

Novel Route for Agmatine Catabolism in *Aspergillus niger* Involves 4-Guanidinobutyrase

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Agmatine, a significant polyamine in bacteria and plants, mostly arises from the decarboxylation of arginine. The functional importance of agmatine in fungi is poorly understood. The metabolism of agmatine and related guanidinium group-containing compounds in *Aspergillus niger* was explored through growth, metabolite, and enzyme studies. The fungus was able to metabolize and grow on L-arginine, agmatine, or 4-guanidinobutyrate as the sole nitrogen source. Whereas arginase defined the only route for arginine catabolism, biochemical and bioinformatics approaches suggested the absence of arginine decarboxylase in *A. niger*. Efficient utilization by the parent strain and also by its arginase knockout implied an arginase-independent catabolic route for agmatine. Urea and 4-guanidinobutyrate were detected in the spent medium during growth on agmatine. The agmatine-grown *A. niger* mycelia contained significant levels of amine oxidase, 4-guanidinobutyraldehyde dehydrogenase, 4-guanidinobutyrase (GBase), and succinic semialdehyde dehydrogenase, but no agmatinase activity was detected. Taken together, the results support a novel route for agmatine utilization in *A. niger*. The catabolism of agmatine by way of 4-guanidinobutyrate to 4-aminobutyrate into the Krebs cycle is the first report of such a pathway in any organism. *A. niger* GBase peptide fragments were identified by tandem mass spectrometry analysis. The corresponding open reading frame from the *A. niger* NCIM 565 genome was located and cloned. Subsequent expression of GBase in both *Escherichia coli* and *A. niger* along with its disruption in *A. niger* functionally defined the GBase locus (*gbu*) in the *A. niger* genome.

The amino acid L-arginine contains a guanidinium group [$-\text{HN}-\text{C}(=\text{NH})-\text{NH}_2$] and occurs universally across all kingdoms of life. Arginase constitutes the major catabolic path for fungal L-arginine (1, 2). Agmatine, a significant polyamine of lower organisms and plants, mostly arises from the decarboxylation of arginine (3, 4). Arginine decarboxylase has been extensively studied in lower organisms (4) and plants (5, 6). On the contrary, with the sporadic exceptions of *Ceratocystis minor* and *Verticillium dahliae* (7), *Gigaspora rosea* (8), and *Panus tigrinus* (9), fungi do not have arginine decarboxylase. Other minor pathways of agmatine metabolism in fruit bodies of *P. tigrinus* have been proposed (9). An alternate route for putrescine biosynthesis via agmatine was engineered in *Saccharomyces cerevisiae* by introducing *Escherichia coli* arginine decarboxylase and agmatinase genes (10). More recently, an arginase-independent catabolism of arginine (also not involving agmatine) was reported in *Kluyveromyces lactis*; an aminotransferase was proposed to convert L-arginine to ketoarginine in the first step (11).

Very little is known about agmatine catabolism in fungi and aspergilli in particular (Fig. 1). Functional agmatinases that hydrolyze agmatine to putrescine are mostly reported from bacteria (12, 13). Published *Aspergillus* genomes do contain a putative agmatinase sequence(s) (Table 1), but none has been functionally annotated. Putrescine in fungi either can form spermidine/spermine or is converted to 4-aminobutyrate (GABA) via 4-aminobutyraldehyde (GABald) (14, 15). Another route to form putrescine from agmatine occurs in plants and archaeobacteria; this involves two enzymes, namely, agmatine deiminase and N-carbamyl-putrescine hydrolase (16, 17). An amine oxidase isolated from *Aspergillus niger* can oxidize agmatine to 4-guanidinobutyraldehyde (GBald) (18). The fate of GBald in fungi is unclear, but it is oxidized to 4-guanidinobutyrate (GB) by an aldehyde dehydrogenase in *Arthrobacter* sp. (19), *Pseudomonas aeruginosa* (20), and *Tinca tinca* (21). 4-Guanidinobutyrase (GBase), which hydrolyzes GB to

GABA, has recently been reported in *A. niger* (GenBank accession no. KF991130 for the nucleotide sequence and GenBank accession no. AHL44994.1 for protein sequence) and *K. lactis* (11). GB could be used as the sole nitrogen source by *Penicillium roqueforti* (22, 23) and *K. lactis* but not by *S. cerevisiae* (11). Finally, fungal GABA metabolism feeds into the Krebs cycle at succinate (15).

Unlike in bacteria, the functional importance of agmatine and its metabolism in fungi is not well understood. Toward this objective, *A. niger* (as well as its arginase-knockout strain) was grown on agmatine and its catabolic route was established by analyzing the metabolites and enzymes. 4-Guanidinobutyrase is an essential ureohydrolase of this pathway. A novel route for agmatine utilization in *A. niger*, by way of GB to GABA, leading to the Krebs cycle, is reported here.

MATERIALS AND METHODS

Chemicals and reagents. The guanidinium compounds (L-arginine, agmatine, GB, 3-guanidinopropionate, guanidinoacetate, L-homoarginine, and D-arginine) and pyrroloquinoline quinone (PQQ) were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. All other chemicals were of analytical grade and were obtained either from Sigma-Aldrich or from

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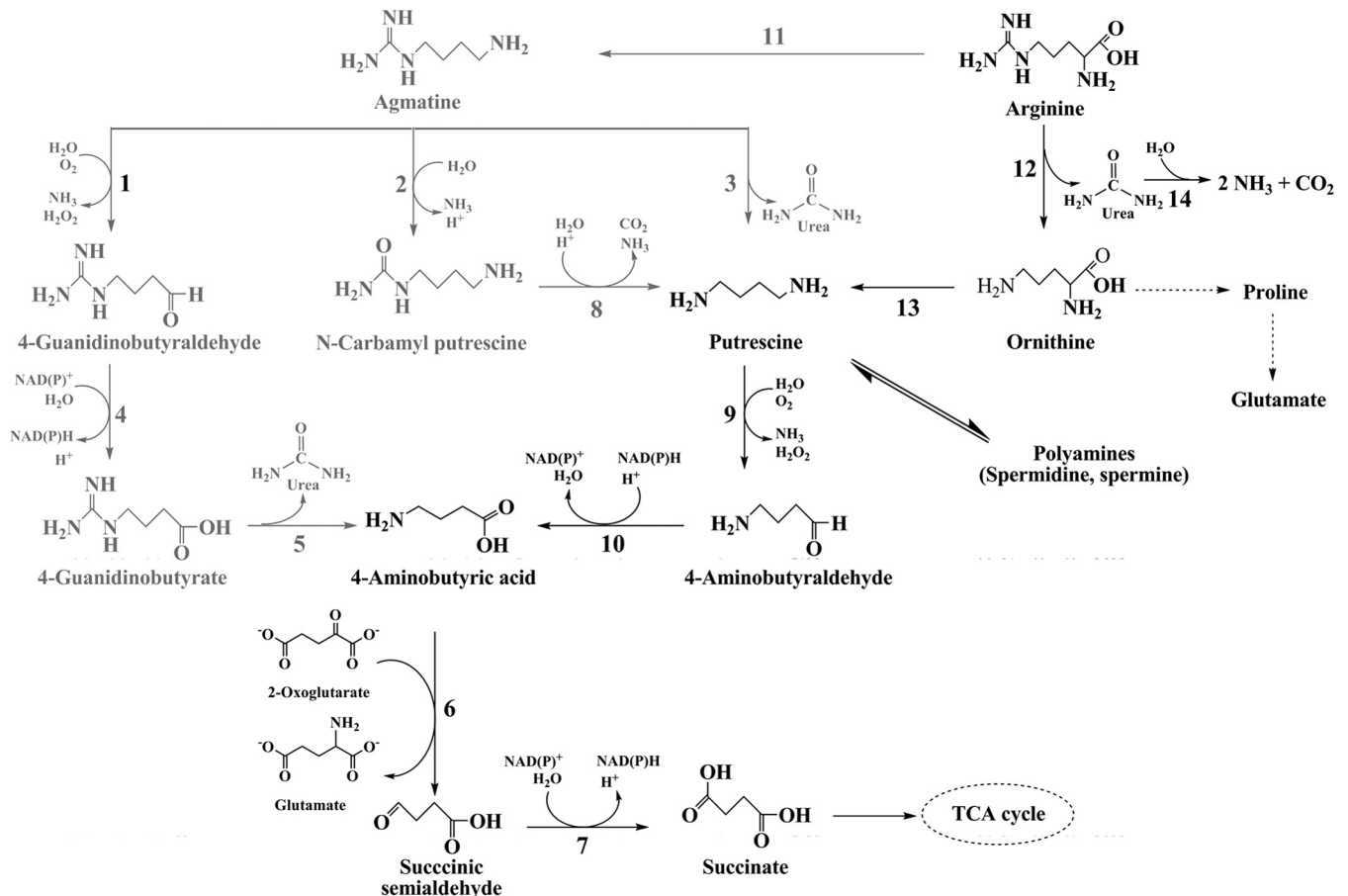


