The choline oxidase (CHOA) and betaine aldehyde dehydrogenase (BADH) genes identified in *Aspergillus fumigatus* are present as a cluster specific for fungal genomes. Biochemical and molecular analyses of this cluster showed that it has very specific biochemical and functional features that make it unique and different from its plant and bacterial homologs. *A. fumigatus* ChoAp catalyzed the oxidation of choline to glycine betaine with betaine aldehyde as an intermediate and reduced molecular oxygen to hydrogen peroxide using FAD as a cofactor. *A. fumigatus* Badhp oxidized betaine aldehyde to glycine betaine with reduction of NAD⁺ to NADH. Analysis of the *AfchoA Δ::HPH* and *AfbadA Δ::HPH* single mutants and the *AfchoA ΔAfbadA Δ::HPH* double mutant showed that *AfChoAp* is essential for the use of choline as the sole nitrogen, carbon, or carbon and nitrogen source during the germination process. *AfChoAp* and *AfBadAp* were localized in the cytosol of germinating conidia and mycelia but were absent from resting conidia. Characterization of the mutant phenotypes showed that glycine betaine in *A. fumigatus* functions exclusively as a metabolic intermediate in the catabolism of choline and not as a stress protectant. This study in *A. fumigatus* is the first molecular, cellular, and biochemical characterization of the glycine betaine biosynthetic pathway in the fungal kingdom.

Glycine betaine (GB) is a small, water-soluble organic molecule that is essential to protect plants, animals, and bacteria against abiotic stress. Two main mechanisms have been proposed for GB’s responsibility for enhanced stress tolerance: (i) osmotic adjustment controlling the absorption of water from the surroundings and (ii) reactive oxygen species (ROS) scavenging (1). In plants, this molecule is essential to fight salt, cold, heat, and drought stresses. The antistress properties of GB have led to the production of transgenic plants that are able to resist abiotic stress and especially to become tolerant to salt and drought (2). It has also been suggested that the same mechanisms would be responsible for the capacity of many human-pathogenic bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis*, to survive and grow in their human host (3–7). In the majority of biological systems, GB is synthesized by a two-step oxidation reaction: choline is first oxidized to betaine aldehyde (BA), which is later oxidized to yield GB. The first oxidation is catalyzed by a choline monooxygenase in plants and by a choline dehydrogenase (CDH) in bacteria, whereas the second reaction is catalyzed by an NADP⁺-dependent betaine aldehyde dehydrogenase (BADH) (1).

Acquisition of osmoprotectants is important for the survival of the conidia and for mycelial growth of filamentous fungal pathogens such as *Aspergillus fumigatus* in their human host (8, 9). However, the mechanisms controlling the osmotic pressure changes during the biological cycle of fungal species remain insufficiently understood. Although trehalose has been repeatedly cited as the major metabolite involved in the osmoprotection of *A. fumigatus*, several arguments suggest that it is not the only one: (i) trehalose is rapidly degraded upon induction of conidial germination and increases again later during mycelial growth; (ii) two metabolic pathways for the biosynthesis of trehalose have been identified, but their respective roles have not been evaluated; and (iii) other polyols have been also suggested to be associated with changes in osmotic pressure (8, 9). This also raised the question of the existence of other molecules that could be associated with osmoprotection during the fungal biological cycle. GB was an obvious candidate to study since (i) it is an osmoprotectant in plants and bacteria and (ii) a transcriptomic study of the conidial germination of *A. fumigatus* showed that a gene orthologous to the bacterial choline oxidase gene was one of the most upregulated genes in the early steps of conidial germination (10).

The GB pathway in *A. fumigatus* was analyzed here to investigate the role of this molecule in the resistance of fungi to abiotic stress. This is the first time that the GB pathway in the fungal kingdom has been analyzed. Biochemical studies have shown that (i) the choline oxidase *AfChoAp* catalyzes the flavin-linked oxidation of choline or betaine aldehyde (BA) to GB, with reduction of molecular oxygen to hydrogen peroxide, and (ii) the betaine aldehyde dehydrogenase *AfBadAp* catalyzes the oxidation of BA to GB with reduction of NAD⁺ to NADH. Studies on single and double deletion mutants showed that GB is not involved in osmotic regulation but is used as a nutrient by this fungal species.

**MATERIALS AND METHODS**

**Strains, media, and culture conditions.** The *A. fumigatus* parental strains CBS 144.89 (= Dal = Af1163) (10) and its *aokB* (11) and the mutant strains used in this study are shown in Table S1 in the supplemental material. *A. fumigatus* strains were maintained on 2% malt agar tubes. Conidial germination and mycelial growth were studied in different 2% agar or shake medium at 37°C. Complex media were Sabouraud (SAB) medium, 2% malt medium, yeast extract-peptone-dextrose (YPD) medium, 1% yeast extract (YE), or RPMI synthetic medium. Mutant and
parental strains of A. fumigatus were also grown in MM medium containing 10 g/l glucose, 0.92 g/l ammonium tartrate, 0.5 g KCl, 0.5 g MgSO\(_4\) · 7H\(_2\)O, 2 g KH\(_2\)PO\(_4\), and 1 ml of trace element solution at pH 6.5. The various media and stresses tested in this study are summarized in Table S2 in the supplemental material. Brieﬂy, media were supplemented with 1.2 M mannitol, 1.2 M sorbitol, or 1 to 2.5 M NaCl to test the effect of osmotic pressure. Growth was also tested at pH 5.0 to 9.0, cold (4 to 10°C) or hot (37 to 50°C) temperatures, and in the presence of 1 to 5 mM hydrogen peroxide or 5 to 80 μM menadione in complex media or in MM without a nitrogen source but containing 20 mM GB. Glucose or/and ammonium tartrate was replaced in the MM medium with 20 mM choline chloride, 20 mM GB, or 6.6 mM L-α-phosphatidylcholine as a sole carbon, nitrogen, or carbon and nitrogen source. Curosurf (Chiesi Farmaceutici, Parma, Italy) was tested at 1 to 20 mg/ml on a 2% agar medium.

