**The Influence of Genetic Stability on Aspergillus fumigatus Virulence and Azole Resistance**

Thaila Fernanda dos Reis,* Lilian Pereira Silva,* Patrícia Alves de Castro,* Pollyne Borborema Almeida de Lima,* Rafaela Andrade do Carmo,† Marjorie Mendes Marini,† José Franco da Silveira,† Beatriz Henriques Ferreira,† Fernando Rodrigues,† Iran Malavazi,§ and Gustavo H. Goldman*,‡

*Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14040-903, Brazil, †Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, 04023-062 Brazil, ‡Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal and ICVS/3B’s - PT Government Associate Laboratory Braga/Guimarães, 4710-057 Portugal, and §Departamento de Genética e Evolução, Centro de Ciências Biológicas e da Saúde, Universidade Federal de São Carlos, São Paulo 13565-905, Brazil

ORCID IDs: 0000-0002-7776-977X (T.F.d.R.); 0000-0002-1497-7440 (B.H.F.); 0000-0002-4526-4961 (I.M.); 0000-0002-2986-350X (G.H.G.)

**ABSTRACT** Genetic stability is extremely important for the survival of every living organism, and a very complex set of genes has evolved to cope with DNA damage upon DNA damage. Here, we investigated the Aspergillus fumigatus atmA (Ataxia-telangiectasia mutated, ATM) and atrA kinases, and how they impact virulence and the evolution of azole resistance. We demonstrated that A. fumigatus atmA and atrA null mutants are haploid and have a discrete chromosomal polymorphism. The ΔatmA and ΔatrA strains are sensitive to several DNA-damaging agents, but surprisingly both strains were more resistant than the wild-type strain to paraquat, menadione, and hydrogen peroxide. The atmA and atrA genes showed synthetic lethality emphasizing the cooperation between both enzymes and their consequent redundancy. The lack of atmA and atrA does not cause any significant virulence reduction in A. fumigatus in a neutropenic murine model of invasive pulmonary aspergillosis and in the invertebrate alternative model Galleria mellonella. Wild-type, ΔatmA, and ΔatrA populations that were previously transferred 10 times in minimal medium (MM) in the absence of voriconazole have not shown any significant changes in drug resistance acquisition. In contrast, ΔatmA and ΔatrA populations that similarly evolved in the presence of a subinhibitory concentration of voriconazole showed an ~5-10-fold increase when compared to the original minimal inhibitory concentration (MIC) values. There are discrete alterations in the voriconazole target Cyp51A/Erg11A or cyp51A/erg11A and/or Cdr1B efflux transporter overexpression that do not seem to be the main mechanisms to explain voriconazole resistance in these evolved populations. Taken together, these results suggest that genetic instability caused by ΔatmA and ΔatrA mutations can confer an adaptive advantage, mainly in the intensity of voriconazole resistance acquisition. Genetic stability is extremely important for the survival of every living organism, and a very complex set of genes has evolved to cope with DNA damage upon DNA damage. These genes encode proteins that sense DNA damage, transduce the signal, and repair the DNA damage. In eukaryotes, the most important DNA damage signal transducers are the AtmA and Rad53-related (ATR) protein kinases, and two other downstream kinases: checkpoint kinases 1 and 2 (Chk1 and Chk2) (Awasthi et al. 2015; Yazinski and Zou 2016). Mammalian ATM and ATR are paralogous phosphatidylinositol-3-kinase (PI-3 kinase)-related protein kinases (PIKK) that lack lipid kinase activity, but phosphorylate substrate proteins on Ser or Thr residues that are followed by Gln (SQ or TQ motifs) (Shiloh 2001, 2003; Bakkenist and Kastan 2004; McKinnon 2004). These kinases possess both overlapping and distinct roles in the regulation of this response, and phosphorylate multiple targets that act collectively to maintain the genome integrity. Upon their activation, both ATM and ATR upregulate cell cycle checkpoint

