Molecular Characterization of the Putative Transcription Factor SebA Involved in Virulence in Aspergillus fumigatus

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Aspergillus fumigatus is a major opportunistic pathogen and allergen of mammals. Nutrient sensing and acquisition mechanisms, as well as the capability to cope with different stressing conditions, are essential for A. fumigatus virulence and survival in the mammalian host. This study characterized the A. fumigatus SebA transcription factor, which is the putative homologue of the factor encoded by Trichoderma atroviride seb1. The ∆sebA mutant demonstrated reduced growth in the presence of paraquat, hydrogen peroxide, CaCl2, and poor nutritional conditions, while viability associated with sebA was also affected by heat shock exposure. Accordingly, SebA::GFP (SebA:green fluorescent protein) was shown to accumulate in the nucleus upon exposure to oxidative stress and heat shock conditions. In addition, genes involved in either the oxidative stress or heat stress response had reduced transcription in the ∆sebA mutant. The A. fumigatus ∆sebA strain was attenuated in virulence in a murine model of invasive pulmonary aspergillosis. Furthermore, killing of the ∆sebA mutant by murine alveolar macrophages was increased compared to killing of the wild-type strain. A. fumigatus SebA plays a complex role, contributing to several stress tolerance pathways and growth under poor nutritional conditions, and seems to be integrated into different stress responses.

Materials and Methods

Strains and culture conditions. The A. fumigatus strains used in this study were CEA17 (pyrG mutant) (for the transformation assays), CEA17-80 (as the wild-type strain), ΔcalA and ΔcrzA (55), ΔsebA::pyrG, and ∆sebA::sebA-::Leu. Three variants of a complete medium were used: YAG (2% glucose, 0.5% yeast extract, 2% agar, trace elements), YUU (YAG supplemented with 1.2 g/liter of both uracil and uridine), and liquid YG medium (consisting of the same compositions but without agar). A modified minimal medium (MM; 1% glucose, the original high

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Aspergillus fumigatus is a major opportunistic pathogen and allergen of mammals that is readily isolated from human habitats and vegetable compost heaps (4, 9, 17, 25, 33). A. fumigatus produces prodigious amounts of conidia; as a consequence, the human respiratory tract is constantly exposed to the fungus, which is increasingly linked to severe asthma and sinusitis (3, 11, 12). The infection of the mammalian host. This study characterized the A. fumigatus SebA transcription factor, which is the putative homologue of the factor encoded by Trichoderma atroviride seb1. The ∆sebA mutant demonstrated reduced growth in the presence of paraquat, hydrogen peroxide, CaCl2, and poor nutritional conditions, while viability associated with sebA was also affected by heat shock exposure. Accordingly, SebA::GFP (SebA:green fluorescent protein) was shown to accumulate in the nucleus upon exposure to oxidative stress and heat shock conditions. In addition, genes involved in either the oxidative stress or heat stress response had reduced transcription in the ∆sebA mutant. The A. fumigatus ∆sebA strain was attenuated in virulence in a murine model of invasive pulmonary aspergillosis. Furthermore, killing of the ∆sebA mutant by murine alveolar macrophages was increased compared to killing of the wild-type strain. A. fumigatus SebA plays a complex role, contributing to several stress tolerance pathways and growth under poor nutritional conditions, and seems to be integrated into different stress responses.

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levels of nitrate salts, trace elements, 2% agar, pH 6.5) was also used. The trace elements, vitamins, and nitrate salts in the media are described in reference 31. Additionally, 10% fetal bovine serum (FBS; Gibco) was used as a medium. For the analysis of growth on lung tissue, the methodology described in reference 21 was followed. Briefly, dissected mouse lungs were placed on the surface of a 1% agarose plate in sterile distilled water. The lung tissue was inoculated with 2,000 conidia in 5 μl of sterile water and grown for 2 days at 37°C. Growth under starvation conditions was determined according to reference 45. Briefly, ~1,000 conidia were spread onto the surface of a YG plate and incubated for 16 h at 37°C. Hyphal plugs containing individual colonies were obtained using a sterile 1-ml tip and transferred onto the center of a 1% agarose plate, and radial growth was determined after 3 days of incubation at 37°C.

**Staining and microscopy.** SebA::GFP (SebA::green fluorescent protein) conidiospores were grown on coverslips in 4 ml of YG medium for 16 h at 30°C. After incubation, subsets of coverslips with adherent germ- lings were left untreated, treated with pararaut (10 mM), H2O2 (2 mM), and CaCl2 (200 mM), or submitted to heat shock (30°C to 37°C and 30°C to 44°C). Subsequently, the coverslips were rinsed with phosphate-buff- ered saline (PBS; 140 mM NaCl, 2 mM KCl, 10 mM NaHPO4, 1.8 mM KH2PO4, pH 7.4) and incubated for 3 min in a solution with Hoechst stain (Life Technologies) (12 μg/ml). After incubation with the dye, the coverslips were washed with PBS and mounted for examination. Slides were visualized on an Observer Z1 fluorescence microscope using a 100× objective oil immersion lens (for GFP, filter set 38—high efficiency [HE], excitation wavelength of 450 to 490 nm, and emission wavelength of 525/50 nm; for Hoechst stain, filter set 49, excitation wavelength of 365 nm, and emission wavelength of 450 to 490 nm, and emission wavelength of 343 nm). DIC (differential interference contrast) images and fluorescent images were captured with an AxioCam camera (Carl Zeiss) and processed using AxiosVision software (version 4.8).