**DNA and RNA analyses.** For DNA extraction, mycelium was grown for 16 h at 37°C in a liquid medium containing 3% glucose and 1% yeast extract. For the RNA extraction, the fungus was grown in YPD liquid medium at 37°C for 16 h. Total DNA and RNA were isolated as previously described (12, 13). Reverse transcriptase PCRs (RT-PCRs) were performed using 2 μg total RNA, using a Thermoscript RT-PCR system kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions with gene-specific primers for AfCHOA and AfBADA (see Table S3 in the supplemental material).

**Production of recombinant A. fumigatus ChoA and BadaA.** The gene AfCHOA was subcloned into pET20b (+), which allows the expression of the enzyme without any tag, whereas the gene AFBAD A was expressed in the pET16 vector, which results in the addition of Histag at the N-terminal region of the protein. The AfCHO A and AFBAD A cDNAs were obtained by PCR amplification using, respectively, the primer pairs Ndel-ATG-AfCHOA-stop-BamHI and Ndel-ATG-AFBADA-stop-BamHI and cDNA from A. fumigatus mycelium as the template (see Table S3 in the supplemental material). The obtained PCR products were gel puriﬁed and digested by Ndel and BamHI. These fragments were introduced into pET16b (Ndel/BamHI). The vector pET20b was used for the ﬁnal expression of the AfCHOA cDNA. For cloning into the pET20b vector, the AfCHOA cDNA was excised with Ndel (5’ site) and BamHI (3’ site) from the pET16b plasmid and inserted with Ndel/BamHI sites of pET20b. After conﬁrming the absence of mutations by DNA sequencing at the DNA Core Facility at Georgia State University, the enzyme was expressed in E. coli Rosetta(DE3)pLysS. After transformation, E. coli Rosetta(DE3)pLysS (Novagen) cells were grown at 37°C in Luria-Bertani medium supplemented with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol, and protein production was induced for 16 h at 20 or 25°C in the presence of 0.2 or 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for AfChoA or AfBadaA, respectively. AfChoA was recovered by sonicating in 50 mM potassium phosphate (pH 7.0) containing 10% glycerol, 10 mM MgCl\(_2\), 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml DNase I, 5 μg/ml RNase, and 0.2 mg/ml lysozyme and puriﬁed by sequential 30 and 65% saturation ammonium sulfate precipitation before loading onto a DEAE-Sepharose Fast Flow column eluted with a linear gradient from 0 to 500 mM NaCl. For best preservation of the activity during storage at −20°C, the enzyme was dialyzed and stored in 100 mM imidazole (pH 7.0) with 10% glycerol. E. coli cells expressing AfBadaA were resuspended in 20 mM sodium phosphate (pH 7.4) containing 500 mM NaCl, 20 mM imidazole, 10% glycerol, 0.2 mg/ml lysozyme, and 1 mM PMSF and sonicated for 20 min. The soluble fraction was loaded onto an nickel-Sepharose column, and elution was with a linear gradient from 20 to 500 mM imidazole. It was veriﬁed by SDS-PAGE that these proteins were puriﬁed to homogeneity (see Fig. S1A in the supplemental material).

**Spectroscopic analysis.** All UV-visible absorbance spectra were recorded using an Agilent Technologies diode array spectrophotometer (model HP 8453). The extinction coeﬃcient of AfChoA was determined in 20 mM Tris–Cl (pH 8.0) and 10% glycerol after denaturation of the enzyme by treatment with 4 M urea at 40°C for 30 min, based upon the ε\(_{450}\) value of 11.3 mM\(^{-1}\) · cm\(^{-1}\) for free FAD (14). To determine the amount of covalently bound flavin, the puriﬁed enzyme was incubated on ice for 30 min after addition of 10% trichloroacetic acid, followed by removal of precipitated protein by centrifugation. The UV-visible absorbance spectrum of the supernatant was recorded again to check for the presence of unbound FAD. AfChoA reduction was achieved by anaerobic substrate reduction carried out with an anaerobic solution of 24 μM enzyme through successive (20 times) evacuations and ﬂushings with O\(_2\)-free argon in an anaerobic cuvette (see Fig. S1B in the supplemental material).

**Biochemical analysis.** AfChoA (24 μM) reduction was achieved by anaerobic incubation with 0.5 mM choline. The enzymatic activity of AfChoA was measured by the method of initial rates as described for the Arthrobacter globiformis wild-type choline oxidase (15). The steady-state kinetic parameters of the enzyme were determined at various concentrations of oxygen (0.1 to 1.1 mM) and choline (0.5 to 10 mM) or BA (0.5 to 10 mM) in 10 mM Tris–Cl (pH 8.0) at 25°C. AfBadA activity was assayed spectrophotometrically by measuring NAD(P)(+) reduction by the increase in extinction at 340 nm (16). The steady-state kinetic parameters of the enzyme were determined at various concentrations of BA (10 to 400 μM) and NAD(P)(+) (0.01 to 10 mM) in 100 mM HEPES-KOH (pH 8.0) at 25°C. In the assay coupling AfChoA and AfBadA, the activity of AfBadA was measured spectrophotometrically as described above at various concentrations of NAD(+) (20 to 740 μM) and ﬁxed concentrations of choline (1 mM) and oxygen (0.25 mM) in 100 mM HEPES-KOH (pH 8.0) at 25°C. The amount of BA produced during catalytic turnover of the enzyme with choline as the substrate was determined spectrophotometrically using 2,4-dinitrophenylhydrazine as described previously (17) (see Fig. S1B in the supplemental material).