**KEYWORDS**

Aspergillus fumigatus

ATM

ATR

azole

DNA damage

Galleria mellonella

genetic instability

PFGE

virulence

voriconazole
strains harboring the association of a tandem repeat sequence and punctual mutation of the Cyp51A gene (TR34/L98H and TR46/ Y121F/T289A) have become widely disseminated all over the world (Hagiwara et al. 2016). Nonetheless, none of these studies have addressed the impact of genomic stability or DNA repair on A. fumigatus azole-resistance and virulence. Here, considering the importance of ATM and ATR in genomic stability and the activation of DNA repair mechanisms, we decided to investigate if ATM and ATR homologs (named AtmA and AtraA) are important for virulence and the evolution of drug resistance in A. fumigatus. Our results indicate that AtmA and AtraA collaborate for A. fumigatus genomic stability. However, A. fumigatus mutants (ΔatmA and ΔatraA) are able to keep their virulence, accompanied by the evolution of voriconazole resistance to higher levels than in the wild-type strain. Surprisingly, Cyp51A mutations or overexpression of cyp51A and/or Cdr1B efflux transporter overexpression are not involved in most of the voriconazole-resistant strains, suggesting that additional mechanisms underlying the evolution of drug resistance prevail in these genetically unstable strains.

MATERIALS AND METHODS

Ethics statement

The principles that guide our studies are based on the Declaration of Animal Rights ratified by the UNESCO in January 27, 1978 in its 8th and 14th articles. All protocols adopted in this study were approved by the local ethics committee for animal experiments from the University of São Paulo, Campus of Ribeirao Preto (Permit Number: 08.1.1277.53.6). Studies on the interaction of A. fumigatus with animals. Groups of five animals were housed in individually ventilated cages, and were cared for in strict accordance with the principles outlined by the Brazilian College of Animal Experimentation and the Guiding Principles for Research Involving Animals and Human Beings, American Physiological Society. All efforts were made to minimize suffering. Animals were clinically monitored at least twice daily and humanely killed if moribund (defined by lethargy, dyspnea, hypothermia, and weight loss). All stressed animals were killed by cervical dislocation.

Strains, media, and growth conditions

The A. fumigatus parental strain used in this study was AF293 (pyrG-). A list of the strains used in this study is described in Supplemental Material, Table S1. All the constructed mutants were grown at 37°C in either MM [1% glucose, 50 ml of a 20x salt solution (120 g/L NaNO₃, 10.4 g/L KCl, 30 g/L KH₂PO₄, and 10.4 g/L MgSO₄), and 1 mL/L of trace elements, pH 6.5] or complete medium [YG: 2% glucose, 0.5% yeast extract, and 1 mL/L of trace elements (22.0 g/L ZnSO₄, 11 g/L boric acid, 5 g/L MnCl₂, 5 g/L FeSO₄, 1.6 g/L CoCl₂, 1.6 g/L CuSO₄, 1.1 g/L (NH₄)₂MoO₄, and 50 g/L ethylendiaminetetraacetic acid)]. The media were made with or without 2% agar in order to obtain the solid or liquid medium, respectively. Additionally, uridine and uracil (1.2 g/L each) were added as a nutritional supplement when necessary, originating YUU (YG + UU) and MM + UU media, respectively. Expression of the atmA gene under the control of the niaA promoter was regulated by nitrogen source: promoter repression was achieved in modified minimal medium (AMM) (1% w/v glucose and 2% w/v agar) supplemented with ammonium tartrate, while induction was achieved in AMM plus sodium nitrate, according to the method of Punt et al. (1991).

Identification of the A. fumigatus AtmA and AtraA, and construction of null mutants