FIG 1 Catabolic steps in agmatine metabolism across various organisms. The different enzymatic steps are numbered as follows: 1, amine oxidase (3, 25, 26); 2, agmatine deiminase (27–31); 3, agmatinase (13, 32); 4, GBald dehydrogenase (33–35); 5, GBase (11, 19, 36, 37); 6, GABA transaminase (15, 38, 39); 7, SSA dehydrogenase (15, 40); 8, *N*-carbamyl putrescine hydrolase (27–30); 9, di-/polyamine oxidase (3, 18, 26); 10, GABald dehydrogenase (19, 20); 11, arginine decarboxylase (7, 9, 41, 42); 12, arginase (2, 43, 45); 13, ornithine decarboxylase (2); 14, urease (2, 46). TCA, tricarboxylic acid. The metabolites and enzymes reported in *A. niger* prior to this work are shown in black.

local suppliers. Water purified from Millipore Milli-Q Ultrapure water purification systems from Millipore, MA, USA, was used throughout this study. Silica thin-layer chromatography (TLC) plates were from Merck (product code 1055540007), and silica gel (60 to 120 mesh) was from Sisco Research Laboratories, India.

Strains and plasmids. *Aspergillus niger* NCIM 565 was obtained from the National Collection of Industrial Microorganisms at the National Chemical Laboratory, Pune, India. *A. niger* strains D-42 (arginase knockout) and Δ XCA-29 (constitutively overexpressing arginase) were derived from the wild-type (WT) parent strain *A. niger* NCIM 565, as described previously (1). The *kusA* disruption strain (*kusA::amdS*; *pyrG*[−]) of *A. niger* (strain MA169.4) (24) was from the Fungal Genetics Stock Center, Manhattan, KS, USA. The plasmids pKA5 (containing *E. coli* arginine decarboxylase and agmatinase [13]) and pETNat (containing *A. niger* arginase cDNA [25]) were used to transform *E. coli* XL1-Blue and BL21(DE3), respectively. Two plasmids, namely, pCB Δ XCGB (with the *A. niger* GBase open reading frame [ORF]) and pETAnGB (with GBase cDNA), were constructed (see below) for GBase expression in *A. niger* and *E. coli* BL21(DE3), respectively.

Media and culture conditions. The *A. niger* strains (except for *A. niger* MA169.4) were maintained on potato dextrose agar (PDA). The inoculum for liquid cultures was generated using spores from such PDA plates. Shake flask cultivation was carried out in either minimal medium (26) or nitrogen-free minimal medium supplemented with the respective guanidinium

compounds (with N in amounts equivalent to that in 28 mM NH₄NO₃); they were individually added after filter sterilization. The final pH of the medium was between 5.5 and 6.0. The mycelia were harvested, blot dried on filter paper, immediately frozen in liquid nitrogen, and stored at −20°C until further use. Stored mycelia were used within 15 days. Enzyme was extracted from the mycelia as described earlier (27). *A. niger* MA169.4 was maintained on minimal agar medium and was cultivated in shake flasks in either complete medium (24) or nitrogen-free minimal medium supplemented with the respective guanidinium compounds (as mentioned above).

Detection of metabolites. *A. niger* samples (5.0 ml) were collected from the growth medium and centrifuged at 2,000 × *g* to remove the mycelia, and the clear spent medium was used to analyze the metabolites. Various guanidinium compounds and metabolites either were resolved on TLC plates or were quantified directly. The TLC plates were developed in two solvent systems (system I, acetone-*n*-butanol-acetic acid-water, 35:35:7:23 [vol/vol]; system II acetone-*n*-butanol-diethylamine-water, 10:10:2:5 [vol/vol]). The amino group-containing compounds were visualized with ninhydrin (1.0% in isopropanol) (28). The Ehrlich reagent (1.0 g *p*-dimethylaminobenzaldehyde dissolved in 25 ml concentrated HCl and 75 ml methanol) was used to detect urea and citrulline. A solution of 2,4-dinitrophenylhydrazine (0.4%) in 2.0 M hydrochloric acid was used to detect aldehydes (29). All guanidinium compounds were detected by spraying the Sakaguchi reagent (30).

TABLE 1 Sequences annotated to be putative agmatinases in *Aspergillus* genomes

<i>Aspergillus</i> sp.	NCBI accession no. of putative agmatinase sequence(s) ^a
<i>A. clavatus</i>	XP_001275257.1 , XP_001274596.1
<i>A. fischeri</i>	XP_001260197.1 , XP_001259366.1
<i>A. flavus</i>	XP_002382185.1 , XP_002381248.1
<i>A. fumigatus</i>	XP_749704.1 , XP_753336.1
<i>A. kawachii</i>	GAA84237.1 , GAA91457.1, GAA89766.1
<i>A. nidulans</i>	CBF79478.1 (XP_680757.1), CBF71628.1
<i>A. niger</i> strains	
CBS 513.88	XP_001400552.1 , XP_001389901.1, XP_001390388.2, XP_001401219.1
ATCC 1015 ^b	EHA23592.1 , EHA21274.1, EHA27215.1, EHA27980.1
<i>A. oryzae</i>	XP_001819186.2 , XP_001823266.2, XP_001824162.1
<i>A. terreus</i>	XP_001215942.1 ^c

^a The NCBI protein database was queried for putative agmatinase sequences from each *Aspergillus* sp. None of these were annotated as GBase. Only sequences that showed $\geq 85\%$ identity (by analysis with the blastp program) with the *A. niger* NCIM 565 GBase sequence (GenBank accession no. [AHL44994.1](#)) are in bold.

^b All four *A. niger* ATCC 1015 sequences are annotated as hypothetical proteins.

^c Annotated as a hypothetical protein.

The guanidinium compounds were also derivatized with benzoin (31), and the fluorescence either was detected (at 312 nm) on TLC plates or was quantified in a spectrofluorometer (RF-5301PC series from Shimadzu; excitation at 325 nm and emission at 443 nm).

4-Guanidinobutyraldehyde (GBald) is not commercially available and, hence, was synthesized (32, 33). GBald was resolved from unreacted L-arginine by silica gel chromatography with methanol as the solvent. The isolated GBald invariably contained traces of GB (a possible oxidation product). GBald was estimated by monitoring its azine derivative at 306 nm (34, 35).