**Mutant constructions.** The cassettes to produce the AfchoA::Hph and AfbadA::Hph single mutants and the AfchoA::afbadA::Hph double mutant were constructed by PCR fusion as described earlier (12) using primers listed in Table S3 in the supplemental material. The resistance marker used to replace AfCHOA, AFBAD A, or both was the HPH gene of E. coli, coding for hygromycin B phosphotransferase. An example of a deletion cassette constructed by PCR fusion of three PCR products (PCR1, PCR2, and PCR3) is shown in Fig. S2 in the supplemental material (deletion of AfCHOA). In a ﬁrst PCR round (see Fig. S2 in the supplemental material), ﬂanking region 1 (PCR1, primers 55-CHOA and 53-CHOA), ﬂanking region 2 (PCR2, primers 35-CHOA and 33-CHOA), and the HPH gene (PCR3, primers 5-CHOA-Hph and 3-CHOA-HPH) were ampliﬁed from wild-type DNA template and pANT7 with the primers listed in Table S3 in the supplemental material. Phusion DNA Polymerase (New England BioLabs) was used, and the PCR conditions were as follows: 98°C for 30 s, 25 cycles of 98°C for 10, 65°C for 20 s, and 72°C for 30 s, and 72°C 10 min. Primers 53-CHOA, 5-CHOA-HPH, 35-CHOA, and 3-CHOA-HPH were 60-bp chlorogenic oligonucleotides containing at the 5’ end a reverse complement sequence (53-CHOA with 5-CHOA-HPH and 35-CHOA with 3-CHOA-HPH) for fusion PCR. The obtained PCR products were gel puriﬁed and used for a second PCR step that allowed fusion of these three separate fragments by using the 55-CHOA and 33-CHOA primers. The PCR conditions were the same as described above. The resulting PCR product was puriﬁed and used to transform A. fumigatus conidia. The AfCHOA::HPH and AfBadA::Hph double mutant and the AfchoAΔ::afbadA::Hph single mutants were constructed in the akuB\(\text{mut}^1\) strain (11). The fusion PCR product (1 to 2 μg) was used to transform A. fumigatus akuB\(\text{mut}^1\) swollen conidia by the electroporation method described previously (18). For transformation experiments, minimal medium MM (1% glucose, 0.092% ammonium tartrate, 0.052% KCl, 0.052% MgSO\(_4\) · 7H\(_2\)O, 0.152% KH\(_2\)PO\(_4\), 1 ml trace element solution, pH 6.8) was used (19). MM containing 250 μg · ml\(^{-1}\) hygromycin (Sigma) was used for the screening of the mutants (AfCHOA::Hph, AfBadA::Hph, and AfchoAΔ::afbadA::Hph), and MM containing 30 μg · ml\(^{-1}\) pleomycin (InvivoGen) was used for the selection of the AfchoAΔ::afbadA::Hph double deletion mutant strain transformed with pAN81.1-eGFP
AfCHOA::promoter::AfBADA-RFP-T plasmid. To check integration of the HPH cassette at the locus, the DNAs of the akuB\textsuperscript{80} and the mutant strains were digested with restriction enzyme (Roche Applied Science) and verified by Southern blotting with probes corresponding to the first flanking region (see Fig. S2A [deletion of AfCHOA], B [deletion of AfBADA], and C [double deletion of AfCHOA and AfBADA] in the supplemental material).

Transcriptional and translational fusion of AfCHOA with eGFP and of AfBADA with RFP-T. The transcriptional fusion (eGFP::promoter::RFP-T) of the AfCHOA and AfBADA promoter (1,650 bp) with enhanced green fluorescent protein (eGFP) and RFP-T was constructed by PCR fusion (Fig. 1C). The eGFP fragment (PCR1) was obtained by PCR amplification using p123 (pOTEF eGFP)-HPH plasmid as the template and the primer pairs NdeI-PCR1-transcript-F and PCR1-transcript-R. The AfCHOA and AfBADA promoter (PCR2) was obtained by PCR amplification using akuB\textsuperscript{80} genomic DNA as the template and the primer pairs PCR2-transcript-F and PCR2-transcript-R. The RFP-T fragment (PCR3) was obtained by PCR amplification using pAL5-lifeact plasmid as the template and the primer pairs PCR3-transcript-F and PCR3-transcript-NdeI-R. The obtained PCR products were gel purified and used for a