The A. fumigatus AtmA and AtraA orthologs were identified through a BLAST search of the A. fumigatus database using the A. nidulans AtmA
and AtrA sequences as queries. Single putative orthologs were identified for each gene: Afu5g12660 (AtmA) and Afu4g04760 (AtrA). AtmA and AtrA protein organization was analyzed by using the SMART interface (http://smart.embl-heidelberg.de/). DNA manipulations and standard genetic approaches were performed according to Sambrook and Russell (2001). The gene replacement cassette was constructed by in vivo recombination using Saccharomyces cerevisiae as a tool (Colot et al. 2006) and the target genes were replaced by the prototrophic marker gene pyrG. Briefly, ~1.0 kb regions on either side of the atmA and atrA genes were PCR-amplified from the gDNA of the Af293 strain using specific primers: atmA (primers P1 and P2) and atrA (primers P5 and P6). Similarly, the 3′-UTR region of each gene was amplified using primers P3 and P4 (for atmA) and primers P7 and P8 (for atrA). The auxotrophic marker pyrG was amplified from pCD2A plasmid (primers P9 and P10) (Table S2). The individual DNA fragments (5′- and 3′-UTRs plus pyrG) were cotransformed with the BamHI-EcoRI-cut pRS426 plasmid into S. cerevisiae SC9721 strain using the lithium acetate method (Schiestl and Gietz 1989). The recombinant yeast candidates were selected in solid YNB-URA medium (7 g/L yeast nitrogen base without amino acids, 0.05 g/L histidine, 0.1 g/L lysine, 0.1 g/L leucine, 0.1 g/L tryptophan, and 2% agar). gDNA of the yeast candidates was extracted, and the deletion cassettes were PCR-amplified using specific primers (P1 and P4 for atmA and P5 and P8 for atrA, Table S2) and transformed into the A. fumigatus Af293 pyrG− background strain (Osmani et al. 1987). The PCR amplifications were performed using Phusion High-Fidelity DNA polymerase (New England Biolabs) or TaKaRa Ex Taq DNA Polymerase (Clontech). Positive A. fumigatus candidates were purified in a selective medium without uridine/uracil, and the gDNA was extracted (Goldman et al. 1995) and checked by southern blot using the AlkPhos DirectLabeling and Detection System (GE Healthcare Life Sciences), according to the manufacturer’s protocol. Two independent transformants for each gene deletion mutant were selected for further analysis. To construct the conditional double mutant ΔatmA ΔniiA::atmA, the ΔatmA strain was treated with 5-fluoroorotic acid, aiming to get a ΔatmA pyrG− strain. Then, the ΔatmA pyrG− strain was transformed with the niiA::atmA cassette (pyrG+). Briefly, the 5′-UTR fragment and the atmA gene were PCR-amplified from Af293 gDNA using the primers P1/P11 and P12/P13, respectively. The pyrG::niiA fragment was PCR-amplified from the niiA::ypkA strain (Colabardini et al. 2013) using the specific primers P18/P19. The replacement cassette was also constructed in S. cerevisiae (exactly as described above) and the A. fumigatus transformation was processed by using ΔatmA pyrG− as a background strain. The conditional double mutant was selected in presence of AMM + 0.2 M NaN3 and checked in the presence of AMM + 0.2 M (induces the promoter) or AMM + 0.2 or 0.4 M NH4 tartrate (represses the promoter). Once more, two independent transformants were selected for further analysis. gDNA extractions from A. fumigatus and S. cerevisiae were performed according to the methods of Goldman et al. (1995) and Schiestl and Gietz (1989), respectively.

Measurements of DNA content per cell

Conidia were collected, centrifuged (and 13,000 rpm for 3 min), and washed with sterile 1× phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4 per liter of sterilized water). For cell staining, the protocol described by Almeida et al. (2007) was followed with modifications. Overnight fixation with 7% ethanol (v/v) at 4°C was carried out. Following fixation, conidia were harvested, washed, and suspended in 850 μL of sodium citrate buffer (50 mM sodium citrate, pH = 7.5). Briefly, sonicated conidia were treated for 1 hr at 50°C with RNase A (0.50 mg/ml; Invitrogen, Waltham, MA) and 2 hr at 50°C with protease K (1 mg/ml; Sigma-Aldrich, St. Louis, MO). Conidia were stained overnight with SYBR Green 10,000× (Invitrogen, Carlsbad, CA) diluted 10-fold in Tris-EDTA (pH 8.0), at a concentration of 2% (v/v) at 4°C. Finally, Triton X-100 (Sigma-Aldrich) was added to samples at a final concentration of 0.25% (v/v). Stained conidia were analyzed in a Fluorescence-activated cell sorting (FACS) LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with a 488 nm excitation laser. Signals from a minimum of 30,000 cells per sample were captured in the FITC channel (530 ± 30 nm) at a low flow rate of ~1000 cells/sec, and an acquisition protocol was defined to measure forward scatter and side scatter on a four-decade logarithmic scale and green fluorescence (FITC) on a linear scale. FACS Diva was used as the acquisition software. Results were analyzed with FlowJo (Tree Star) software, version 10, and with ModFit LT Software (Verity Software House, Topsham, ME).