Enzyme and protein estimations. To assay different enzyme activities, the crude *A. niger* mycelial extracts were prepared in appropriate buffers. Ureohydrolases were extracted in 200 mM potassium phosphate buffer (with 2.0 mM 2-mercaptoethanol and 1.0 mM phenylmethylsulfonyl fluoride at a final pH of 7.2), whereas for aldehyde dehydrogenases, 100 mM sodium pyrophosphate (with 14 mM 2-mercaptoethanol, 1.0 mM EDTA, and 0.1% Triton X-100 at a final pH of 9.0) was used. These enzyme extracts were routinely desalted (on Sephadex G-25) to eliminate assay interference by small molecules. The protein concentration was estimated (36) using bovine serum albumin as a reference. For each enzyme monitored, specific activity is defined as the number of units per milligram of protein.

The arginase standard assay mixture at pH 10.6 consisted of L-arginine (150 mM), glycine (150 mM), MnSO₄ (1.0 mM), and an appropriate amount of enzyme. For other ureohydrolases, standard assay mixtures at pH 7.5 consisted of HEPES (100 mM), MnSO₄ (1.0 mM), the corresponding guanidinium substrate (25 mM), and an appropriate amount of enzyme. The urea formed was estimated by a modified Archibald method (37, 38). One unit of ureohydrolase activity was defined as amount of enzyme required to produce 1 μ mol of urea per min in the standard assay. Amine oxidase (with benzylamine as the substrate) was routinely assayed as reported earlier (18). One unit of amine oxidase was defined as the amount of enzyme that produced 1.0 nmol of benzaldehyde per min in the standard assay. Amine oxidase (with agmatine as the substrate) was also assayed by monitoring the azine derivative of GBald at 306 nm (34, 35); however, an acetaldehyde standard curve was employed, as pure GBald was not available. One unit of enzyme activity is defined as the amount of enzyme required to produce 1.0 nmol of GBald (acetaldehyde equivalents) per min in the standard assay. Arginine decarboxylase was assayed with L-arginine as described before (39). Both arginine and agmatine (the product) were monitored on TLC plates as their fluorescent derivatives

TABLE 2 Primers used in this work

Primer	Sequence (5'-3') ^a	Restriction site
GBNdF1	5'-CAAATTCATATGAGATCCCTCACT TGGG-3'	NdeI
GBNoR1	5'-CATAACTGCGGCCGCATACTTCAT TCACAGC-3'	NotI
GBEco2	5'-CGGAATTCAGAAGCTGAACATTG-3'	EcoRI
GBXhR2	5'-GCAGTACAAGCTCAAGGACG-3'	
GBXbF1	5'-TACAAATCTAGAAAGCACTATGG-3'	XbaI
GBEco1	5'-GTCGAATTCGTGATCGGGATG-3'	EcoRI
GBM1F1	5'-GATTGCTGCTCCGAATTTCC-3'	
GBM1R1	5'-GCTATCCGTCTCACTCTTAC-3'	
GBRTR1	5'-TGGTGTGATCGGGATGTGC-3'	

^a Restriction sites are underlined.

(31, 40). The *E. coli* extract containing arginine decarboxylase and agmatinase served as the respective enzyme-positive controls; *E. coli* XL1-Blue transformed with pKA5 was used for this purpose (13). The *A. niger* succinic semialdehyde (SSA) dehydrogenase (SSADH) activity was determined by monitoring the formation of NADH at 340 nm (41). GBald dehydrogenase was assayed by a method essentially similar to that used for SSA dehydrogenase, except that GBald was used as the substrate in place of SSA.

The enzyme activity data presented are typical reproductions of at least three independent measurements. Experimentally determined values are presented as points, while the lines join two data points.

MS/MS analysis of GBase protein. GBase protein was purified to homogeneity from GB-grown *A. niger* mycelia. The purified GBase was desalted, concentrated, and taken in water with an Amicon Ultra-15 centrifugal filter unit with an Ultracel-10 membrane (molecular mass cutoff, ~ 10 kDa; Millipore) before loading on an SDS-polyacrylamide gel. In-gel digestion with trypsin was done as reported previously (42), with some modification. The trypsin-digested GBase was mixed with matrix solution (α -cyano-4-hydroxycinnamic acid [CHCA]; λ maxima at 337 nm and 355 nm). The sample was air-dried at room temperature before it was subjected to matrix-assisted laser desorption/ionization-tandem time of flight mass spectrometry (MS) analysis (AB Sciex, Framingham, MA). A frequency-tripled neodymium-doped yttrium aluminum garnet laser (355 nm, 3.49 eV) was used as the laser source. The acquired spectra were processed using attached 4000 series Explorer software (version 3.5.3). The peptide fragment ions were subjected to similarity searches (MS/MS ion search) using the Mascot server (version 2.1; Matrix Science) against the sequences in the NCBI database with fungi as the queried organism. The number of missed cleavages was set at 2. MS/MS spectra with a Mascot score higher than the significant score, using a confidence interval greater than 95%, were considered.

Cloning and expression of the GBase ORF in *A. niger*. The peptide fragment data from MS/MS analysis of the purified GBase protein were used to identify the GBase locus (*gbu*) in the *A. niger* genome (<http://genome.jgi-psf.org/Aspni5/Aspni5.home.html>). The corresponding GBase ORF from *A. niger* NCIM 565 genomic DNA (1,384 bp) was PCR amplified using primers GBNdF1 and GBNoR1 (Table 2). The PCR product (obtained by use of a 25- μ l reaction mixture containing 3.5 mM MgCl₂, 200 μ M deoxynucleoside triphosphates, 0.5 μ M primers, and 5 units of *Pfu* DNA polymerase from MBI Fermentas) was purified with a GeneJET gel extraction kit (MBI Fermentas, Lithuania). The NdeI-NotI fragment from this amplicon was cloned in front of the constitutive *A. niger citA* promoter to obtain pCB Δ XCG (see Fig. S1 in the supplemental material). This plasmid was linearized with ScaI and used to transform *A. niger* NCIM 565 protoplasts (43). The transformants were single spored and characterized for GBase expression.

Construction of *A. niger* GBase knockout. A total of 1,442 bp (the terminal 253 bp of the GBase ORF along with 1,189 bp of the 3' flanking