FIG 1 Organization and expression of BADA and CHOA gene cluster in A. fumigatus. (A) Organization of BADA and CHOA gene cluster in A. fumigatus. The BADA and CHOA tail-to-tail cluster is found in most ascomycetes (panel a). In 3 species, two (for Blastomyces dermatitidis and Paracoccidioides brasiliensis) (panel c) or three (for Histoplasma capsulatum) (panel b) genes are inserted between the CHOA and BADA genes (dotted lines). (B) Gene expression during growth was determined by RT-PCR in akuB\textsuperscript{80} resting conidia (0 h) and in conidia incubated in YPD broth at 37°C for 0.5, 2, or 4 h (swollen conidia), for 8 h (germinated conidia), and for 20 and 30 h (mycelia). Specific primer pairs designed to include an intron in the PCR product to verify the lack of genomic DNA amplification (see Table S3 in the supplemental material) were used for cDNA amplification. The sizes of amplified products are indicated. The EF1α gene was constitutively expressed and used as a control of constitutive expression. H2O, negative control; G, A. fumigatus genomic DNA. (C) Transcriptional fusion was performed by fusion of three PCR fragments: the PCR1 fragment encoding eGFP, the PCR2 fragment corresponding to the promoter of the AfCHOA and AfBADA genes (1,650 kb), and the PCR3 fragment encoding RFP-T. (D and E) eGFP (D) and RFP-T (E) fluorescences were observed in cytosol of swollen conidia, germ tubes, and mycelium. Cultures were grown in liquid MM with choline chloride (20 mM) as the sole nitrogen source. (F) Bright-field image of the same hyphae. Note the resting conidia (small conidia, inset F) are not fluorescent in panels D and E, confirming that the AfCHOA and AfBADA genes are not expressed in resting conidia.
second PCR step that allowed fusion of these two separate fragments by using NdeI-PCR1-transcript-F and PCR3-transcript-NdeI-R primers. The resulting PCR product was purified and digested with NdeI, whose restriction site is present in the NdeI-PCR1-transcript-F and PCR2-transcript-R-NdeI primers. This fragment was introduced into pAN8.1 plasmid (20) to obtain the pAN8.1-eGFP::promoter::RFP-T plasmid (NdeI). One to 2 μg of linear pAN8.1-eGFP::promoter::RFP-T (BgIII) was used to transform the swolen conidia of the wild-type Dαl strain. The revertant cassette corresponding to the translational fusion (eGFP::AfCHOA::promoter: AbADA::RFP-T) of the AfCHOA open reading frame (ORF) (1,720 bp) with eGFP (720 bp) and the AfBADA ORF (1,602 bp) with the RFP-T (735 bp) under the control of the AfCHOA and AfBADA promoter (1,650 bp) was obtained by PCR fusion (see Fig. S2C in the supplemental material). In a first PCR round (see Fig. S2C in the supplemental material), the eGFP (PCR1, primers NdeI-PCR1-translational-F and PCR1-translational-R), the fragment corresponding to the AfCHOA ORF and AfCHOA and AbADA promoter and the AfBADA ORF (PCR2, primers PCR2-translational-F and PCR2-translational-R), and the RFP-T (PCR3, primers PCR3-translational-F and PCR3-translational-NdeI-R) were amplified, respectively, from the pL23 (pOTEF eGFP)-H16 plasmid, wild-type DNA template, and the pAL5-ill-eat plasmid. The primers are listed in Table S3 in the supplemental material. The obtained PCR products were gel purified and used for a second PCR step that allowed fusion of these four separate fragments by using the NdeI-PCR1-translational-F and PCR3-translational-NdeI-R primers. The resulting PCR product was purified and digested with NdeI, whose restriction site is present in the NdeI-PCR1-translational-F and PCR3-translational-NdeI-R primers. This fragment was introduced into pAN8.1 plasmid to obtain the pAN8.1-eGFP::AfCHOA::promoter: AbADA::RFP-T plasmid (NdeI). One to 2 μg of linear pAN8.1-eGFP::AfCHOA::promoter::AbADA::RFP-T (BgIII) was used to transform the swolen conidia of the AfchoAΔAfbadAΔ::H16 plasmid. Double mutant. Fluorescence light microscopy observations were performed after the fungus was grown in MM medium supplemented with choline to boost the fluorescence.

Nuclear magnetic resonance (NMR) analysis. Mycelium grown for 16 h on MM at 37°C under shaking conditions was disrupted with 1-mm diameter glass beads for 2 min in a Fastprep cell breaker (MP Biomedical). After removal of the polysaccharides and proteins by the addition of 2 volumes of ethanol, the supernatants were submitted to one-dimensional (1D) 1H- and 2D 13C-edited heteronuclear single quantum correlation (HSQC), 2D 1H,13C-heteronuclear multiple bond correlation (HMBC), and 2D 1H,15N-HSQC spectroscopies (21). As described below, the supernatants were dried in vacuo and then reconstituted with the minimum amount of water. After removing the residual insoluble materials by centrifugation, these obtained supernatants were lyophilized to the minimum amount of water. After removing the residual insoluble materials by centrifugation, these obtained supernatants were lyophilized to the minimum amount of water.

For assays of survival of parental and mutant strains in the lungs of infected animals, 6 × 105 fluorescent isothiocyanate (FITC)-labeled conidia in 30 μl of 0.05% Tween 20 were inoculated intranasally in 7-week-old C57Bl/6 male mice (Charles River Laboratory, L’Arbresle, France) as described previously (13). After an infection period of 36 h, bronchoalveolar lavages were performed (4 mice per fungal strain). The percentage of germination was quantified after incubation of the bronchoalveolar lavage (BAL) fluid in an equal volume of 2× Sabouraud (4% glucose–2% mycopeptone) culture medium at 37°C for 7 h.

RESULTS

A gene cluster encodes the choline oxidase and betaine aldehyde dehydrogenase of A. fumigatus. Two genes involved in GB metabolism in A. fumigatus were identified by BLAST analysis. They were located nearby on chromosome 8 and arranged in a head-to-head configuration at a distance of 1.7 kb between their ORFs. The cDNAs corresponding to the AfCHOA and AfBADA genes were sequenced in order to determine the structures (intron/exon) of these genes. The first one coded for a choline oxidase, AchoAp (AFUB_083470). It was a 1,723-bp gene that contained two exons of 247 and 1,382 bp separated by an intron of 94 bp coding for a 542-amino-acid protein. The second one encoded a betaine dehydrogenase (AbBadAp; AFUB_083480); it was 1,605 bp long and contained two exons of 485 and 1,015 bp separated by an intron of 105 bp coding for a 499-amino-acid protein. AchoAp displays 37% identity and 54% similarity with ChoAp of Arthrobacter globiformis (AAP68832.1), which is the only bacterial species that has a unique CHOIA gene. All relevant amino acid genes of the active site previously identified in A. globiformis CHOIA were present in all fungal CHOIA genes (data not shown). The percentages of...
TABLE 1 Steady-state kinetic parameters for AfChoAp and AfBadAp