**Construction of A. fumigatus sebA disruption mutant and SebA::GFP.** A gene replacement cassette was constructed by in vivo recombin- ation in S. cerevisiae as previously described in reference 14. Briefly, ~2.0-kb regions on either side of the sebA open reading frame (ORF) (Afusgi09800) were selected for primer design. For construction, the primers (named 5F and 5R) were used to amplify the flanking 5′ untranslated region (5′-UTR) of the targeted ORF. Likewise, primers 3F and 3R were used to amplify the 3′-UTR ORF flanking region. The primers 5F and 3R also contained a short sequence homologous to the multiple- cloning site (MCS) of the pRS426 plasmid. Both fragments (the 5′-UTR and 3′-UTR) were amplified from genomic DNA (gDNA) by PCR using A. fumigatus gDNA as a template. The A. fumigatus pyrG selectable marker for uridine or uracil prototrophy was used for generating the ΔsebA strain.

Deletion cassettes were generated by transforming each fragment along with the cut plasmid, pRS426 BamHI-EcoRI, into S. cerevisiae SC94721 via the lithium acetate method (14, 49). The DNA of the S. cerevisiae transformants was extracted as described in reference 23, dia- lyzed, and transformed by electroporation into Escherichia coli DH10B to rescue the pRS426 plasmid harboring the deletion cassette. The cassette was PCR amplified from the plasmids and used for A. fumigatus transfor- mation. Southern blot analysis was used to demonstrate that the cassettes had integrated homologously at the targeted A. fumigatus locus. To com- plement the ΔsebA strain, a region of ~2.0 kb flanking the sebA gene was amplified using gDNA from CEA10 as a template. Protoplasts were cotransformed with the 6.8-kb PCR product and the hygromycin B resistance vector (pHAT-α) and then plated on media containing hygromycin B (150 μg/ml).

To generate the SebA::GFP strain, the sebA ORF was cloned in frame with the green fluorescent protein (GFP) gene. The construct links GFP to the C terminus of sebA and is separated by four additional codons that, after translation, produce a four-amino-acid linker (glycine-threonine-arginine-glycine) (59). The S. cerevisiae in vivo recombination system was used for production of the transformation cassette, and then the frag- ments were PCR amplified. First, the sebA ORF and its 5′-UTR flanking region were amplified from gDNA of the wild-type strain by the use of the primers sebA pRS426 5f and sebA SPACER GFP Rev. The stop codon of the sebA gene was omitted in this construction. The GFP ORF was amplified from the pMCB17apx plasmid (provided by Vladimir P. Efimov) by the use of the primers Spacer GFP Fwd and GFP V3′ AF. The last frag- ment was PCR amplified from the sebA deletion cassette and contained the selective marker pyrG. The primers used for this PCR amplification were GFP pyrG Fwd and pyrG Rev. The amplification of the 3′-UTR was done with the Afu sebA 3F and Afu sebA 3r primers. S. cerevisiae in vivo recombination was performed as previously described. The PCR-ampli- fied cassette was transformed into the A. fumigatus wild-type strain.

**Heat and oxidative stress treatments.** The heat shock viability assay (41) was carried out with wild-type and ΔsebA strains. Conidia (1 × 10⁶ ml⁻¹) were incubated in YG for 3 h at 30°C in a shaker (250 rpm). After this period, the germlings were exposed to 37°C and 48°C for 15, 30, and 60 min. The untreated control group was maintained at 30°C. Conidia were collected, diluted, and plated on YAG plates. Plates were incubated at 37°C for 48 h. Viability was determined as the percentage of colonies on treated plates compared with untreated controls.

For real-time analysis, heat shock treatment was done according to reference 40. For temperature-shift experiments, conidia (5 × 10⁶ ml⁻¹) from the wild-type strain were incubated in YG medium for 17 h at 30°C. Cultures were then transferred to 37°C or 48°C for continued growth, for 15, 30, 60, and 120 min. Total RNA extracted from samples before (0 min) and after (15, 30, 60, and 120 min) the two temperature shifts (30°C to 37°C and 30°C to 48°C) were used to profile the sebA gene expression.

The sebA gene expression was also analyzed under different osmotic and oxidative stress conditions. Cultures containing 10⁶ wild-type or ΔsebA conidia were grown overnight in YG media and then treated with either 2 mM H2O2 or 10 mM paraquat for 15 and 30 min to assess oxida- tive stress tolerance and 1.2 M KCl or 1.2 M sorbitol for 30, 60, 120, and 180 min to assess osmotic stress tolerance.