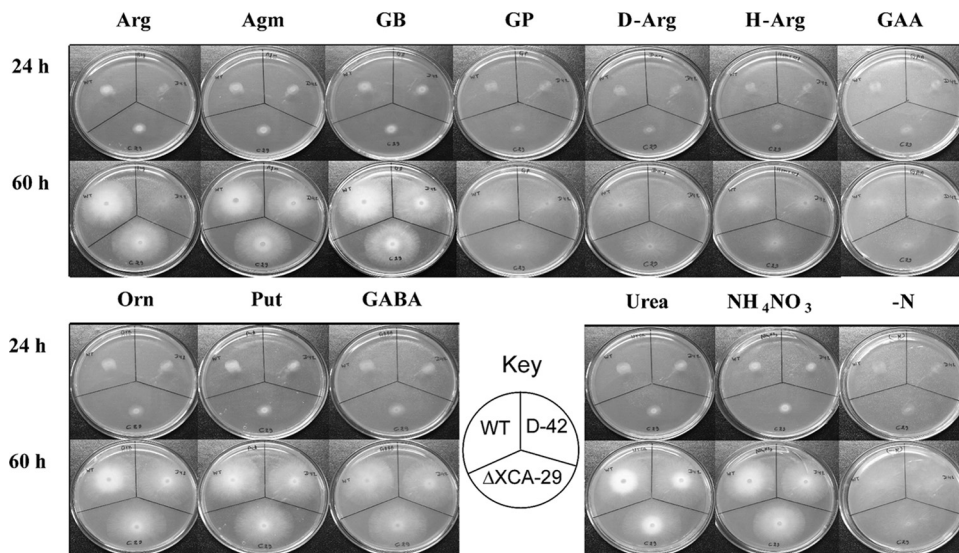


FIG 2 Growth of *A. niger* on various guanidinium compounds. (Top) Spores (equal numbers) of *A. niger* (the WT, D-42, and Δ XCA-29 strains) were spot inoculated on media containing different nitrogen sources (20 mM with respect to total N), namely, L-arginine (Arg), agmatine (Agm), 4-guanidinobutyrate (GB), 3-guanidinopropionate (GP), D-arginine (D-Arg), L-homoarginine (H-Arg), and guanidinoacetate (GAA). (Bottom) The corresponding growth patterns on urea and the respective amino products (namely, L-ornithine [Orn], putrescine [Put], and 4-aminobutyric acid [GABA]) of ureohydrolase action. The results for controls with ammonium nitrate (NH_4NO_3) and no nitrogen source (-N) are also shown. Growth was recorded at two time points.

region) was PCR amplified from *A. niger* NCIM 565 genomic DNA with primers GBeco2 and GBXhr2 (Table 2). The amplicon was cloned into pBSGCDH (44) between the EcoRI and XhoI sites to obtain pBSGB3'F. At the same time, 1,548 bp (the initial 514 bp of the GBase ORF along with 1,034 bp of the 5' flanking region) was also PCR amplified (with primers GBXbF1 and GBeco1; Table 2) and cloned into pBSGB3'F between the EcoRI and XbaI sites to obtain pBSGB5'3'F. *A. niger pyrA* was inserted at the EcoRI site of pBSGB5'3'F to form the *gbu* disruption cassette (pBSG-BPyrADel). *A. niger* MA169.4 was transformed with either a circular or an XmnI-linearized disruption cassette. The transformants (*gbu::pyrA*) were selected on YDA (yeast nitrogen base without amino acids and ammonium sulfate but containing dextrose, ammonium nitrate, and agar) plates without uracil, thus scoring for uracil prototrophy.

Cloning and expression of GBase cDNA in *E. coli*. Total RNA was extracted from *A. niger* mycelia grown on GB by use of an RNeasy plant minikit (Qiagen, Leusden, The Netherlands). Reverse transcriptase PCR (RT-PCR) was performed with 6.5 μg of total RNA using a Transcriptor first-strand cDNA synthesis kit (Roche, Mannheim, Germany). Two short introns were predicted in the early part of the GBase ORF (GenBank accession no. KF991130). Only this region was reverse transcribed with a combination of primers GBnDF1 and GBRTR1 (Table 2). The NdeI-BmtI fragment of this amplicon was moved along with the BmtI-NotI fragment from pCB Δ XCGb to construct complete GBase cDNA (1,269 bp) in pETAnGB (see Fig. S1 in the supplemental material). This plasmid was transformed into competent *E. coli* BL21(DE3) cells, and GBase expression was followed (45).

RESULTS AND DISCUSSION

Arginase provides the sole catabolic route for arginine in *A. niger* (1). An *in silico* analysis retrieved four more putative ureohydrolase sequences from the genome of this fungus (Table 1). Such sequences from *A. niger* and other aspergilli are mostly annotated as putative agmatinases. The possible occurrence of additional ureohydrolases and the catabolism of other guanidinium compounds were thus anticipated. The metabolism of agmatine and related guanidinium group-containing compounds by *A. niger*

was therefore explored through growth, metabolite, and enzyme studies.

Growth on various guanidinium compounds. The nitrogen-free minimal medium supplemented with L-arginine, agmatine, or GB supported good growth of *A. niger*. Growth on NH_4NO_3 as the nitrogen source served as a positive control. Scant or negligible growth was observed on media containing 3-guanidinopropionate, guanidinoacetate, and two arginine analogs, namely, D-arginine and L-homoarginine (Fig. 2). The *A. niger* strains D-42 (arginase knockout) and Δ XCA-29 (constitutively overexpressing arginase) (1) were chosen to assess the arginase contribution to guanidinium compound metabolism in this fungus. The growth phenotypes of these two strains were similar to the growth phenotype of the parent strain (WT), except that strain D-42 was unable to grow on arginine. The results are consistent with the fact that the fungus is able to utilize agmatine and GB in an arginase-independent manner.

Ureohydrolases are known to act on L-arginine, agmatine, and GB to form ornithine, putrescine, and GABA, respectively, with the other product always being urea (Fig. 1, steps 3, 5, and 12). The suitability of these metabolites as potential nitrogen sources was examined. The three strains of *A. niger* were able to grow equally well on ornithine, putrescine, GABA, and urea (Fig. 2). The possible occurrence of agmatinase and GBase in this fungus could therefore be anticipated and was tested (see below). For this, the fungus was conveniently grown in liquid culture to observe possible metabolites appearing in the culture medium and detect enzyme activities in the mycelia.

Metabolites observed in culture media. The culture media were sampled periodically during the growth of *A. niger* (WT and D-42 strains) on different nitrogen sources. These samples were analyzed for the presence of various metabolites.