Pulsed-field gel electrophoresis (PFGE), running conditions, and membrane transference

The agarose blocks containing chromosomal DNA from A. fumigatus isolates (wild strain and mutant strains) were submitted to. The Gene Navigator System (Pharmacia) was used under the conditions described by Sasaki et al. (2014) with minor modifications. The gels were prepared with Seakan agarose (FMC-Bioproducts), 1.1% in 1× TAE (Tris-acetate-EDTA) according to the electrophoretic run protocol, with a duration of 168 hr with constant voltage of 42 V at 10°C. The best electrophoretic resolution was achieved with homogeneous pulses of 900 sec for 24 hr, 1800 sec for 24 hr, 2700 sec for 48 hr, 3600 sec for 48 hr, and 4500 sec for 24 hr, and were applied with interpolation. Schizosaccharomyces pombe and S. cerevisiae chromosomal DNA was used as the molecular size standard in each of the electrophoretic runs. After this process, the gel was incubated in ethidium bromide solution (0.5 μg/ml) for 30 min and photographed under ultraviolet (UV) light. The chromosomal bands were transferred to N+ nylon membranes (Amersham) using the VacuGene XL system (Pharmacia). Gels were deposited on the membrane and treated with three different solutions for 40 min each: solution 1 (depurination) containing 0.25 M HCl; solution 2 (denaturation) composed of 0.5 M NaOH and 1 M NaCl; and solution 3 (neutralization) containing Tris-base 1 M and 0.5 M NaCl pH 8.0. The gel was treated with 20× SSC (175.3 g/L NaCl and 88.2 g/L sodium citrate, pH 7.0) over 3 hr. The membranes were then irradiated with ultraviolet light (150 ml) for DNA fixation using the GS Gene Linker UV Chamber (Bio-Rad) and stored at −20°C.

Phenotypic characterization of the ΔatmA and ΔatrA null mutants

A total of 105 conidia of each strain tested were grown in solid MM in the presence or absence of camptothecin (CPT: 10, 25, and 50 μM), bleomycin (BLEO: 1, 2, and 4 μg/ml), methyl methanesulfonate (MMS: 0.001, 0.005, and 0.02%), 4- nitroquinoline-1-oxide (4-NQO: 0.01, 0.1, and 1 μg/ml), menadione (0.015 and 0.03 mM), and paraquat (0.25 mM). The plates were incubated for 120 hr at 37°C and the radial growth was measured. The radial growth of each strain in the presence of drugs was normalized by the growth of each strain in MM without any drug. All plates were grown in triplicate and the average ± SD is plotted. Alternatively, 105 conidia of each strain were inoculated in 500 μl of liquid MM medium supplemented or not with 1.5, 2, 2.5, 3, and 4 mM of H2O2.

UV light viability assays

In order to determine the viability of the nondividing cells, a total of 100 fresh conidia for each strain were plated on the surface of solid YG.
using a spreader. Subsequently, the plates were irradiated with UV light using a UV Stratalinker 1800 (Stratagene, La Jolla, CA) and incubated at 37° for 48 hr to determine the UV sensitivity of G1 quiescent conidia (Bergen and Morris 1983). Additionally, to determine the UV survival of already dividing cells, 100 conidia for each strain were also spread on solid YG plates, but incubated for 4.5 hr at 37° before UV exposure (Malavazi et al. 2008). Cell viability was measured by determining the percentage of colonies on treated plates compared to the untreated controls. These experiments were done in triplicate for each strain and averages ± SD are shown.

Mitosis assay
A total of 1 × 10^5 conidia from wild-type, ΔatmA, and ΔatrA strains were inoculated in a 30 mm petri dish containing 3 ml of liquid YG supplemented with 100 mM of hydroxyurea (HU) (37°). After 5 hr of incubation, the germlings were released from cell cycle arrest by four washes with YG medium, and then incubated in fresh YG (without HU) at 37° for the indicated periods of time. For nuclei visualization, cells were treated for 5 min with 20 μg/ml of Hoechst 33342 (Invitrogen). After staining, coverslips were washed twice with PBS and analyzed on an Observer Z1 fluorescence microscope using a 100× objective oil immersion lens.