**RNA extraction and real-time PCRs.** After the different conditional treatments, mycelia were harvested by filtration, washed twice with water, and immediately frozen in liquid nitrogen. Total RNA was isolated from germlings disrupted by grinding in liquid nitrogen with a pestle and mor- tar and extracted using TRIzol reagent (Invitrogen). RNA (10 μg) from each treatment was fractionated on a 2.2 M formaldehyde–1.2% (wt/vol) agarose gel, stained with ethidium bromide, and visualized under UV light. The presence of intact 25S and 18S rRNA bands was used to assess RNA integrity. RNase-free DNase I treatment was carried out as previ- ously described (52). A 20-μg volume of total RNA was treated with DNase and purified using an RNEasy kit (Qiagen). cDNA was synthesized using a SuperScript III First Strand Synthesis system (Invitrogen) and oligo(dT) primers according to the manufacturer’s protocol.

All PCRs were performed using an ABI 7500 Fast real-time PCR sys- tem (Applied Biosystems) and a TaqMan Universal PCR Master Mix kit (Applied Biosystems). The reactions and calculations were performed ac- cording to reference 52. The primers and Lux fluorescent probes (Invitro- gen) used are described in Table S1 in the supplemental material.

**Murine model of pulmonary aspergillosis.** Outbred female mice (BALB/c strain; body weight, 20 to 22 g) were housed in vented cages containing 5 animals. Mice were immunosuppressed with cyclophospha- mide at 150 mg/kg of body weight administered intraperitonally on days −4, −1, and 2 prior to and postinfection. Hydrocortisoneacetate (200 mg/kg) was injected subcutaneously on day −3 (modified from reference 39). A. fumigatus spores used for inoculation were grown on Aspergillus complete medium for 2 days prior to infection. Conidia were freshly har- vested in PBS and filtered through a Miracloth (Calbiochem). Conidial suspensions were spun for 5 min at 3,000 × g, washed three times with PBS, counted using a hemocytometer, and resuspended at a concentra- tion of 2.5 × 10⁶ conidia/ml. Viable counts of the administered inocula were determined, following serial dilution, by plating on Aspergillus complete medium, and the conidia were grown at 37°C. Mice were anes- thetized by halothane inhalation and infected by intranasal instillation of
5.0 × 10⁴ conidia in 20 μl of PBS. As a negative control, a group of 5 mice received PBS only. Mice were weighed every 24 h from the day of infection and visually inspected twice daily. In the majority of cases, the endpoint for survival experimentation was identified when a 20% reduction in body weight was recorded, at which time the mice were sacrificed. The statistical significance of comparative survival values was calculated using log rank analysis and the Prism statistical analysis package. Additionally, at 3 days postinfection, 2 mice per strain were sacrificed and the lungs were removed, fixed, and processed for histological analysis.

**Lung histopathology and fungal burden.** After sacrifice, the lungs were removed and fixed for 24 h in 3.7% formaldehyde–PBS. Samples were washed several times in 70% alcohol before dehydration in a series of alcohol solutions of increasing concentrations. Finally, the samples were diafanized in xylol and embedded in paraffin. For each sample, sequential 5-μm-thick sections were collected on glass slides and stained with Gomori methenamine silver (GMS) or hematoxylin and eosin (HE) stain following standard protocols (25). Briefly, sections were deparaffinized, oxidized with 4% chromic acid, stained with methenamine silver solution, and counterstained with picric acid. For HE staining, sections were deparaffinized and stained first with hematoxylin and then with eosin. All stained slides were immediately washed, preserved with mounting medium, and sealed with a coverslip. Microscopic analyses were done using an Axioplan 2 imaging microscope (Zeiss) at the stated magnifications under bright-field conditions.

To investigate fungal burden in murine lungs, mice were immunosuppressed with cyclophosphamide at 150 mg/kg of body weight administered intraperitoneally on days −4 and −1, while hydrocortisoneacetate was injected subcutaneously at 200 mg/kg on day −3. Five mice per group (wild-type, ΔsebA, and ΔsebA::sebA strains and PBS control) were intranasally inoculated with 1 × 10⁶ conidia/20 μl of suspension. A higher inoculum, in comparison to the survival experiments, was used to increase fungal DNA detection. Animals were sacrificed 72 h postinfection, and both lungs were harvested and immediately frozen in liquid nitrogen. Samples were homogenized with glass beads by performing a vortex procedure for 10 min, and DNA was extracted via the phenol-chloroform method. DNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). At least 500 μg of total DNA from each sample was used for quantitative real-time PCRs. A primer and a Lux probe (Invitrogen) were used to amplify the 18S rRNA region of *A. fumigatus* (primer, 5′-CTTAAATAGCCCGGTCCGCATT-3′; probe, 5′-CATCACAGACCTGTTATTGCCG-3′) and an intronic region of mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (primer, 5′-CATCACAGACCTGTTATTGCCG-3′; probe, 5′-GGGCAAGGCTAAAGGTCAGCG-3′). Six-point standard curves were calculated using serial dilutions of gDNA from all the *A. fumigatus* strains used and the noninfected mouse lung. Fungal and mouse DNA quantities were obtained from the threshold cycle (C_T) values from an appropriate standard curve. Fungal burden was determined as the ratio between picograms of fungal and micrograms of mouse DNA.