(i) **GB as an intermediate in agmatine catabolism.** L-Arginine,

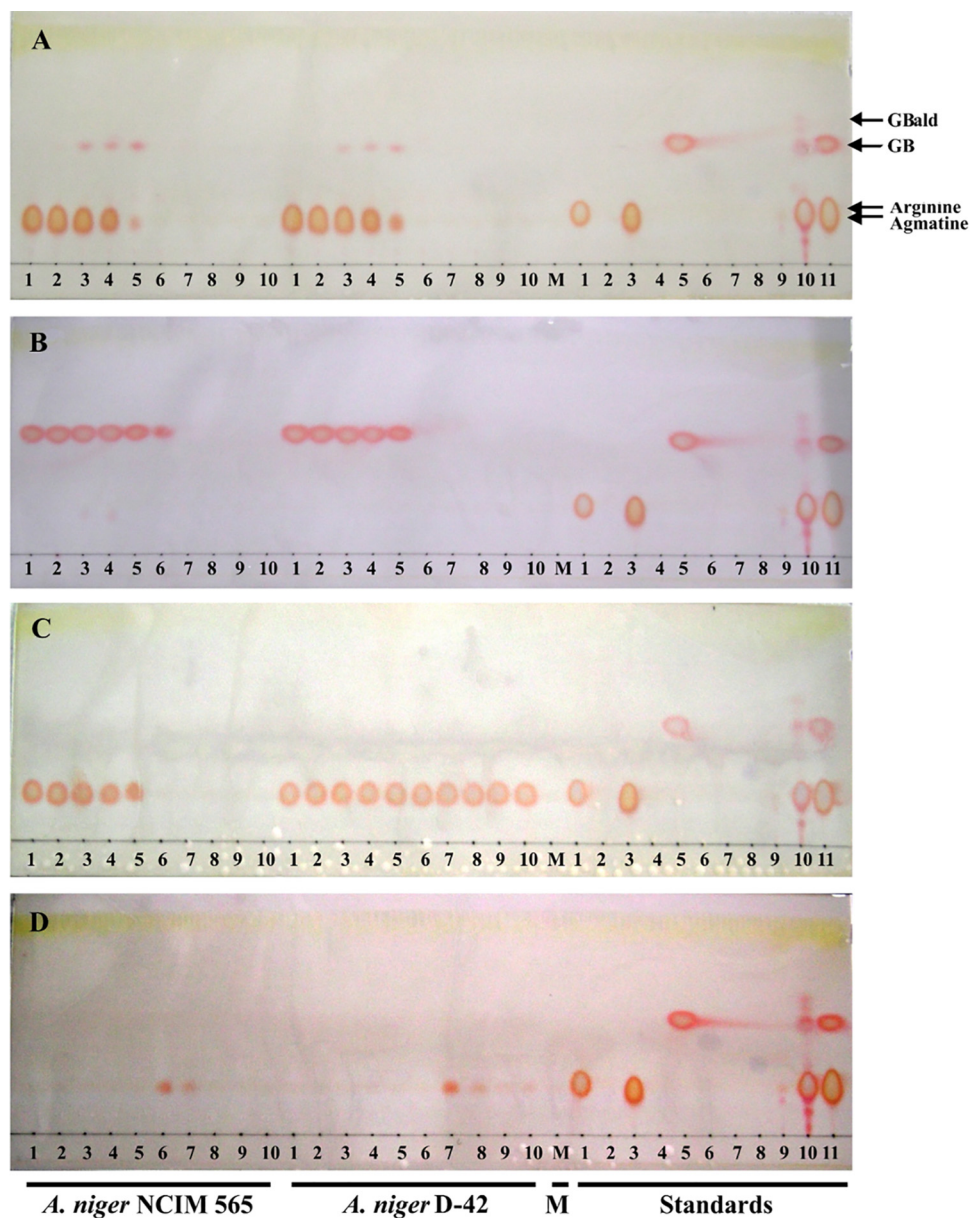


FIG 3 Detection of guanidinium compounds by TLC. *A. niger* strains (the WT and D-42 strains) were grown in liquid culture with agmatine (A), GB (B), L-arginine (C), and NH_4NO_3 (D) as the sole nitrogen source. Aliquots of these culture media (2.0 μl each) were spotted on TLC plates, developed in solvent system I, and visualized by use of the Sakaguchi reagent. Results for samples obtained after every 10 h of growth (lanes 1 to 10) are shown. Culture media with no nitrogen (lane M), the standards (L-arginine, ornithine, agmatine, putrescine, GB, GABA, urea, citrulline, SSA, and GBald [lanes 1 to 10, respectively]), and a mixture of L-arginine, agmatine, and GB (lane 11) were tested.

agmatine, and GB disappeared from the *A. niger* culture medium with time (Fig. 3); since strain D-42 was unable to grow on arginine, only agmatine and GB disappearance was observed in this case (Fig. 4). The results are consistent with the growth of *A. niger* on these nitrogen sources (Fig. 2). A transient appearance of GB was observed (at between 30 and 50 h) only in the agmatine-containing culture medium. No GB could be found in the culture media when the fungus was grown on arginine or ammonium nitrate (Fig. 3). As agmatine and L-arginine could not be directly resolved, their fluorescent benzoin derivatives were analyzed (see Fig. S2 in the supplemental material). Agmatine was not detected in the spent media of arginine-grown samples. A faint fluorescent

spot (R_f 0.94) was, however, observed in agmatine-grown samples at 30 h; the R_f of this spot corresponds to that of standard GBald, thereby suggesting that it may also be a metabolic intermediate in the agmatine catabolic pathway.

Potential metabolic intermediates, like citrulline, SSA, putrescine, and GABA, were not detected in the culture media at any time point. Amino compounds (except for agmatine or arginine, whenever one of these was added to the media) were not detected. Interestingly, urea was found in the culture media when the organism was grown on L-arginine, agmatine, or GB; urea transiently appeared when these compounds disappeared from the media (Fig. 4). Besides urea as the common product, ureohydro-

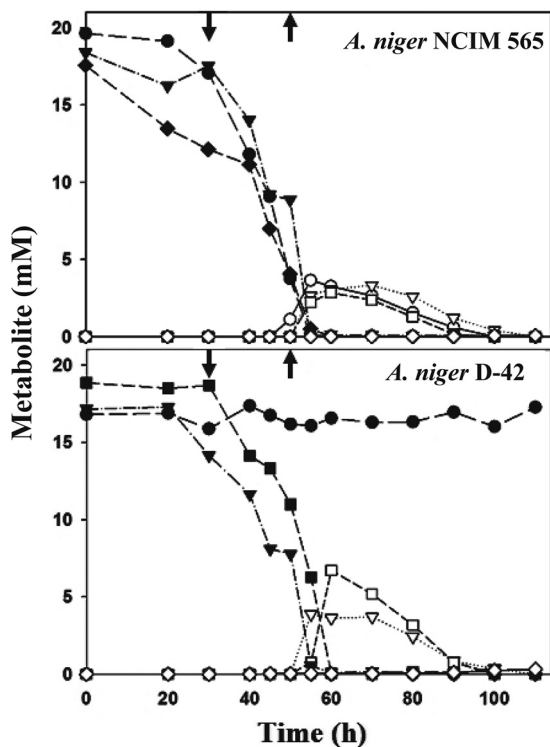


FIG 4 Fate of guanidinium compounds and detection of urea in *A. niger* culture media. *A. niger* strains (the WT and D-42 strains) were grown in liquid culture with L-arginine (circles), agmatine (inverted triangles), GB (squares), or NH_4NO_3 (diamonds) as the sole nitrogen source. Aliquots of the culture media were sampled at various time points and analyzed for the respective guanidinium compounds (filled symbols) and urea (the corresponding open symbols). GB appeared (\downarrow) and disappeared (\uparrow) from the culture medium (Fig. 4) only when the fungus was grown on agmatine.

lase action does produce an amine, such as ornithine (by arginase), putrescine (by agmatinase), or GABA (by GBase). As none of these products appeared in the respective culture media, the transient accumulation of urea (and not ornithine or GABA) suggests that *A. niger* prefers the prior catabolism of amino products over urea. However, aspergilli do possess a functional urease, and the *A. niger* enzyme has a reasonable K_m for its substrate (3.0 mM) (46). Deferred urea utilization could be due to the paucity of urease activity *per se* under those growth conditions.

Table 3 summarizes the metabolites found in the culture media. The data suggest that GB and also, possibly, GBald are intermediates in the catabolism of agmatine. Urea was the common metabolite detected on L-arginine, agmatine, and GB. Urea formation from agmatine and GB (even with *A. niger* strain D-42) clearly implied the possible presence of a ureohydrolase(s) other than arginase.

(ii) **Detection of PQQ in the culture medium.** Only when *A. niger* (and also the D-42 strain) was grown on agmatine as the sole nitrogen source, the culture medium turned pink at later time points. The possible identity of this pink product as pyrroloquinoline quinone (PQQ) was considered. The pink product was purified on a DEAE-Sephacel column and spectrally characterized (Fig. 5). The UV-visible (UV-Vis) and fluorescence spectra of this compound matched those reported for PQQ (47, 48). Upon electrospray ionization MS analysis, the m/z value (313.89) of the compound was the same as that of authentic PQQ. PQQ is a cofactor for some amine oxidases (47–49). Detection of PQQ, only when grown on agmatine, implicates a role for amine oxidase in *A. niger* agmatine catabolism.

Enzymology of agmatine metabolism. The detection of relevant enzymes constitutes important evidence in support of an operational metabolic pathway. Therefore, various enzyme activities were monitored in the desalted crude mycelial extracts (see Materials and Methods) of *A. niger* grown (for 24 h) on L-arginine, agmatine, GB, or NH_4NO_3 .