RNA extraction and qPCR reactions
A total of 1 × 10^7 conidia of the indicated A. fumigatus strains were inoculated in 50 ml of liquid MM and incubated in a reciprocal shaker at 37°, 180 rpm. After 16 hr of growth, the voriconazole was added to a final concentration of 0.35 μg/ml and incubated for an additional 4 hr. Then, mycelia were harvested by filtration, washed twice with sterile H2O, and immediately frozen in liquid nitrogen. For total RNA isolation, mycelia were ground in liquid nitrogen and the total RNA was extracted using Trizol (Invitrogen). The RNA integrity was analyzed using an Agilent 2100 Bioanalyzer system. For real-time PCR experiments, RNase-free DNase I treatment was carried out as previously described by Semighini et al. (2011) and cDNA was generated using the SuperScript III First Strand Synthesis system (Invitrogen) with oligo(dT) primers, according to the manufacturer’s instructions. All of the qPCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems). Primers P14 and P15 (Table S2) were used to evaluate the mRNA accumulation of the cyp51A/erg11A gene.

Virulence analysis of the ΔatmA and ΔatrA mutants
In the murine model of pulmonary aspergillosis, lung histopathology, and fungal burden, outbred female mice (BALB/c strain; body weight, 20–22 g) were housed in vented cages containing five animals. Mice were immunosuppressed and infected with 1 × 10^5 conidia contained in 20 μl of suspension, according to a previously described protocol (Dinamarco et al. 2012). The statistical significance of the comparative survival values was calculated using Mantel–Cox and Gehan–Breslow–Wilcoxon log rank analysis, and the Prism statistical analysis package.

To investigate fungal burden and the histopathological pattern in the murine lungs after infection, mice were similarly immunosuppressed with cyclophosphamide but infected with 1 × 10^6 conidia/20 μl of suspension. All the animals were killed 72 hr postinfection and the lungs were harvested. One of the lungs from each animal was immediately frozen in liquid nitrogen and used for fungal burden detection, while the other lung was immediately fixed in 10% formaldehyde solution and subjected to histopathology processing.

Larvae of Galleria mellonella were obtained by breeding adult moths (Fuchs et al. 2010) weighing 275–330 mg, kept under starvation in petri dishes at 37° in darkness for 24 hr prior to infection. All the selected larvae were in the final (sixth) instar larval stage of development. Fresh conidia from each A. fumigatus strain were harvested from YAG plates in PBS solution and filtered through a Miracloth (Calbiochem). For each different strain, the spores were counted using a hemocytometer and the stock suspension was done at 2 × 10^9 conidia/ml. The viability of the administered inoculum was determined by plating a serial dilution of the conidia on YAG medium at 37°. A total of 5 μl (1 × 10^6 conidia/larva) from each stock suspension was inoculated per larva. The control group was composed of larvae inoculated with 5 μl of PBS to observe the killing due to physical trauma. The inoculum was performed by using Hamilton syringe (7000.5KH) via the last left proleg. After infection, the larvae were maintained in petri dishes at 37° in the dark and were scored daily. Larvae were considered dead by presenting the absence of movement in response to touch.

Fungal burden
For DNA extraction, lungs were ground in liquid nitrogen, homogenized by vortexing with glass beads for 10 min, and DNA was extracted via the phenol–chloroform method. DNA quantity and quality was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). At least 500 μg of total DNA from each sample was used for qPCR. A primer and a Lux probe (Invitrogen) was used to amplify the 18S rRNA region of A. fumigatus (primer, 5’-CTTAAAAATGCGGCGTGTTTGTCTATTGGG-3’; probe, 5’-CATCACAGACCTGTGATTGTGGCG-3’) and an intronic region of mouse glyceraldehyde-3-phosphate dehydrogenase (primer, 5’-CGAGGGACTGGAGCCAGACAG-3’; probe, 5’-GGGCCTGCTAAAGGTCAGCG-3’). Six-point standard curves were calculated using serial dilutions of gDNA from all the A. fumigatus strains used and the uninfected mouse lung. Fungal and mouse DNA quantities were obtained from the threshold cycle (CT) values from an appropriate standard curve. Fungal burden was determined as the ratio between picograms of fungal and micrograms of mouse DNA.
Histopathology

After they had been fixed in 10% formaldehyde solution for the histopathology methodology, the lungs were diaphanized and embedded in paraffin, and then sliced in serial sections of ~5 µm thickness. The staining was done with hematoxylin and eosin or GMS (Sigma-Aldrich GMS Kit). The slides were analyzed and the images were recorded using the microscope (Leica DM6000 B).