**Phagocytosis index.** To estimate the percentage of phagocytosed conidia, alveolar macrophages were harvested from lungs of BALB/c mice (8 to 10 weeks old) by using an intravenous catheter (Angiocath; Becton Dickinson) (18 gauge, 1.88 in. long). Six mice per experiment were sacrificed; the trachea was exposed and the catheter attached. A 1-ml volume of RPMI 1640 medium (Sigma-Aldrich) was introduced and subsequently removed from the lungs three times. Macrophages from all six animals were mixed and centrifuged at 4,000 rpm for 5 min. The supernatant was removed, and the pellet was washed with 5 ml of RPMI 1640 medium and resuspended in 1 ml of RPMI 1640–10% fetal calf serum (PCS) (Gibco). Cells were counted in a hemocytometer. The phagocytic assay was per-

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**FIG 1** Growth phenotypes of the ΔsebA mutant strain. The wild-type, the ΔsebA::sebA⁰, and the ΔsebA mutant strains were grown for 72 h in YAG medium (A), YAG medium plus paraquat at different concentrations (B), or YAG medium or YAG medium plus paraquat at 4 mM (C) or for 48 h at 37°C in YAG medium plus different concentrations of H₂O₂ (D).
formed according to Mech and coworkers (38) with some changes. Briefly, in a 24-well plate containing one 15-mm-diameter coverslip per well, 5 × 10^5 macrophages were incubated with 1 ml of RPMI 1640–10% FCS (assay medium) at 37°C with 5% CO_2 for 1 h. Afterward, the wells were washed with 1 ml of assay medium to remove nonadherent cells. In each well, 1 ml of assay medium containing 2.5 × 10^5 conidia (5 conidia/macrophase) was added. Duplicate wells were assayed for each strain. The samples were incubated at 37°C with 5% CO_2 for 80 min, after which the supernatant was removed and 500 μl of 3.7% formaldehyde–PBS was added. After 15 min, the samples were washed with 1 ml of ultrapure water and incubated for 20 min with 495 μl of water–5 μl of calcofluor (10 mg/ml). The samples were washed and mounted on slides with 50% glycerol. A Zeiss Observer.Z1 fluorescence microscope was used to assess the percentage of phagocytosed spores. Macrophage cells were not permeable; hence, conidia inside the macrophages were not stained, while conidia outside the macrophages were stained, by calcofluor. At least 100 spores were counted per sample, and a phagocytosis index was calculated. The experiments were repeated in triplicate.

Conidial killing by alveolar macrophages. To assess conidial killing by alveolar macrophages, the phagocytic cells were obtained as described above. In a 96-well plate, 5 × 10^4 macrophages were added with 200 μl of RPMI 1640–10% FCS per well and incubated at 37°C with 5% CO_2 for 1 h. Afterwards, 2.5 × 10^5 conidia (5 conidia/macrophase) were added and incubated at 37°C with 5% CO_2 for 4 h. As a positive control, wells containing medium and spores without macrophages were used. Triplicate wells were assayed for each strain with and without macrophages. After incubation, the plate was centrifuged at 3,500 rpm for 10 min, supernatant was removed without disturbing the pellet, and 100 μl of 1% Triton X-100 was added. After 10 min at room temperature, samples were removed from the plate and serially diluted in sterile water. The dilutions were plated on A. fumigatus complete medium (38) and incubated at 37°C for 2 days. The percentage of conidial killing was calculated by comparing CFU numbers from samples incubated with macrophages to CFU numbers from those incubated without macrophages. The experiments were repeated three times.