(i) **Ureohydrolases.** The ability of *A. niger* to utilize arginine, agmatine, and GB (Fig. 2) in association with the detection of urea in the culture media (Fig. 4 and Table 3) points to the possible occurrence of more than one ureohydrolase. Of these, arginase is well studied (1, 25), and it was detected on all the nitrogen sources tested (Table 4). Scoring for the presence of agmatinase (on agmatine) and GBase (on GB) activities was therefore of interest. Since agmatinase activity was not found in agmatine-grown mycelia, an alternate route(s) for agmatine catabolism was anticipated. However, GBase activity was consistently observed when the fungus was grown on either GB or agmatine (Table 4). Under similar conditions, the *A. niger* D-42 strain (arginase knockout) was devoid of agmatinase and arginase activities, but GBase activity was readily detected. Arginase therefore may not have a direct role to play in agmatine/GB catabolism. Arginase-independent catabolism of arginine was recently reported in *K. lactis*; this pathway involved ketoarginine and GBase, but no agmatine was in-

TABLE 3 Summary of metabolites observed in the culture medium of *A. niger* WT and D-42 strains

Metabolite	Detection of metabolite with the following nitrogen source and strain ^a :							
	L-Arginine		Agmatine		GB		NH_4NO_3	
	WT	D-42	WT	D-42	WT	D-42	WT	D-42
L-Arginine	+	+	–	–	–	–	(+)	(+)
Agmatine	–	–	+	+	–	–	–	–
GABA	–	–	–	–	–	–	–	–
GB	–	–	+	+	+	+	–	–
GBald	–	–	+	+	–	–	–	–
SSA	–	–	–	–	–	–	–	–
L-Ornithine	–	–	–	–	–	–	(+)	(+)
Putrescine	–	–	–	–	–	–	–	–
Urea	+	–	+	+	+	+	–	–

^a + and –, presence and absence of the particular metabolite, respectively; (+), detection of traces. The D-42 strain does not grow on L-arginine.

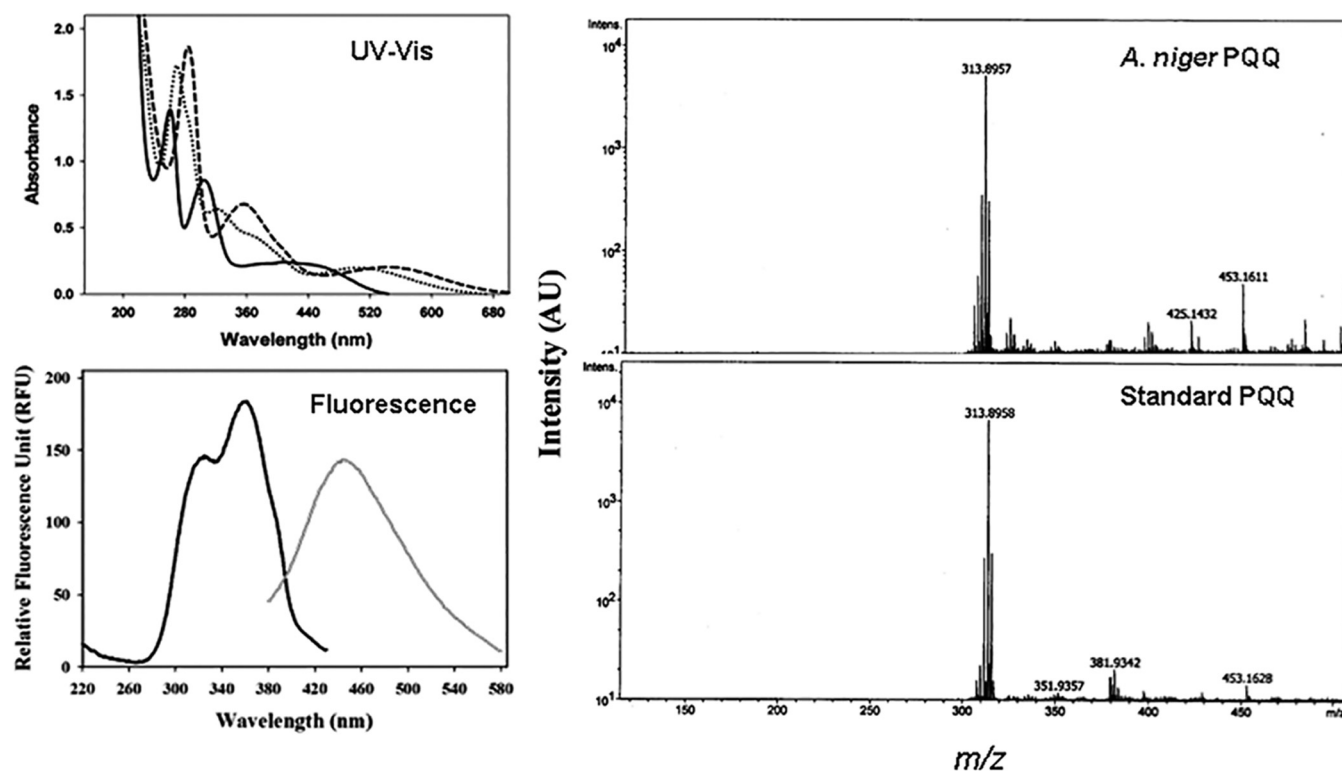


FIG 5 Identification of PQQ in *A. niger* spent medium. (Left) The UV-Vis spectra (solid line, pH 1.0; dotted line, pH 7.0; dashed line, pH 12.0) and the fluorescence spectra at pH 7.0 (black line, emission λ at 448 nm; gray line, excitation λ at 323 nm) of the isolated pink compound are shown. (Right) The mass spectrum of the pink compound is compared with that of standard PQQ. AU, relative abundance units.

involved (11). The *A. niger* ureohydrolase data presented here clearly implicate a role for GBase in agmatine catabolism.

(ii) **Amine oxidase.** The utilization of agmatine without an agmatinase contribution (see above) and the associated detection of PQQ in the spent medium suggested that an amine oxidase may participate in the first step of its catabolism (Fig. 1). *A. niger* ex-

presses amine oxidases to utilize amines as the nitrogen source; many of them are well characterized. A broadly specific amine oxidase acting on agmatine is reported from *A. niger* (18, 50). *A. niger* crude extracts, obtained from agmatine-grown mycelia, contained an amine oxidase that could oxidize agmatine and benzylamine (Table 4). The absence of this activity in mycelia grown on other nitrogen sources, like GB, arginine, or NH_4NO_3 , is strongly indicative of its role in initiating agmatine catabolism in *A. niger*. Detection of GBald (Table 3), the product of agmatine oxidation, lends further support to this view.

(iii) **Aldehyde dehydrogenases.** An amine oxidase acting on agmatine forms GBald. Consecutive steps catalyzed by amine oxidase and GBald dehydrogenase define a metabolic path from agmatine to GB (Fig. 1). A dehydrogenase converting GBald to GB could be anticipated from the unambiguous detection of GB in the culture medium (Fig. 2). Its biochemical characterization was limited by the fact that GBald is not commercially available. Claiming a specific *A. niger* GBald dehydrogenase activity is fraught with two difficulties: (i) some GB was always found in synthesized and purified GBald samples; GBald may be unstable and susceptible to air oxidation. (ii) *A. niger* displays significant dehydrogenase activities with promiscuous substrate specificity (51). While NAD-dependent GBald oxidation could be demonstrated in extracts of mycelia grown on agmatine, substantial dehydrogenase activity was also observed with acetaldehyde and propionaldehyde as the substrates (Table 4). Resolving/purifying these aldehyde dehydrogenases is required to establish the presence of a GBald-specific enzyme in *A. niger*. Such a GBald-

TABLE 4 Enzyme activities in *A. niger* mycelia

Enzyme	Activity (U mg^{-1} protein) ^a			
	L-Arginine	Agmatine	GB	NH_4NO_3
Ureohydrolase				
4-Guanidinobutyrase	ND	0.7	0.2	ND
Arginase	10.0	2.3	4.0	5.0
Agmatinase	ND	ND	ND	ND
Amine oxidase				
Benzylamine	ND	81.0	ND	ND
Agmatine	ND	5.0	ND	ND
Aldehyde dehydrogenase^b				
GBald	0.3	11.8	9.6	ND
Propionaldehyde	1.9	64.9	21.5	ND
Acetaldehyde	1.1	59.3	18.8	ND
SSA	ND	5.0	28.4	ND

^a The data on enzyme activity in crude extracts are representative of those from three independent experiments (performed in duplicate), and the variation was less than 10%. ND, not detected.

