FIG. 6. **Transcription of *azgA* and *uapA* permeases in the presence of various nitrogen sources.** *A*, Northern blot. Mycelia were grown for 8 h at 37 °C on urea as nitrogen source and transferred to the different metabolites indicated below for two further hours: "−," transfer to no nitrogen source; *NH_4*, ammonium L (+) tartrate; *Ur*, urea; *Gu*, guanine; *Ad*, adenine; *Hx*, hypoxanthine; *AU*, uric acid. *azgA*, *uapA*, and *acnA* probes are defined under "Experimental Procedures." *B*, quantification of the *azgA* and *uapA* transcripts: *uapA* and *azgA* data were corrected using the corresponding *acnA* transcript signal intensity as a measure of RNA loading. All values are expressed in relation to the hypoxanthine-induced condition, given the arbitrary value of 100. *nd*, no signal detected.



B

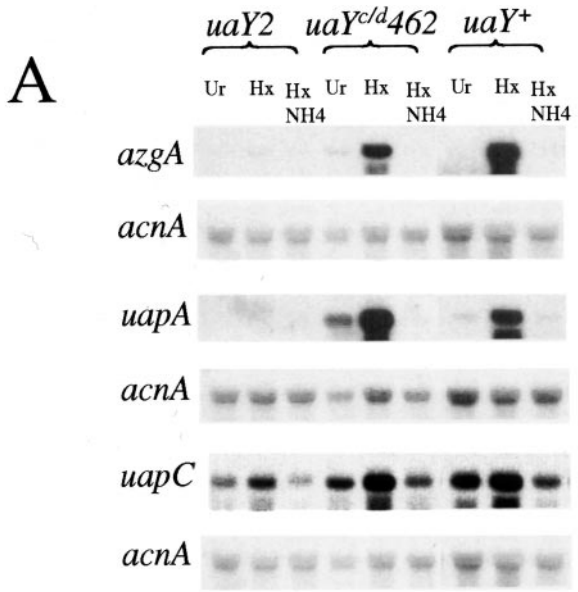
	hxB20 xanA1					*hxB⁺ xanA⁺*				
	urea	Hx	UA	Hx/NH_4	UA/NH_4	urea	Hx	UA	Hx/NH_4	UA/NH_4
azgA	7	21	102	12	12	6	100	62	16	12
uapA	nd	nd	56	nd	nd	nd	100	178	nd	nd
uapC	15	45	81	21	21	25	100	71	34	25
uaZ	nd	nd	98	nd	nd	nd	100	169	nd	nd

FIG. 7. **Comparison of *azgA*, *uapA*, and *uapC* mRNA levels in wild type and *hxB20 xanA1* mutant strains.** *A*, Northern blot: *hxB20 xanA1*: RNA extracted from a strain carrying loss of function alleles in the *hxB* and *xanA* genes; *hxB^+xanA^+*: mRNA extracted from a strain carrying the wild type alleles of these genes. All strains were grown for 8 h as described in the legend to Fig. 6 and for an additional 2 h in media containing the following metabolites: *Ur*, urea; *Hx*, hypoxanthine; *UA*, uric acid; *Hx/NH_4*, hypoxanthine + ammonium L (+) tartrate; *UA/NH_4*, uric acid + ammonium L (+) tartrate. Probes and concentration of metabolites are defined under "Experimental Procedures." *B*, quantification of the *azgA*, *uapA*, *uapC*, and *uaZ* transcripts: *uapA*, *uapC*, *azgA*, and *uaZ* data were corrected according to the corresponding *acnA* transcript intensity. *uapA* and *uaZ* hybridized in the same membrane. All values are expressed in relation to the wild type induced by hypoxanthine, given the arbitrary value of 100. The values shown for the expression of *azgA* in the wild type are the average of four different experiments, other values correspond to the experiment shown. *nd*, no signal detected.

tionships between the proteins of the *azgA* family and those of the NAT family.

Molecular Characterization of azgA Mutants—We amplified PCR fragments corresponding to the entire *azgA* sequence from mutants *azgA4*, *azgA201*, *azgA212*, and *azgA217*. These were sequenced. The *azgA201* allele corresponds to a deletion of 1.2 kb from nucleotides Cyd^{494} to Cyd^{1646} including promoter sequences and amino acids 1 to 206. This strain shows no *azgA* mRNA (not shown). Therefore it is a total loss of function of the *azgA* gene. The phenotype of *azgA201* is identical to the *azgA4* mutant strain. Thus, the proposal that the residual growth of *$azgA^-$* strains is because of the activity of other transporters including UapC (1–3) is correct. This validates earlier work carried out with *azgA4* as a loss of function mutant (this article, and Refs. 2, 3, and 5) before *azgA201* was characterized. The *azgA4* mutation was shown to be a TTC deletion leading to loss of Phe^{257}. This amino acid is located in the 5^{th} putative transmembrane segment according to most of the secondary structure prediction programs used. The *azgA212* mutation is a Gly^{2188} deletion leading to a frameshift at amino acid 369. The *azgA217* allele is a GT to AG change leading to the replacement of Ser^{436} by Lys. This residue is located in a transmembrane segment according to 4 of 6 of the programs used. The position of the mutations is shown in Fig. 3.