| Protein | Substrate | Ligand | $k_{cat}$ s$^{-1}$ | $K_m$ mM | $k_{cat}/K_m$ M$^{-1}$ s$^{-1}$ | $K_m$ mM | \(k_{cat}/K_{O2}\) M$^{-1}$ s$^{-1}$ | $K_{NAD(P)^+}$ mM | \(k_{cat}/K_{NAD(P)^+}\) M$^{-1}$ s$^{-1}$ | $K_m$ mM |
|---------|-----------|--------|-------------------|-----------|--------------------------|---------|--------------------------|-----------------|--------------------------|---------|
| AfChoAp | Choline   |        | 40.0 ± 0.2        | 1.8 ± 0.1 | 22.200 ± 700             | 0.69 ± 0.01 | 58,000 ± 1,000          | 1.4 ± 0.1       |                         |         |
|         | Betaine-aldehyde | | 18.2 ± 0.4       | 1.6 ± 0.1 | 11,400 ± 800             | 0.36 ± 0.02 | 52,000 ± 3,200          | 3.8 ± 0.3       |                         |         |
| AfBadAp | NAD$^+$   |        | 10.2 ± 0.4        | 0.025 ± 0.001 | 408,000 ± 20,000       | 0.037 ± 0.004 | 276,000 ± 11,000       | 50 ± 1         |                         |         |

$^a$ Parameters are for AfChoAp with oxygen and choline or betaine aldehyde as substrates and for AfBadAp with betaine aldehyde and NAD(P)$^+$ as substrates. Values are sample means and standard errors. $K_m$ is the $K_m$ for either choline or betaine aldehyde. Data for reactions with no ligand were fitted to the equation \(v = k_{cat}AB/(K_B + AB + K_A)\) (Equation 1) and those for AfChoAp with choline and NAD$^+$ ligand were fitted to the equation \(v = k_{cat}AB/(K_A + AB + K_B)\) (Equation 1) and those for AfBadAp with betaine aldehyde and NAD$^+$ ligand were fitted to the equation \(v = k_{cat}AB/(K_A + AB + K_B)\) (Equation 1).


doxygen and NAD(P)$^+$, respectively, and $k_{cat}$ is the turnover enzyme (c) saturated with both substrates. The choline oxidase activity was measured in 20 mM Tris-HCl (pH 8.0) at 25°C and the betaine aldehyde dehydrogenase activity in 100 mM HEPES-KOH (pH 8.0) at 25°C.

identity and similarity between the BadAp of *A. fumigatus* and the best-characterized BadAp bacterial protein, from *Pseudomonas aeruginosa* (NP_254060.1), were 57% and 70%, respectively, and those between AfBadAp and BadAp of *Arabidopsis thaliana* (AEE35649.1) were 35% and 54%, respectively.

Interestingly, BADA clustered in the opposite orientation with respect to the CHOAO gene in *A. fumigatus* and other ascomycete fungi, suggesting the presence of a common promoter (Fig. 1). No N-terminal secretion signal (N-terminal peptide signal) or transmembrane domains were predicted in either protein. Both AfCHOAO and AfBADAO mRNAs were expressed in germinating conidia (immediately after their incubation in a nutritive medium) and growing mycelia but not in resting conidia (Fig. 1). The transcriptional fusion of the AfCHOAO promoter with eGFP and of the AfBADAO promoter with RFP-T also showed that fluorescence of both eGFP and RFP-T was seen in swollen and germinating conidia and mycelia but not in the dormant conidia (Fig. 1).

Biochemical characterization of recombinant AfChoAp and AfBadAp. Both recombinant AfChoAp and AfBadAp were produced in *E. coli*, with a molecular masses of 60 and 52 kDa, respectively (see Fig S1A in the supplemental material). The UV-visible absorbance spectra of the oxidized and reduced forms of the AfChoAp are shown in Fig. S1B in the supplemental material. With AfChoAp, the absorbance in the visible region of the oxidized species is typical of that of a flavoprotein, with bands at 361 and 448 nm and shoulders at 420 and 460 nm. The UV-visible absorbance spectrum of the reduced enzyme obtained through anaerobic reduction with choline showed a well-resolved maximum at ~360 nm, consistent with the enzyme-bound flavin being present in the anionic form (27). The flavin was shown to be covalently bound to the enzyme as observed after protein precipitation with 10% trichloroacetic acid and removal of the denatured protein through centrifugation. The extinction coefficient of the enzyme at 448 nm was calculated to be 11,500 M$^{-1}$ cm$^{-1}$ from the absorbance of FAD after protein denaturation by urea. The absorbance spectrum of AfBadAp from 200 to 600 nm showed a single peak at 280 nm with no absorption in the visible region, consistent with the absence of a bound chromophore.

AfChoAp was able to catalyze the oxidation of choline to G8 with BA as an intermediate and to reduce molecular oxygen to hydrogen peroxide. Under the experimental conditions tested, recombinant AfChoAp had an average specific activity of 9 µM O$_2$ min$^{-1}$ mg E$^{-1}$, using 10 mM choline and atmospheric oxygen at pH 7.0 and 25°C. The steady-state kinetic mechanism and the associated kinetic parameters were determined by measuring the initial rates of oxygen consumption at various concentrations of both oxygen and choline or BA as the substrate. Initial velocity data yielded a series of lines converging to the left of the y axis in double-reciprocal plots for both choline and BA, indicating a mechanism in which a ternary complex must be formed before release of the first product of the reaction. In accordance with the observed kinetic pattern, the data described a sequential steady-state kinetic mechanism (Table 1). To test if BA was released from the active site of the enzyme, the amount of BA accumulated during catalytic turnover of AfChoAp with saturating choline and 1.1 mM oxygen dissolved in aqueous buffered solution was determined using 2,4-dinitrophenylhydrazine (see Fig S1C in the supplemental material). The amount of BA released and the amount of BA expected to be released based on the kinetic parameters were similar, showing that BA was predominantly released from the active site during turnover of the enzyme with choline as the substrate.