^b A high background dehydrogenase activity (NADH appearance) was observed with agmatine.

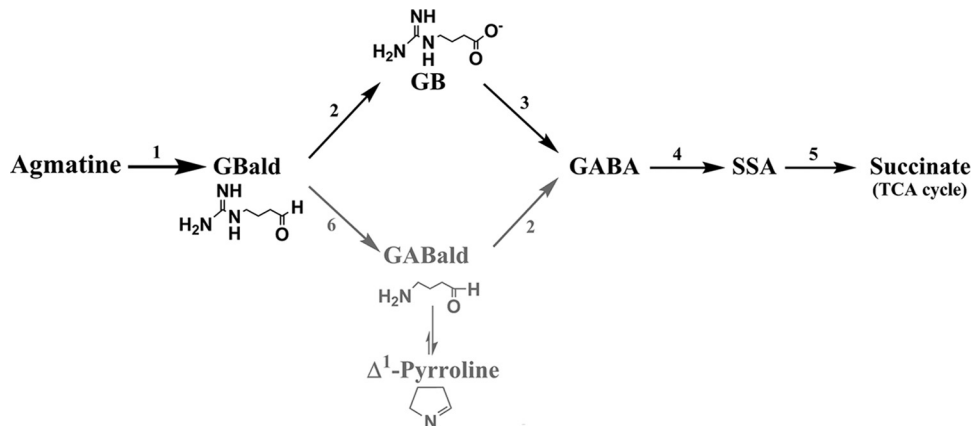


FIG 6 Proposed pathway for agmatine catabolism in *A. niger*. The different enzymatic steps are numbered as follows: 1, agmatine oxidase; 2, GBald/GABald dehydrogenase; 3, GBase; 4, GABA transaminase; 5, SSA dehydrogenase; 6, GBald ureohydrolase. Likely steps and metabolites in the alternate path for GBald are in gray.

specific aldehyde dehydrogenase is known in *Pseudomonas* spp. (52, 53).

GABA may arise from glutamate, agmatine, or 4-guanidinobutyrate, but its catabolism is well established in *A. niger* and involves GABA transaminase and SSADH (15). GB (provided externally or arising internally from agmatine; Table 3) is converted by *A. niger* GBase to GABA (Table 4). SSADH is thus the second aldehyde dehydrogenase expected to participate in agmatine catabolism. Indeed, SSADH activity was found in *A. niger* mycelia grown on agmatine or GB (Table 4). A specific SSADH from GABA-grown *A. niger* has already been characterized (41, 51).

Deconstructing *A. niger* agmatine metabolism. Spermidine is the major polyamine in ascomycete fungi, while putrescine and spermine contribute as minor polyamines (14). The presence of agmatine is not documented in these fungi. With the rare exceptions of *Ceratocystis minor* and *Verticillium dahliae* (7) and of *Panus tigrinus* (9), there are no reports on arginine decarboxylase (and, by implication, agmatine biosynthesis) in the fungal kingdom. In several attempts, we did not detect any arginine decarboxylase activity in arginine-grown *A. niger* mycelia. Also, when queried with known arginine decarboxylase sequences, no promising hits were retrieved from the *A. niger* genome (<http://genome.jgi-psf.org/Aspni7/Aspni7.home.html>). Both biochemical data and the results of *in silico* analysis suggest that *A. niger* may not have a functional arginine decarboxylase, the major route for agmatine biosynthesis (Fig. 1). The inability of strain D-42 to grow on arginine is also consistent with this idea.

Constitutive overexpression of arginase did not impart any growth advantage to *A. niger* on other guanidinium compounds (Fig. 2, strain Δ XCA-29). Agmatine is not a substrate for purified *A. niger* arginase. A highly specific arginase and the absence of agmatinase rule out the formation of putrescine as the first product of agmatine in *A. niger*. The presence of significant amine oxidase activity in agmatine-grown *A. niger* mycelia along with the detection of PQQ (a cofactor associated with some amine oxidases) in the corresponding spent media is consistent with agmatine oxidation by an amine oxidase. Agmatine is converted to GBald by this step. There are two possible routes from GBald to GABA (Fig. 6), whereas the subsequent metabolism of GABA in *A. niger* is well established. The detection of GB (only in the culture

medium supplemented with agmatine; Table 3) and the induction of GBald dehydrogenase, GBase, and SSADH activities on agmatine strongly favor the GB route (Fig. 6). Without firm evidence to exclude the possibility of the presence of a GBald-specific ureohydrolase, the existence of the alternate path for GBald cannot be discounted. Both GBald and GABald dehydrogenase were ascribed to the same enzyme in *P. aeruginosa* (52), whereas GBald and GABald dehydrogenase represent two distinct enzymes in other systems (53, 54). A well-designed isotope-labeling study could substantiate this aspect further. Regardless of how GBald is processed, GABA is the common downstream metabolite. The sequential action of GABA transaminase and SSADH converts GABA to succinate, which in turn enters the Krebs cycle (15).

While *A. niger* may not biosynthesize agmatine, this fungus is capable of growth by assimilating externally provided agmatine (Fig. 2). This metabolic versatility is consistent with its saprophytic lifestyle. Three alternate routes of agmatine catabolism are possible across organisms. GB and GBase participate in only one of these pathways; incidentally, this route begins with agmatine oxidation. The growth, metabolite, and enzyme activity data, taken together, define a novel pathway for agmatine catabolism in *A. niger*. Although individual enzymatic steps are reported in diverse organisms (Fig. 1), *A. niger* provides the first functional example of this agmatine catabolic pathway.

Functional annotation of *A. niger* GBase. The detection of GBase activity in *A. niger* mycelia grown on either GB or agmatine (Table 4) further led to its purification and characterization. The peptide fragments of the GBase protein (see “MS/MS analysis of GBase protein” in Materials and Methods) matched those of hypothetical protein AN7488.2 (from *A. nidulans* FGSC A4; NCBI nr database for fungi). A blastp analysis of this sequence retrieved a highly similar sequence (Table 5) from the *A. niger* genome (<http://genome.jgi-psf.org/Aspni5/Aspni5.home.html>; annotated as a putative arginase/agmatinase/formiminoglutamase). The corresponding ORF from *A. niger* NCIM 565 genomic DNA (1,384 bp) was cloned in front of the *citA* promoter for its constitutive expression (see Fig. S1 in the supplemental material). When transformed with linearized pCB Δ XCG (see Materials and Methods), 28 stable *A. niger* transformants were obtained. Four of them were analyzed for GBase expression in detail. These transformants were