RESULTS
There is a single Seb1 homologue in A. fumigatus. A BLASTp search of the A. fumigatus genome database (http://www.aspgd.org) performed using T. atroviride seb1 as a query revealed a single open reading frame with significant similarity. The potential homologue, Afu4g09080 (here named sebA), is predicted to be a 618-amino-acid zinc finger C2H2 protein with homology to the T. atroviride Seb1 (from residue 477 to residue 616; e-value, 1.2e-55; 70.2% similarity and 61.7% identity). SebA shares identity, only in the zinc finger domain, with the S. cerevisiae TFs Msn2p (from residue 375 to residue 440; identity, 70.2% similarity and 61.7% identity), Msn4p (from residue 493 to residue 548; e-value, 4.2e-20; 72% similarity and 68% identity), and Com2p (from residue 375 to residue 440; e-value, 1.3e-15; 70.2% similarity and 61.7% identity). SebA shares identity, only in the zinc finger domain, with the S. cerevisiae TFs Msn2p (from residue 483 to residue 544; e-value, 7.2e-17; 72% similarity and 54% identity), Msn4p (from residue 437 to residue 544; e-value, 1.3e-15; 57% similarity and 38% identity), and Com2p (from residue 375 to residue 440; e-value, 4.2e-20; 72% similarity and 68% identity). Identity with these S. cerevisiae homologues is mainly in vivo S. cerevisiae fusion-based approach (see Materials and Methods). Replacement of the sebA gene in several transformants was verified by Southern blot analysis; one of the transformants was used for further phenotypic characterization (see Fig. S1 in the supplemental material). The ΔsebA phenotype
was complemented with sebA from the wild-type strain, producing the ΔsebA::sebA⁺ strain (see Fig. S1 in the supplemental material). All strains were able to grow on uracil, uridine, and hygromycin. The ΔsebA mutant showed radial growth comparable to that of the wild-type and complemented strains, except at lower temperatures (such as 25°C and 30°C), when the radial growth was reduced (Fig. 1A). The ΔsebA mutant was more sensitive to oxidative stress factors, such as paraquat and H₂O₂ (at 37°C), than the wild-type and complemented strains, as shown by the reduction in growth in liquid complete medium (Fig. 1B and D) and solid complete medium (Fig. 1C). No growth reduction was observed when the ΔsebA mutant was grown under osmotic stress conditions, such as in the presence of increasing concentrations of KCl, NaCl, or sorbitol (data not shown). The reduction in radial growth was greater when the ΔsebA mutant was grown on minimal medium (at 37°C) and even greater at different temperatures on solid FBS (10%) medium (Fig. 1A). Growth of the wild-type, ΔsebA::sebA⁺, and ΔsebA strains in FBS (10%) liquid medium from 24 to 96 h was evaluated (Fig. 2B). The ΔsebA mutant demonstrated a dramatic (between 60 and 70%) reduction in dry mycelial weight compared to the wild-type and complemented strains (Fig. 2B). Growth characteristics on YAG medium and on dissected mouse lung tissue were also compared. As previously shown (Fig. 1), the wild-type and ΔsebA and ΔsebA::sebA⁺ mutant strains had the same radial diameter after 48 h of growth on YAG medium (Fig. 2C); however, the ΔsebA mutant strain had reduced growth on lung tissue after 48 h (Fig. 2C). Growth of the ΔsebA strain was also impaired on lung tissue, in vivo, as shown by the histopathology of infected lungs (see Fig. 9B) and fungal burden quantifications (see Fig. 9C). To verify whether SebA is important for growth under nutrient-limiting conditions, the wild-type, ΔsebA::sebA⁺, and ΔsebA strains were grown under starvation conditions as described in reference 45. Both the wild-type and ΔsebA::sebA⁺ strains were able to produce restricted growth in the absence of extracellular nutrients, while the ΔsebA strain had significantly reduced growth under starvation conditions (Fig. 2D). The ΔsebA mutant showed no differential susceptibility to antifungal agents, such as amphotericin, azoles, and caspofungin, in E-tests or susceptibility to cell wall-targeting agents, such as Congo red (data not shown).

To assess the subcellular location of SebA, we constructed a SebA::GFP strain. This strain behaves exactly like the wild-type strain (data not shown). When grown on YG for 16 h at 30°C, it showed a weak and diffuse fluorescent signal in the cytosol (Fig. 3A). In contrast, when this strain was transferred to YG–2 mM H₂O₂ (Fig. 3B) or YG–10 mM paraquat (Fig. 3C) for 15, 30, or 60 min, SebA::GFP progressively accumulated in the nucleus, as shown by Hoechst costaining (Fig. 3B and C).

Taken together, these results suggest that SebA is important for the oxidative stress response and plays a role during growth under poor nutrient conditions.

**The transcriptional basis for A. fumigatus ΔsebA sensitivity to oxidative stress.** The A. fumigatus ΔsebA strain was shown to be...
more sensitive to paraquat and H$_2$O$_2$ (Fig. 1B). Therefore, a transcriptional approach was used to characterize the transcriptional basis of the oxidative stress sensitivity of the \(\Delta sebA\) mutant. Several \(A. fumigatus\) homologues involved in oxidative stress tolerance were identified, Lux fluorescent probes were designed for these genes, and then gene expression in response to H$_2$O$_2$ and paraquat was determined in the wild-type, \(\Delta sebA\), and \(\Delta sebA::sebA^+\) strains via real-time reverse transcription-PCR (RT-PCR) (Fig. 4 and 5). In the wild-type strain, \(sebA\) transcription did not increase in the presence of H$_2$O$_2$ (2 mM) or paraquat (10 mM) for 15 and 30 min or when the strain was exposed to increasing concentrations of KCl and sorbitol (data not shown). The transcriptional response of several \(A. fumigatus\) genes involved in the oxidative stress response (http://www.aspgd.org), including \(Af yap1\) and \(Afatf1\) (Afu6g09930 and Afu3g11330), which encode TFs required for resistance to oxidative stress (26, 34, 65), \(Af ccp1\) (Afu4g09110), which encodes a cytochrome c peroxidase (1, 34, 307), \(Af c a t 1\) (Afu3g02270), \(Af c a t 2\) (Afu8g01670), \(Afs o d 1\) (Afu5g09240), which encodes a Cu/Zn superoxide dismutase (30, 32, 34, 42, 62), and \(Afs o d 2\) (Afu4g11580), which encodes a manganese-superoxide dismutase (32, 57), was assessed via real-time RT-PCR.