AfBadAp activity was measured with the method of initial rates by measuring the rate of NAD(P)$^+$ reduction at various concentrations of both BA and NAD(P)$^+$. No enzyme inhibition by BA or NAD(P)$^+$ was observed with concentrations as high as 200 µM NAD$^+$, 5 mM NADP$^+$, or 400 µM BA. The lines in the double-reciprocal plots converged to the left of the y axis, indicating a sequential mechanism. In accordance, the best fit of the data was achieved with equation 1 (Table 1). The enzyme could use either NAD$^+$ or NADP$^+$ as the oxidizing substrate for the reaction, but NAD$^+$ was clearly preferred, as indicated by the ratio of the second-order rate constants for substrate capture ($k_{cat}/K_{O2}$) being larger than 300 (Table 1). The recombinant AfBadAp had a specific activity of 33 µM NAD$^+$ min$^{-1}$ mg E$^{-1}$, using 1 mM NAD$^+$ and 0.5 mM BA at pH 8.0 and 25°C.

To evaluate whether NAD$^+$ had an effect on the oxidation of choline by AfChoAp, the steady-state kinetics of AfChoAp with choline as the substrate was determined in the presence of 300 µM NAD$^+$. With choline as the substrate, the best fit of the data was obtained with equation 2 (Table 1), which describes a steady-state mechanism with the formation of a ternary complex where the $K_m$ value for choline is significantly smaller than the term $K_m/K_{Oxygen}$ that is present in equation 1 (28). If one considers a $K_{choline}/K_{Oxygen}$ ratio of at least 0.05 as being required to simplify equation 1 to equation 2, then a $K_m$ value for choline of 26 µM can be estimated from the data in Table 1. Consequently, the $k_{cat}/K_m$ ratio for choline can be estimated to be $\geq 9 \times 10^6$ M$^{-1}$ s$^{-1}$. Thus, in the presence of NAD$^+$, the $K_m$ value for choline decreased by at least 70 times.
AfChoAp and AfBadAp both catalyzed the oxidation of BA to GB, but only AfChoAp catalyzes the oxidation of choline to BA. Thus, to test if AfBadA was able to oxidize the aldehyde released from the active site of AfChoAp in turnover with choline, the production of NADH was measured in a reaction mixture of the two enzymes, choline, oxygen, and NAD$^+$. Reduction of NAD$^+$ by AfBadAp was observed, suggesting that the BA released from AfChoAp could be utilized by AfBadAp. The limiting rate for NADH production at saturating NAD$^+$ with 1 mM choline, 0.25 mM oxygen, and 87 nM AfChoAp was 10.7 s$^{-1}$. This value is comparable to the turnover number of 10.4 s$^{-1}$ for AfBadAp at saturating BA and NAD$^+$ concentrations.

This study in A. fumigatus is the first biochemical characterization of a GB biosynthetic pathway in the fungal kingdom. AfChoAp and AfBadAp function in a coordinated fashion to synthesize GB from choline, as illustrated in Fig. 2. The first step in the biosynthetic pathway is the conversion of choline to BA catalyzed by AfChoAp. This reaction requires the presence of molecular oxygen, which is used to oxidize the enzyme-bound flavin cofactor with concomitant production of hydrogen peroxide. BA is then released from the active site of AfChoAp and undergoes a fast hydration reaction in aqueous solution that results in the reversible formation of gem-diol-choline (boxed step in Fig. 2). The gem-diol-choline is further oxidized to GB in a reaction catalyzed by AfBadAp. The aldehyde is instead further oxidized to GB by AfBadAp in a reaction that requires NAD$^+$ as the oxidizing substrate for the enzyme. Note that the methyl groups have been represented as lines in the intermediate steps of the reaction.

Functional characterization of the AfCHOA/AfBADA cluster. In order to understand the functional roles of these proteins, the AfchoA::HPH and AfbadA::HPH single mutants and the AfchoA::AfbadA::HPH double mutant were constructed. The replacement of the AfCHOA gene, the AfBADA gene, and both the AfCHOA and AfBADA genes by the HPH gene at the right locus is shown in Fig. S2 in the supplemental material. Mycelial growth and conidial germination were analyzed in agar (Fig. 3A) or liquid MM (Fig. 3B) supplemented with 20 mM choline chloride, BA, or GB or 6.6 mM phosphatidylcholine in the absence of glucose, ammonium tartrate, or both as the sole carbon and nitrogen source. In contrast to the case for the akub$^{34}$ parental strain and the AfBadA::HPH mutant, the growth and germination of the AfchoA::HPH and AfchoA::AfbadA::HPH mutants were severely impaired at 37°C when choline chloride, BA, or phosphatidylcholine was used as the sole nitrogen source (Fig. 4 and data not shown). The addition of 20 mM GB restored the growth defect observed in the AfchoA::HPH mutant and in the AfchoA::AfbadA::HPH double mutant (Fig. 4). Similar growth defects of the mutants were observed when choline was used as the carbon source or as carbon and nitrogen sources (see Fig. S3 in the supplemental material). However, the growth was much higher when choline was used as a nitrogen source. These results showed that GB biosynthesis in A. fumigatus is more important in the case of nitrogen starvation than in carbon starvation. It also suggested that the catabolic pathway from GB to ammonium could be a salvage pathway in the case of nitrogen starvation.