Maintenance of genetic stability in atmA and atrA after sequential mitotic divisions

For the sequential mitotic divisions in solid MM without stress, the wild-type and null mutants ΔatmA and ΔatrA were divided into four independent populations, each originating a total of 20 different populations: WT0, WT1, WT2, WT3, ΔAtmA-1, ΔAtmA-2, ΔAtmA-3, ΔAtmA-2-0, ΔAtmA-1-2, ΔAtmA-2-1, ΔAtmA-2-3, ΔAtmV1, ΔAtmV2, ΔAtmV3, ΔAtmV1-0, ΔAtmV2-0, ΔAtmV3-0, ΔAtmV1-2, ΔAtmV2-1, ΔAtmV2-2, and ΔAtmV2-3. Then, a total of $1 \times 10^8$ spores from each strain listed above (except the parents: WT0, ΔAtmA-1, ΔAtmA-2, ΔAtmA-3, ΔAtmA-2-0, ΔAtmA-1-2, ΔAtmA-2-1, ΔAtmA-2-3, ΔAtmV1, ΔAtmV2, ΔAtmV3, ΔAtmV1-0, ΔAtmV2-0, ΔAtmV3-0, ΔAtmV1-2, ΔAtmV2-1, ΔAtmV2-2, and ΔAtmV2-3) were inoculated in solid MM plus a subinhibitory concentration of voriconazole (0.2 µg/ml) and incubated for 5 d at 37°C. Then, a total of $1 \times 10^8$ spores from each strain listed above (except the parents: WT0, ΔAtmA-1, ΔAtmA-2, ΔAtmA-3, ΔAtmA-2-0, ΔAtmA-1-2, ΔAtmA-2-1, ΔAtmA-2-3, ΔAtmV1, ΔAtmV2, ΔAtmV3, ΔAtmV1-0, ΔAtmV2-0, ΔAtmV3-0, ΔAtmV1-2, ΔAtmV2-1, ΔAtmV2-2, and ΔAtmV2-3) were inoculated in solid MM plus a subinhibitory concentration of voriconazole (0.2 µg/ml) and incubated for 5 d at 37°C. The procedure above was repeated five times for each strain, except the parents (WT0, ΔAtmA-1, ΔAtmA-2, ΔAtmA-3, ΔAtmA-2-0, ΔAtmA-1-2, ΔAtmA-2-1, ΔAtmA-2-3, ΔAtmV1, ΔAtmV2, ΔAtmV3, ΔAtmV1-0, ΔAtmV2-0, ΔAtmV3-0, ΔAtmV1-2, ΔAtmV2-1, ΔAtmV2-2, and ΔAtmV2-3). By the end, a total of 15 evolved populations (WT1, WT2, WT3, ΔAtmA-1, ΔAtmA-2, ΔAtmA-3, ΔAtmA-2-0, ΔAtmA-1-2, ΔAtmA-2-1, ΔAtmA-2-3, ΔAtmV1, ΔAtmV2, ΔAtmV3, ΔAtmV1-0, ΔAtmV2-0, ΔAtmV3-0, ΔAtmV1-2, ΔAtmV2-1, ΔAtmV2-2, and ΔAtmV2-3) were obtained and used for further assays.