When the wild-type and the complemented strains were exposed to no or 2 mM H$_2$O$_2$ for 15 or 30 min, all the investigated genes showed elevated transcription (Fig. 4). The transcriptional response of the \(\Delta sebA\) strain was lower than that of the wild-type and complemented strains (Fig. 4), except for \(Afs o d 1\) and \(Afs o d 2\), which had transcription similar to or higher than that seen with the wild-type strain (Fig. 4F to G). The wild-type, the \(\Delta sebA::sebA^+\) strains were also exposed to 10 mM paraquat for 15 and 30 min, and gene expression was determined (Fig. 5). Again, all the genes showed increased transcription in the wild-type and the \(\Delta sebA::sebA^+\) strains when exposed to paraquat (Fig. 5).

**FIG 4** The transcriptional basis for \(A. fumigatus\) \(\Delta sebA\) sensitivity to hydrogen peroxide. The wild-type, \(\Delta sebA\), and \(\Delta sebA::sebA^+\) strains were grown for 16 h in YG at 37°C and transferred to fresh YG medium with either 0 or 2 mM H$_2$O$_2$ and grown for a further 15 or 30 min. The relative quantitations of \(Af yap1\) (A), \(Afatf1\) (B), \(Af ccp1\) (C), \(Af c a t 1\) (D), \(Af c a t 2\) (E), \(Afs o d 1\) (F), and \(Afs o d 2\) (G) and tubulin gene expression were determined by a standard curve (i.e., \(C_T\) values plotted against a logarithm of the DNA copy number). Data shown represent the means (± standard deviations) of the results of four biological replicate experiments (*, \(P < 0.001\)).
while the transcriptional response of the ΔsebA strain, for the majority of genes, was lower than in the wild-type and complemented strains (Fig. 5), except for Afyap1, Afatf1, and Afsod1, which did not show any increase in transcription compared to the control (Fig. 5A, B, and F). Interestingly, in the absence of either H2O2 or paraquat, the ΔsebA strain showed higher transcription than the wild-type and complemented strains for the majority of the genes, except Afcat1 and Afsod2 (Fig. 4 and 5).

Collectively, these data suggest that SebA contributes to the transcriptional regulation of several genes related to *A. fumigatus* oxidative stress adaptation.

*A. fumigatus sebA* is important for heat shock recovery. The fact that SebA was involved in the oxidative stress response and provided greater fitness under poor nutrient conditions suggested that SebA could be involved in other kinds of stress responses. Despite our not seeing any growth defects for the ΔsebA mutant at 37 and 44°C (Fig. 1), the involvement of sebA in heat adaptation was evaluated by exposing the wild-type, the ΔsebA::sebA+ strains to heat shock and measuring the sebA gene expression (Fig. 6A). Transcription of sebA increased in the wild-type strain after exposure to 37°C and 48°C (approximately 6.5- and 4.0-fold after 30 and 60 min and 2.0-fold after 60 and 120 min; Fig. 6A). Accordingly, when all three strains were grown for 5 h at 30°C and then exposed to 37°C for 60 min, there were decreases of 40%, 35%, and 70% in the viability of the wild-type, the ΔsebA::sebA+ strains, and the ΔsebA strains, respectively (Fig. 6B). Both heat shock treatments induced the SebA::GFP protein to accumulate in the nucleus (Fig. 6C to E). However, SebA::GFP was seen only after 60 min of heat shock (Fig. 6C to E).

To evaluate if SebA is able to control directly or indirectly the transcriptional response of *A. fumigatus* to heat shock, several homologues recognized as being responsible for the heat shock response in *S. cerevisiae* (http://www.yeastgenome.org), including...
the HSFI homologue Aflsf1 (Afu5g01900), which encodes a TF that regulates the response to heat shock, the HSP30 homologue Aflsp30 (Afu6g06470), the HSP90 homologue Aflsp90 (Afu5g04170), and the SSP1 homologue Afssp1 (Af8g03930), which encodes a chaperone that is also a member of the HSP70 protein family, were assessed via real-time RT-PCR. All these genes, except Af2g09960, have been demonstrated to have increased transcription during heat shock in A. fumigatus (1, 19). In this study, all these genes showed increased expression in the wild-type and ΔsebA::sebA+ strains upon heat shock, for at least a single time point, during one or both heat shock transitions (Fig. 7; see also Fig. S2 in the supplemental material). In contrast, much more variable behavior was observed for the ΔsebA mutant strain. Some of the genes had a smaller increase in expression (such as Aflsf1; Fig. 7A) or a much greater increase in expression (such as Aflsp30 and Aflsp90; Fig. 7B and C) or even reduced expression (such as Afssc1 and Afssp1; Fig. 7D and E). Taken together, these data suggest that SebA may also participate in the modulation of the transcriptional regulation of several genes involved in the A. fumigatus heat shock response.
The *A. fumigatus* ΔsebA mutant strain is more sensitive to calcium. In an attempt to further identify which biochemical pathways SebA is involved in, and/or regulates, the calcineurin/CrzA pathway was evaluated to determine if it is required for the action of the SebA mechanism. First, the ΔsebA strain was shown to be more sensitive to CaCl$_2$ than the corresponding wild-type and ΔsebA::sebA strains (Fig. 8A). Interestingly, ΔsebA was as sensitive to CaCl$_2$ as the ΔcrzA mutant (Fig. 8B).
tional dependence of sebA on calcium was evaluated by real-time RT-PCR. The expression of sebA in the wild-type, ΔcaIA, and ΔcrzA mutant strains, in response to a short pulse of CaCl₂ (200 mM) for 10 and 30 min (Fig. 8C), was determined. In the wild type, sebA expression increased 2-fold following exposure to CaCl₂ (Fig. 8C). The increases were approximately 3- and 8-fold in the ΔcrzA mutant strain and 2- and 3-fold in the ΔcaIA mutant strain for CaCl₂ exposures of 10 and 30 min, respectively (Fig. 8C). Collectively, these results suggest that SebA is involved in the calcium metabolism and that it possibly interacts with the calcineurin-CrzA pathway.