NMR studies confirmed that in MM medium no GB was present in the intracellular fraction of the AfchoA::HPH mutant, whereas it was identified in the parental strain. The NMR analysis was originally based on$^1$H,$^1$C-HSQC and$^1$H,$^1$C-HMBC experiments (see Fig. S4 in the supplemental material) and was further confirmed by use of a$^1$H,$^1$N-HSQC method that was appropriate to detect choline derivatives NCH$_3$ and CH$_3$ (Fig. 4) (21).

The mutants were complemented by the ectopic integration of the translational fusion construct eGFP::AfCHOA::promoter::AfBADA::RFP (see Fig. 5 in the supplemental material).

The GFP-tagged AfChoAp and RFP-tagged AfBadAp restored the growth and germination defects observed in the AfchoA::AfbadA::HPH double mutant (Fig. 3). This result indicated that these fusion proteins complement the GB biosynthesis pathway. The fluorescence of the AfChoAp-eGFP and AfBadAp-RFP-T fusion proteins was seen in the cytoplasm of mycelium and germinating conidia, demonstrating that AfChoAp and AfBadAp were cytoplasmic proteins (Fig. 5). In contrast, no fluorescence was seen in resting conidia, which, like for the transcriptional fusion, became positive as soon as they were plunged into a nutritive medium (data not shown).

In contrast to the role of GB in bacteria, the GB mutants were not affected by stress and especially osmotic stress: no difference in growth was seen when the parental and mutant strains were grown on solid complex rich or solid MM in the presence of high concentrations of osmolytes (1.2 M mannitol, 1.2 M sorbitol, or 1 to 2.5 M NaCl). In addition, the addition of 0.6 M NaCl did not change the expression of the CODA and BADA genes (see Fig. S5 in the supplemental material). The degradation of the nonreducing sugar trehalose and the polyols mannitol and arabinitol of the resting conidia was similar during the conidial germination of the wild type and the AfchoA::AfbadA::HPH double mutant (see Fig. S6 in the supplemental material). In addition, no changes in the glyceroipentitol of the resting and germinated conidia were seen in the parental and mutant strains. In agreement with these data, the expression of genes coding for trehalose and mannitol biosyntheses was similar in resting, swollen, and germinated conidia of the wild type and the AfchoA::AfbadA::HPH double mutant (see Fig. S7 in the supplemental material, and data not shown). These results showed that there was no interconnection between the metabolism of the osmoprotectant trehalose and polyols and the GB pathway.

The mutants were no more sensitive to other stresses, such as...
ROS, from which bacteria and plants are known to be protected by GB. For example, no growth differences were seen between the parental strain and the GB mutants of *A. fumigatus* on solid MM in the presence of reactive oxidants (hydrogen peroxide at 1 to 5 mM and menadione at 5 to 80 μM). Similarly, the growth of the mutant was identical to that of the parental strain on solid MM at pH 2.0 and 9.0 and at constant temperatures between 4 and 50°C. The conidial viability of the mutant was not more altered than that of the parental strain after a freezing shock (24 h at -20°C) or heat shock (5 min at 75°C) or after drying at room temperature for 2 years (data not shown).

Since *A. fumigatus* CHOAs and BADA were expressed in vivo (see Fig. S8A in the supplemental material) and the composition of the lung surfactant is phosphatidylcholine rich, a mutant such as the *AfchoAΔ::HPH* mutant, which is unable to use choline, could be less fit in a lung environment than its parental strain. In reality, the conidia of the *AfchoAΔ::HPH* and *AfbadAΔ::HPH* single mutants and the *AfchoAΔAfbadAΔ::HPH* double mutant survived in the lungs of immunocompetent mice like the parental strain (see Fig. S8B in the supplemental material). Moreover, the mutants were able to grow on Curosurf, which is a naturally derived surfactant from porcine lung that is rich in phospholipids (mainly phosphatidylcholine) with 1% hydrophobic surfactant proteins B and C (data not shown). These results suggested that the GB pathway is not required for the germination and the establishment of *A. fumigatus* in the lung. These results also showed that in *A. fumigatus*, GB was not used as a protectant against various abiotic and biotic stresses.

**FIG 3** Growth of *AfchoAΔ::HPH*, *AfbadAΔ::HPH*, and *AfchoAΔAfbadAΔ::HPH* mutants of *A. fumigatus* at 37°C on MM agar with choline or BA as the sole nitrogen source. (A) The *akuBku80* parental and the *AfchoAΔ::HPH*, *AfbadAΔ::HPH*, and *AfchoAΔAfbadAΔ::HPH* mutants and revertant *AfchoAΔAfbadAΔ::AfCHO-eGFP+AfBADA-RFP* were grown on agar plates with MM with glucose and ammonium tartrate, MM with glucose and without a nitrogen source, or MM with glucose and without ammonium tartrate, which is replaced by choline chloride (20 mM), BA (20 mM), or GB (20 mM). (B) Microscopic observation of the *akuBku80* parental and the *AfchoAΔ::HPH*, *AfbadAΔ::HPH*, and *AfchoAΔAfbadAΔ::HPH* mutant cultures in liquid MM without a nitrogen source and in liquid MM with choline chloride (20 mM) or BA (20 mM) as the sole nitrogen source. Experiments were repeated three times with similar results.
DISCUSSION

The gene cluster for the synthesis of glycine betaine is unique in *A. fumigatus*. In higher plants, the two-enzyme reaction involved in the oxidation of choline to GB is catalyzed by a ferredoxin-dependent choline monooxygenase (EC 1.14.15.7) and an NAD(P)⁺-dependent betaine aldehyde dehydrogenase (BADH) (EC 1.2.1.8) (29). In mammals and Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, or *Sinorhizobium meliloti*, the two-enzyme biosynthesis of GB is catalyzed by a choline dehydrogenase (CDH) (EC 1.1.99.1) and a betaine aldehyde dehydrogenase (30–32). In Gram-positive bacteria, such as *Bacillus subtilis*, an alcohol dehydrogenase (EC 1.1.1.1) produces the BA, which is then converted to GB by BADH (33). A choline oxidase activity was previously shown in a few filamentous
fungi (34–36), but the enzymatic activity and the encoding genes were not characterized.