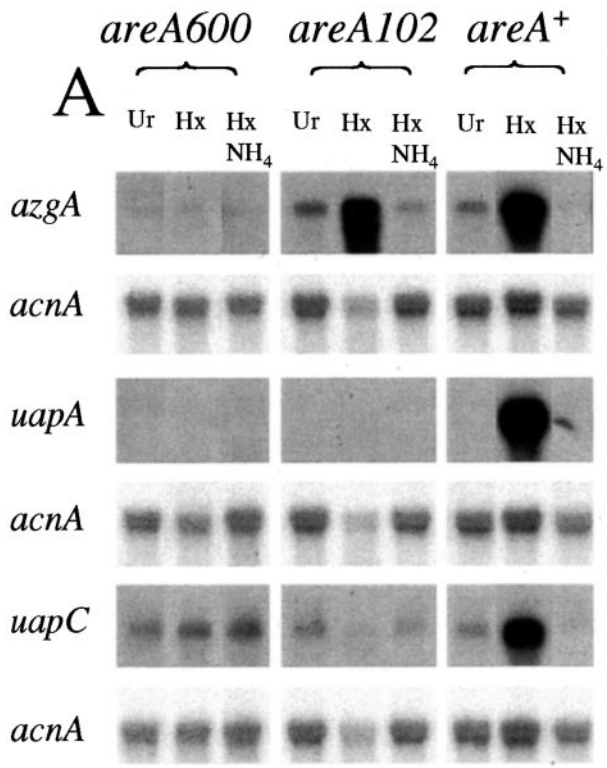
Regulation of azgA Expression—The genes encoding enzymes or transporters of the purine utilization pathway are induced by uric acid and repressed by ammonium and require the UaY and AreA transcription factors (2, 3, 15, 16, 41, 42). Fig. 6 shows that the expression of *azgA* is induced by adenine, guanine, hypoxanthine, and uric acid. A significant expression is seen when the mycelium is starved for nitrogen. The above results suggest that uric acid is the physiological inducer of the *azgA* gene. This is the case for every other gene of the purine catabolism pathway tested to date, other purines inducing through their conversion to uric acid (43–46). To establish this unequivocally, we determined the *azgA* mRNA steady state levels in an *hxB20 xanA1* double mutant. This strain is completely impaired in the conversion of hypoxanthine and xanthine to uric acid, and in this background hypoxanthine is completely unable to act as an inducer of enzymes of purine catabolism (43). Fig. 7 compares the induction by hypoxanthine and uric acid in the wild type and the double mutant strain for the three transporter genes, *uapA*, *uapC*, and *azgA*. We have also included a Northern blot revealing the *uaZ* (urate oxidase) mRNA, where induction by uric acid but not by hypoxanthine in strains unable to convert hypoxanthine to uric acid had first been shown at the level of enzyme activity (44). For *uapA* and *azgA* we can state that hypoxanthine induces only after its conversion to uric acid, as it has been shown for the *hxA* (xanthine dehydrogenase), *uaZ* (urate oxidase), *alX* (allantoinase), and *aaX* (allantoicase) genes (43–45). Surprisingly, we do



B

	uaY2			uaY^c/d 462			uaY^+		
	urea	Hx	Hx/NH4	urea	Hx	Hx/NH4	urea	Hx	Hx/NH4
azgA	9	14	3	15	105	5	11	100	5
uapA	5	13	7	54	181	5	4	100	2
uapC	28	43	18	64	112	29	47	100	29

FIG. 8. **Transcription of *azgA*, *uapA*, and *uapC* in uaY mutants.** *A*, Northern blot. *uaY2*, loss of function allele; *uaY^c/d462*, constitutive-derepressed allele; *uaY^+*, wild type allele. All strains were grown for 8 h on urea as nitrogen sources and transferred for 2 additional hours to: urea (*Ur*), non-induced; hypoxanthine (*Hx*), induced; hypoxanthine + ammonium L (+) tartrate (*Hx/NH4*), induced repressed. For concentrations of metabolites, see "Experimental Procedures." Probes were as described in the legend to Fig. 7. *B*, quantification of the *azgA*, *uapA*, and *uapC* transcripts as described in the legend to Fig. 7.