The A. fumigatus ΔsebA mutant strain has decreased virulence in a low-dose murine infection. To assess the role of SebA in pathogenicity, the A. fumigatus deletion mutant was assessed in a neutropenic murine model for invasive pulmonary aspergillosis, comparing virulence of the A. fumigatus ΔsebA mutant strain (n = 10) to the virulence of the wild-type strain (n = 10) and the complemented strain (n = 10) (Fig. 9A). Wild-type infection resulted in a mortality rate of over 90% at 5 days postinfection, while infection with the ΔsebA strain resulted in a significantly reduced mortality rate of approximately 50% after 9 to 15 days postinfection (P < 0.0005). With respect to the kinetics of infection, mice succumbing to infection with the ΔsebA mutant had a median survival of 7 days, compared to 3 days for mice infected with the wild-type strain. To directly link the observed attenuation of infection with the loss of the sebA gene, an independent strain reconstituted with the wild-type sebA gene into the ΔsebA strain was assessed (see Fig. S1 in the supplemental material). This reintegration restored full virulence to the ΔsebA mutant (Fig. 9A). Histological examinations were performed to further elucidate the basis of the attenuated virulence of the ΔsebA strain. Infected tissues were collected during early infection with the aim of identifying differences in growth rate, tissue invasion, and the inflammatory response between the two strains. At 72 h postinfection, the lungs of mice infected with the wild-type isolate contained multiple foci of invasive hyphal growth, representing penetration of the pulmonary epithelium into the major airways (Fig. 9B) and pockets of branched invading mycelia originating from the alveoli (Fig. 9B). In contrast, infection resulting from ΔsebA inoculations was typified by contained inflammatory infiltrates in bronchioles (Fig. 9B), some of which contained fungal elements in the form of poorly germinated or ungerminated spores. Fungal burden was determined by real-time PCR and showed that the ΔsebA mutant strain did not grow as well as the wild-type or the complemented ΔsebA strain (Fig. 9C; P < 0.0001). Taken together, these data strongly indicate that SebA plays a role in A. fumigatus virulence.

Alveolar macrophages (AM) play an essential role in clearing A. fumigatus conidia from the lung (44). Approximately 50% of A. fumigatus wild-type, ΔsebA, and ΔsebA::sebA+ conidia were internalized after 80 min of incubation with AM (Fig. 10A). After a 4 h incubation, in vitro killing of resting conidia reached about 40%, 70%, and 40% for the A. fumigatus wild-type, ΔsebA, and ΔsebA::sebA+ strains, respectively (Fig. 10B). These data suggest that there are no differences in the uptake of ΔsebA conidia by AM, while the ΔsebA strain is more sensitive to AM killing.

DISCUSSION

The first member of the SebA/Seb1 family in filamentous fungi was isolated from T. atroviride and named the seb1 (stress response element binding) gene because the zinc finger domain specifically recognizes the 5'-AGGGG-3' sequence (43). In S. cerevisiae, this motif is recognized and bound by Msn2p/Msn4p (48). The T. atroviride seb1 gene encodes a C2H2 zinc finger protein that is approximately 63% identical to the S. cerevisiae proteins Msn2p-Msn4p and Com2p in their zinc finger regions. Msn2p and Msn4p are activated under several different sets of stress conditions, causing their rapid accumulation in the nucleus (6, 8, 10, 13, 18, 22, 24, 28, 36, 47, 50, 60). However, Seb1 may not be the direct Msn2p-Msn4p homologue, since seb1 overexpression in S. cerevisiae was

![FIG 8](image-url)
A. fumigatus sebA contributes to virulence in neutropenic mice. (A) Comparative analysis of wild-type, ΔsebA, and ΔsebA::sebA− strains in a neutropenic murine model of pulmonary aspergillosis. Mice in groups of 10 per strain were infected intranasally with a 20-μl suspension of conidiospores at a dose of 5.0 × 10⁴. (B) Histological analysis of the infected murine lung 72 h after infection with the wild-type strain reveals invasion of the murine lung epithelium. (C) Fungal burden was determined by real-time RT-PCR based on the measured quantity of the 18S ribosomal DNA (rDNA) in each of the treated samples was normalized using the Ct values obtained for an intronic region of the mouse GAPDH DNA amplifications. Data shown represent the means (± standard deviations) of the results determined with lungs from five mice for each treatment.