AfChoAp is a flavin-dependent choline oxidase, as indicated by the ability of the recombinant enzyme to catalyze the oxidation of choline or BA to GB with molecular oxygen as the oxidizing substrate. The percentage of identity between the choline oxidases of *A. fumigatus* and *Arthrobacter globiformis*, which is the only bacterial species that has a unique CHOA gene, was 37% (37). In addition, the amino acids in the catalytic site of the *A. globiformis* choline oxidase are conserved in *A. fumigatus* and other fungi, as follows: H99, site of flavin covalent attachment (38); H310, proton relay with H466; E312, substrate binding (39); H351, hydrogen bonding OH of choline (40); V464, substrate positioning and oxygen reactivity (41); H466, positive charge stabilization of the alkoxide intermediate of the reaction (42); and N510, involved in the reductive and oxidative half reaction (44). Both the fungal and bacterial enzymes display steady-state kinetic mechanisms with formation of a ternary complex with either choline or BA as the substrate, with oxygen reacting with the reduced flavin before the organic product of the reaction is released from the enzyme active site (37).

AfBadAp oxidizes BA to GB with reduction of NADH. The percentage of identity between the AfBadAp and the best-characterized PaBADH bacterial protein from *P. aeruginosa* was 57%. All relevant amino acids of the active site previously identified from the crystal structure of the PaBADH enzyme were present in BadAp from *A. fumigatus* and other fungi (45); a catalytic glutamate (E252), a catalytic cysteine (C286), and the NAD⁺ binding motif (G-x(3)-[ST]-G). The fungal enzyme is also able to utilize NADPH as the reducing substrate, but NAD⁺ is clearly preferred as indicated by a \( \frac{k_{cat}}{K_{NAD^+}} \) ratio larger than 300. From a kinetic standpoint, AfBadAp displays a sequential steady-state kinetic mechanism with BA and either NAD⁺ or NADPH⁺, as previously reported for bacterial betaine aldehyde dehydrogenases (45, 46). AfBadAp is exquisitely efficient in the oxidation reaction, as indicated by the second-order rate constant for substrate capture \( k_{cat}/K_m \), with a value of \( 4 \times 10^5 \) M⁻¹ s⁻¹. This is essential for the depletion of BA from the intracellular milieu, thereby abating the potential toxic effect of having an aldehyde reacting nonspecifically with a number of intracellular nucleophiles (47).

The GB pathway in *A. fumigatus* presents several unique features exclusively specific to fungal enzymes: (i) the fungal ChoAp is the only one to use NAD⁺ as a cofactor, whereas bacterial enzymes preferentially use NADPH⁺; (ii) in contrast to the bacterial enzyme, which retains BA in the active site during catalysis, the fungal enzyme releases it to the solution, where it is further oxidized to GB by AfBadAp; and (iii) AfBadAp is not inhibited by BA, NAD⁺, or NADPH⁺.

GB is used as a nutrient in fungi. In plants and bacteria, GB has been shown to enhance tolerance to a variety of stresses, such as high salt concentration, freezing, chilling, drought, or oxidants, because it stabilizes the quaternary structure of enzymes and maintains the highly ordered state of the membranes at nonphysiological temperatures and salt conditions (1, 48–52). All these abiotic stresses also induce an oxidative burst, the damages from which can be reduced by GB (1). In halophilic fungi, such as *Penicillium fellutanum*, it was suggested that GB was associated with osmotic protection, but there was no biochemical and molecular analysis of the GB pathway in this species (35). In contrast, in *A. fumigatus* the production of GB does not seem to be associated with a tolerance to stress.

![Localization of AfChoAp-eGFP and AfBadAp-RFP-T fusion proteins.](https://example.com/image)

**FIG 5** Localization of AfChoAp-eGFP and AfBadAp-RFP-T fusion proteins. (A and B) The AfchoAΔAfbadAΔ:HPH mutant transformed with pAN8.1 containing the AfCHOA::eGFP (A) and AfBADA::RFP-T (B) fusions under the control of the AfCHOA and AfBADA gene promoter. (C) Bright-field image of the same germinating conidia (upper panel) and hyphae (lower panel). The fungus was grown in liquid MM with choline chloride (20 mM) as the sole nitrogen source.
Several reports mention the fact that in bacteria GB has a dual function: it can be used alternatively as an antistress molecule or as an energy source (53–56). Interestingly, *A. fumigatus* seems to use GB as a sole source of carbon, nitrogen, and energy through successive demethylations to glycine. Choline and GB, with methionine, are the most important carriers of methyl groups in different metabolic pathways. However, methionine is used for protein synthesis, whereas choline serves predominantly for the formation of cell membranes (57–59). Moreover, GB may be directly used as a methyl group donor, whereas choline needs to be converted in a two-step oxidation reaction catalyzed by AChoAp (57–59). The GB biosynthesis could be a rescue pathway for the use of unusual nitrogen (or carbon) sources under nutrient starvation conditions. Transcriptome data for *A. fumigatus* during early conidial germination indeed revealed an upregulation of genes involved in lipid catabolism (10). The link between lipid catabolism and GB biosynthesis may be relevant for fungal physiology, since use of GB would be a quick catabolic way to mobilize choline for amino acid synthesis, which was shown to be upregulated during germination (10). However, despite being highly upregulated during germination, catabolism of choline to produce GB during germination does not seem to be essential to this morphological event. Lipidome analysis of the conidial germination should improve our understanding of lipid metabolism during conidial germination and of the interconnections of GB with the other lipid components of the fungus.

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