B

	areA600			areA102			areA^+		
	urea	Hx	Hx/NH4	urea	Hx	Hx/NH4	urea	Hx	Hx/NH4
azgA	5	4	5	9	136	4	6	100	4
uapA	nd	nd	nd	nd	nd	nd	nd	100	nd
uapC	28	36	36	16	24	13	16	100	11

FIG. 9. **Transcription of *azgA*, *uapA*, and *uapC* in areA mutants.** *A*, Northern blot. *areA600*, loss of function allele; *areA102*, mutation conferring altered specificity; *areA^+*, wild type allele. Symbols of growth conditions are as described in the legend to Fig. 8. Probes are the same as described in the legends to Figs. 7 and 8. *B*, quantification of the *azgA*, *uapA*, and *uapC* transcripts as described in the legend to Fig. 8.

see a low but significant induction by hypoxanthine of *uapC*. Fig. 7 also shows that the transcription of the three transporter genes is repressed by ammonium.

Fig. 8 shows the expression of the transporter genes in *uaY* mutants. Induction was checked with both uric acid and hypoxanthine as inducers (only hypoxanthine shown, identical results with uric acid). The *uaY2* mutation abolishes the inducibility of *azgA*. The *uaY^c462* mutation, a Ser to Leu change in residue 222, affects differentially the expression of different genes under UaY control, resulting in various degrees of constitutive and ammonium derepressed expression. *azgA* and *uapC* are not affected in contrast to what was reported for *uapA* (47), the partial constitutivity of the latter being confirmed here.

Fig. 9 shows the expression of *azgA* in the null mutant *areA600* (17, 48) and the specificity mutant *areA102* where expression of *uapA* is abolished and that of *uapC* strongly impaired (confirmed on Fig. 8, Refs. 2, 3, 14, and 17). The former strongly impairs the expression of *azgA*, whereas the latter has no effect on its expression. Hypoxanthine is used as inducer in this experiment, as *areA102* mutations are drastically impaired in the uptake of uric acid but not of hypoxanthine (49).

Thus *azgA*, as all other purine catabolic genes, is under the control of both UaY and AreA. However, these results could be explained either at the level of direct interaction of these transcription factors with the *azgA* promoter or at the level of inducer exclusion. This point will be dealt with below.

DISCUSSION

Uptake studies have established that AzgA is a high affinity, high capacity hypoxanthine transporter. Competition studies strongly suggest that AzgA also transports with high capacity and high affinity adenine. Hypoxanthine transport by AzgA is competed by, and thus AzgA probably transports, albeit with reduced capacity and affinity, guanine, guanosine, 6-thioguanine, and 8-azaxanthine. Competition by 8-azaxanthine was unexpected as xanthine is not a substrate of AzgA. It is also surprising that 8-azaguanine, which is very toxic for strains with a wild type *azgA* gene does not compete hypoxanthine uptake. The K_m values of AzgA for its major substrates, hypoxanthine and adenine, ranges between 1.4 and 3.0 μM, which are lower than those of UapA (7–10 μM) and UapC (8–24 μM) for their cognate substrates (5). This makes teleonomic sense. The substrates of the AzgA transporter can serve both as sources of purines through the salvage pathway and as nitrogen sources, whereas xanthine and uric acid can serve only as nitrogen sources.

AzgA is the first physiologically characterized member of a related family or subfamily of transporters. AzgA and its homologues have no obvious extended similarities with any nucleobase transporter characterized previously. This includes transporters of the PRT family (4) as the *S. cerevisiae* FCY2 adenine-hypoxanthine-cytosine transporter (50) and its homologues in *C. albicans*, the plant PUP family like the AtPUP1

adenine hypoxanthine cytosine transporter of *A. thaliana* (51), the mammalian ENT2 nucleoside facilitated diffusion protein, which is also able to transport nucleobases (52), and the *Trypanosome* transporter TbNT5 (53).

We have indicated some differences and similarities with members of the NAT family. These suggest that the two families may be related. It is an arbitrary decision whether to place the two groups in separate families of proteins or in one family with two highly divergent subfamilies. The non-conservation of residues involved in substrate recognition comforts the divisive rather than the gathering taxonomy. In fact, the methodology used to construct the tree shown in Fig. 5 (sequences alignment using ClustalW) maximizes identities and thus possible phylogentic relationships. The COG data base phylogenetic trees define the *azgA*-like proteins as a separate family, whereas the TransportDB data base includes them in the NAT family but in a clearly separate cluster.