FIG 9 A. fumigatus sebA contributes to virulence in neutropenic mice. (A) Comparative analysis of wild-type, ΔsebA, and ΔsebA::sebA− strains in a neutropenic murine model of pulmonary aspergillosis. Mice in groups of 10 per strain were infected intranasally with a 20-μl suspension of conidiospores at a dose of 5.0 × 10⁴. (B) Histological analysis of the infected murine lung 72 h after infection with the wild-type strain reveals invasion of the murine lung epithelium. (C) Fungal burden was determined by real-time RT-PCR based on the measured quantity of the 18S ribosomal DNA (rDNA) in each of the treated samples was normalized using the Ct values obtained for an intronic region of the mouse GAPDH DNA amplifications. Data shown represent the means (± standard deviations) of the results determined with lungs from five mice for each treatment.

There is a great deal of speculation about which factors are responsible for efficient A. fumigatus virulence and pathogenicity (for reviews, see references 15, 37, and 61). Thermotolerance is the most important genetic trait for fungal survival during mammalian and avian infection and within compost (1, 19, 40). Thus, the genetic determinants involved in heat and stress resistance might also contribute to the virulence of this opportunistic pathogen. Few A. fumigatus genes that have been shown to be involved in thermotolerance were not linked to virulence (5, 12, 66). Thus, thermotolerance is a polygenic trait, and the most direct approach to identify genes involved in this genetic program is the isolation of transcription factors that activate signal transduction pathways related to thermal adaptation. As expected, most of the analyses have shown that heat shock proteins are very important for the thermotolerance, but in addition, several proteins involved in oxidative stress response, signal transduction, transcription, translation, and carbohydrate or nitrogen metabolism were observed (1, 19, 40). In S. cerevisiae, the heat shock response is mostly controlled by Hsf1, Msn2/Msn4, and Hac1, and targets for these transcription factors have already been identified (22, 27, 63–66). Upon heat shock, the A. fumigatus Hsf1 homologue has increased transcription (1, 22). Albrect et al. searched for putative A. fumigatus Hsf1 binding signatures and found 17 genes with a potential heat shock element in the promoter region (1). These genes encode not only proteins that are involved directly in the heat shock response (such as chaperones) but also enzymes involved in the oxidative stress response, signal transduction, and carbohydrate and nitrogen metabolism, among others. In S. cerevisiae, there is a link between the heat shock response, oxygen respiration, the formation of reactive oxygen intermediates, and the ac-
activation of the oxidative stress response (56). *A. fumigatus* SebA has a very complex role, contributing to several pathways of stress tolerance and growth under poor nutrient conditions. The *A. fumigatus* SebA seems to be one of the components that integrates responses to different stresses, such as heat shock and oxidative stress, during growth into the mammalian host. Not only does the ∆sebA mutant have decreased viability under oxidative and heat shock stress conditions, but also several genes that are important for coping with these stresses have heterogeneous transcription compared to those of the wild-type strain. The influence of the Ca²⁺-calcinurin–CrzA pathway on sebA is evidence of this complex regulation. The ∆sebA mutant is more sensitive to calcium and sebA has increased transcription upon calcium exposure in the ∆crzA mutant strain. It is possible that CrzA controls the SebA levels in the nucleus; this kind of regulation has already been observed for *S. cerevisiae* Msn2/Msn4 (58). It is also possible that there is a connection between *A. fumigatus* SebA and calcineurin under poor nutrient conditions, considering the fact that the *A. fumigatus* ∆calA mutant cannot grow in FBS (16). However, the increased expression of SebA in the ∆calA and ∆crzA mutants does not affect oxidative stress, heat shock stress, and conidial viability in these mutant strains.

Consistently, the multiple defects of the ∆sebA mutant affected the ability of the pathogen to cope with different stresses and nutritional conditions, which resulted in the attenuated virulence seen in the neutropenic murine model of invasive pulmonary aspergillosis and the decreased viability during AM phagocytosis. The attenuated virulence of the ∆sebA could be due to a global reduction in its fitness, while the decreased viability after AM phagocytosis could be due to the increased sensitivity of the mutants to reactive oxygen and nitrogen species present in the AM phagolysosomal compartments. However, there are no differences between the mutant and the wild-type strains during their internalization by the AM, which could suggest that the sebA null mutation does not have a considerable impact on the chemical composition of the conidia surface. The reduced ability of the ∆sebA mutant to survive within the mammalian environment may be the result not only of the decreased capacity to deal with stress conditions but also of the incapacity to metabolize nutrients. We have observed a reduction in growth of this mutant in poor nutrient media, such as FBS and murine lung. Nevertheless, there was no reduction in proteolytic activity in the ∆sebA mutant strain (data not shown). Thus, the combination of these phenotypic defects associated with fungal growth and response to stressing conditions led to a significant decrease in its ability to establish disease even in an immunocompromised host.

It remains to be demonstrated which signal transduction pathways and genetic programs are being affected by SebA. However, the current work demonstrated that a transcription factor could have a dramatic impact on virulence and survival in the mammalian host. It is tempting to speculate how SebA homologues would affect virulence and pathogenicity in other plant and mammalian fungal pathogens.

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