TABLE 5 *A. niger* GBase sequence retrieved by MS/MS analysis

Sequence type	Sequence
Peptide fragments retrieved by MS/MS analysis ^a	DLGGWYGRK
	QMAGTSYNTR
	SPVTPASSEYGSK
	LVTLGGDHSVALPALR
	SKLVTLGGDHSVALPALR
	DCGDIPITPFDNGLAER
	LNIVGADIVEVSPSYDNK
	ALYQIQKIPITVLHFDAHLDTWNPVR
Retrieved GBase sequence from <i>A. niger</i> genome ^b	MRSLTWAAACLALAGTAFAHAHHDHEEVPEHVREELLRKWDQEFTFSGIASFAHLKPKVCLIEPDERYDIAVIGAP FDTAVSYRPGARFGPRISIRAASARQMAGTSYNTRAGINPYASWATVKDCGDIPITPFDNGVAERQMYEAFLE LGSRPALTSADSKYGTKGISAGKSKLVTLGGDHSVALPALRALYQIQKIPITVLHFDAHLDTWNPVRYSAWYWT SEQAHFNHGSFFHKASREGLICNSTSAHAGLRTRLTGVDSDYTNPGPEQGFMRHADDIDDLGPMG IVNRIERIGLSDQPVYLSVDIDVLDPATAPGTGTPEPGWTTREFIRILRGIEKLNIVGADIVEVSPSYDNK GETTALAAAQVAFEIITSMVKAGAGEELGGWYGRKVTPEETVVVEEGEDPVEKKEAKDEL

^a The eight peptide fragments are presented in order of frequency of their occurrence (highest to lowest).

^b The *A. niger* GBase sequence was retrieved (protein identifier, 55649; location, chr_4_2:3573655-3575104) by querying the *A. niger* genome database (<http://genome.jgi-psf.org/Aspni5/Aspni5.home.html>) with the AN7488.2 hypothetical protein of *A. nidulans* (see Materials and Methods). Peptide fragments identified through MS/MS analysis are highlighted in gray. The single underlining and double underlining indicate that the overlapping SKLVTLGGDHSVALPALR and LVTLGGDHSVALPALR sequences were found as peptide fragments; both are distinct from the subsequent fragment, ALYQIQKIPITVLHFDAHLDTWNPVR.

constitutive for GBase and did not require induction with GB or agmatine for expression (Table 6).

Disruption of the *A. niger* *gbu* locus was also attempted in a $\Delta kusA$ background (24). First, it was ensured that *A. niger* MA169.4 (*kusA::amdS*; *pyrG*⁻) grows on GB as the sole nitrogen source and displayed GBase activity (Table 6). Upon transformation with pBSGBPyrADel (see Materials and Methods), more than 150 transformants were obtained. Twelve of them were unable to grow on GB as the sole nitrogen source; one of them (the $\Delta gbu-90$ strain) was chosen for detailed characterization. Integration of the disruption cassette in the *A. niger* $\Delta gbu-90$ genome was confirmed by genomic PCR using the primer pair GBM1F1 and GBM1R1 (which produced an amplicon of 2.8 kb from *A. niger* $\Delta gbu-90$ versus a 1.6-kb product from the *A. niger* MA169.4 genome); this amplicon was also confirmed through restriction digestion with EcoRV (see Fig. S3 in the supplemental material). The growth of the $\Delta gbu-90$ strain on various metabolites of the proposed agmatine catabolic pathway was tested. As expected, the $\Delta gbu-90$ strain was unable to grow on GB but did grow on urea and GABA (the two products of the GBase reaction) (Fig. 7). In contrast, *A. niger* MA169.4 was able to utilize GB as the sole nitrogen source. This confirms that GBase is the second functional ureohydrolase in the *A. niger* genome (Table 1) and it is solely responsible for GB uti-

lization. The growth (albeit poor; Fig. 7) of the $\Delta gbu-90$ strain on agmatine indicates that the agmatine amino group alone can serve as a source of nitrogen for this fungus. Agmatine metabolism by the $\Delta gbu-90$ strain in liquid culture was accompanied by the concomitant accumulation of GB but the absence of urea. However, about 5 mM urea was found in the medium after 60 h of *A. niger* MA169.4 growth. Taken together, these observations strongly indicate that efficient growth on agmatine requires a functional GBase as well.

Two small introns (127 to 183 bp and 311 to 368 bp) were predicted in the *A. niger* GBase ORF (GenBank accession no. KF991130). These were confirmed by GBase cDNA synthesis by RT-PCR and subsequent expression of this cDNA (pETANGB) in *E. coli*. Upon isopropyl- β -D-1-thiogalactopyranoside induction, *E. coli* cells transformed with pETANGB displayed significant GBase activity (~ 3.0 U mg⁻¹ protein); no activity was detected in

TABLE 6 GBase activity in *A. niger* transformants grown in minimal medium

Transformant	Sp act (U mg ⁻¹ protein)
XCGB-13	0.36
XCGB-03	0.30
XCGB-19	0.25
XCGB-17	0.31
<i>A. niger</i> NCIM 565	0.00
<i>A. niger</i> NCIM 565 ^a	0.16
<i>A. niger</i> MA169.4 ^a	0.23
<i>A. niger</i> $\Delta gbu-90$ ^b	0.00

^a With GB as the sole nitrogen source.

^b No growth on GB.

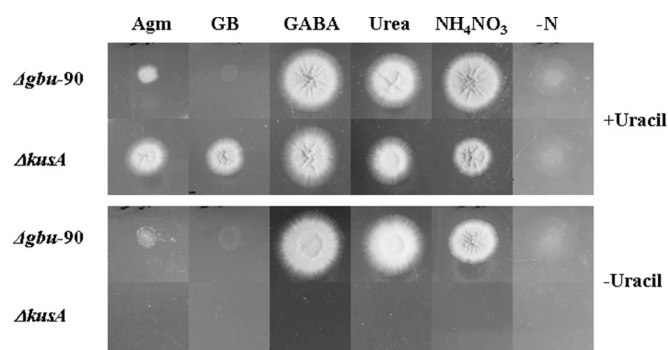


FIG 7 Phenotypic analysis of *A. niger* $\Delta gbu-90$ (GBase disruptant) and its parent, *A. niger* MA169.4 ($\Delta kusA$). Spores (equal numbers) were spotted on minimal media containing different nitrogen sources (15 mM with respect to total N), namely, agmatine (Agm), 4-guanidinobutyrate (GB), 4-aminobutyric acid (GABA), and urea. Controls for growth on ammonium nitrate (NH_4NO_3) and no nitrogen source (-N) are also shown. Growth was recorded at 60 h. Medium without uracil (10 mM) served to show that *A. niger* $\Delta gbu-90$ is a uracil prototroph and also for utilization of uracil as a nitrogen source.

uninduced transformants or cells without the plasmid. It may be noted that the only ureohydrolase activity reported in *E. coli* is that of agmatinase (13). The heterologous expression of GBase activity in *E. coli* thus functionally annotated the *gbu* ORF of *A. niger* NCIM 565.

Conclusions. The results presented here support a novel route for agmatine utilization in *A. niger*. The catabolism of agmatine proceeds by way of 4-guanidinobutyrate to 4-aminobutyrate into the Krebs cycle. This study also led to the discovery of GBase as the second ureohydrolase activity from *A. niger*. The corresponding GBase ORF was cloned and functionally expressed. An *A. niger* Δ *gbu* strain was constructed, and it was unable to utilize GB. *A. niger* GBase orthologs (with amino acid sequence identities of $\geq 85\%$) were found to be present in all the available *Aspergillus* genomes (Table 1, accession numbers in bold). Finally, the similar patterns of expression of ureohydrolase activities in *A. nidulans* (see Table S1 in the supplemental material) lend support to the possibility that the *A. niger* agmatine catabolic pathway presented here may be common across aspergilli.

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