The transcriptional regulation of this transporter in growing mycelium is identical to that of well characterized genes of purine catabolism (reviewed in Ref. 42). The transcription of AzgA is inducible by uric acid, repressible by ammonium, and dependent on the GATA factor AreA and the pathway specific factor UaY. This seems to contradict the fact that 8-azaguanine is toxic on agar plates in the presence of ammonium, in the absence of effectors of UaY (uric acid and its thioanalogues, Refs. 1 and 44), and in strains carrying null *uaY* (16) or *areA* (17) mutations, all conditions where the *azgA* gene is very poorly expressed. We have no simple explanation for this contradiction. The constitutive expression of the AzgA transporter in pre-germinating conidia[2] does not seem a sufficient explanation, as 8-azaguanine is also toxic to mycelia grown under non-inducing conditions. It is possible that 8-azaguanine is highly toxic and even the low, constitutive levels of AzgA protein are sufficient to import the analogue into the cell.

The non-inducibility of *azgA* in *uaY* and *areA* null mutations could be because of a direct participation of these factors or to exclusion of the inducer. The results of Fig. 7 confirms that hypoxanthine acts as inducer only via its conversion to uric acid. When uric acid is used to induce, what is blocked in *uaY* mutants is its uptake via UapA and UapC, when hypoxanthine is used to induce what is blocked in *uaY* mutants is its intracellular conversion to uric acid, whereas there is strong evidence that hypoxanthine is taken up in *uaY*$^{-}$ mutations (13, 41, 42). As a *uaY*$^{-}$ null mutant is not induced for *azgA*, *uapA*, or *uapC* by either hypoxanthine or uric acid, the minimal interpretation of these results is that their induction necessitates uric acid and thus most probably, the protein that mediates uric acid induction, UaY (16, 42). Two canonical (CGG-6X-CCG) and one aberrant (CGG-6X-GCG) UaY binding sites are present in the *azgA* putative promoter region (not shown). Sites responding to these sequences are found in all genes known to be regulated by UaY and have been shown to bind *in vitro* to the UaY DNA binding domain (16, 54, 55). Preliminary *in vivo* methylation protection experiments strongly suggest that UaY binds to at least the UaY promoter proximal site only in the presence of inducer.[10]

In an AreA null mutant neither uric acid can be taken up nor can hypoxanthine be converted to uric acid. Thus the impairment of expression of *azgA* in an *areA600* mutation could be because of inducer exclusion, direct involvement of AreA in *azgA* transcription, or both mechanisms. Area binds to sequences responding to the HGATAR consensus (14, 56, 57). The *areA102* mutation increases substantially binding to TGATAR sites and decreases drastically binding to A/CGATAR sites (14) but does not alter significantly the expression of *azgA*. Five putative AreA binding sites, both of the TGATAR and the A/CGATAR type are present upstream of the *azgA* open reading frame (not shown). Only directed mutagenesis of these sites can distinguish between inducer exclusion and a direct action of AreA in *azgA* transcription. The direct involvement of UaY and AreA in the *uapA* and *uapC* promoters had been shown by *cis*-acting mutations affecting UaY and AreA binding (2, 3, 14).[11]

The expression of *azgA* is higher under nitrogen starved conditions than when urea, reputed to be a neutral (non-inducing, nonrepressing) nitrogen source, is present in the medium. This may be because of sufficient free ammonium accumulating in urea-grown mycelia to afford partial repression, to an increase in intracellular uric acid arising from nucleic acid degradation under starvation conditions or to a specific regulation mechanism. These non-exclusive possibilities have not been investigated further.

The cloning of *azgA* by insertional mutagenesis constitutes the first use of a recently constructed transposon tool for *A. nidulans*, and at the same time illustrates the paucity of transposon insertion in coding regions. It is surprising that an insertion in the *azgA* gene is at least 2 orders of magnitude lower than a spontaneous mutation in the same gene. Similar results were obtained for other mutational insertions of *impala*.[12] This technique allowed us to clone a gene that seemed to be absent in a number of gene libraries available to us, and by this doing, define a new group of membrane proteins, present in plants and in both prokaryotic and eukaryotic microorganisms.

Acknowledgment—G. C. thanks M. Soubes for constant encouragement and discussion.

Note Added in Proof—The work reporting the expression of *azgA* in pre-germinating conidia (see "Discussion" and Footnote 2) is now in press (Amilis, A., Cecchetto, G., Sophianoupolou, V., Koukaki, M., Scazzocchio, C., and Daillinas, G. (2004) *Mol. Microbiol.*, in press).

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