For the sequential mitotic divisions in solid MM in the presence of voriconazole, the wild-type and null mutants ΔatmA and ΔatrA were divided into four independent populations, each originating a total of 20 different populations: WT0, WT1, WT2, WT3, ΔAtmA-1, ΔAtmA-2, ΔAtmA-3, ΔAtmA-2-0, ΔAtmA-1-2, ΔAtmA-2-1, ΔAtmA-2-3, ΔAtmV1, ΔAtmV2, ΔAtmV3, ΔAtmV1-0, ΔAtmV2-0, ΔAtmV3-0, ΔAtmV1-2, ΔAtmV2-1, ΔAtmV2-2, and ΔAtmV2-3. Then, a total of $1 \times 10^8$ spores from each strain listed above (except the parents: WT0, ΔAtmA-1, ΔAtmA-2, ΔAtmA-3, ΔAtmA-2-0, ΔAtmA-1-2, ΔAtmA-2-1, ΔAtmA-2-3, ΔAtmV1, ΔAtmV2, ΔAtmV3, ΔAtmV1-0, ΔAtmV2-0, ΔAtmV3-0, ΔAtmV1-2, ΔAtmV2-1, ΔAtmV2-2, and ΔAtmV2-3) were inoculated in solid MM plus a subinhibitory concentration of voriconazole (0.2 µg/ml) and incubated for 5 d at 37°C. The procedures above were repeated 10 times for each strain. By the end of the process, a total of 15 evolved populations (WT1, WT2, WT3, ΔAtmA-1, ΔAtmA-2, ΔAtmA-3, ΔAtmA-2-0, ΔAtmA-1-2, ΔAtmA-2-1, ΔAtmA-2-3, ΔAtmV1, ΔAtmV2, ΔAtmV3, ΔAtmV1-0, ΔAtmV2-0, ΔAtmV3-0, ΔAtmV1-2, ΔAtmV2-1, ΔAtmV2-2, and ΔAtmV2-3) were obtained and used for further assays.

Aspergillus fumigatus ATM and ATR
DAtmA2V1, DAtmA2V2, and DAtmA2V3) were obtained and used for further assays.

**MIC**

The susceptibility of the indicated strains was assessed by using antifungal MIC or minimal effective concentration (MEC) methods (Clinical and Laboratory Standards Institute 2008). The experiments were done in microtiter plates, which were filled up with 200 µl of liquid MM and inoculated with a total of $1 \times 10^4$ conidia/well. Plates were incubated at 37°C for 48 hr. MICs of the assayed drugs were determined visually as a no-growth endpoint at 48 hr of incubation. MECs gave the lowest caspofungin concentration that led to the growth of small, rounded, compact microcolonies compared to the growth control (caspofungin-free MM) after 48 hr incubation. The different drugs were diluted with the following range: (i) caspofungin (0–2 µg/ml), (ii) voriconazole (0–2 µg/ml), and posaconazole (0–2 µg/ml). Three repetitions were performed for each treatment.
Data availability

Strains are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

Molecular and functional characterization of A. fumigatus ATM and ATR orthologs

A. nidulans AtmA and AtrA orthologs were used as queries to identify the A. fumigatus AtmA and AtrA orthologs: Afu5g12660 (e-value: 0.0; identity: 65.7%; and similarity: 78.6%) and Afu4g04760 (e-value: 0.0; identity: 64.4%; and similarity: 78.3%), respectively. A. fumigatus atmA and atrA encode putative proteins with 2895 and 2451 amino acids and molecular weights of 325.7 and 276.1 kDa, respectively. The organization of protein domains analyzed by using the SMART interface (http://smart.embl-heidelberg.de/) showed that both A. nidulans and A. fumigatus structure and organization are very conserved (Figure S1). The AtmA has the following domains: a TAN domain (telomere-length maintenance and DNA damage repair; 3.1e-63; SM0001342) from amino acids 1–153, a FAT domain (present in the PIK-related kinases; e-value: 4.3e-6; PF2259) from amino acids 1969–2884, a P13Kc domain (phosphoinositide 3-kinase, catalytic domain; e-value: 2.09e-81; SM000146, from amino acids 2567–2884, and a FATC domain (possibly playing a role in redox-dependent structural and cellular stability; e-value: 1.68–9; SM001343). AtrA has the following domains: a UME domain (found in nucleolar proteins; e-value: 2.43e-51; SM0008092) from amino acids 905–1011, a FAT domain (e-value: 2.9–44) from amino acids 1570–1900, a P13Kc domain (e-value: 1.35–93) from amino acids 2143–2440, and a FATC domain (e-value: 7.14–13) from amino acids 2419–2451.

In order to further investigate the role of AtmA and AtrA in A. fumigatus, Afu5g12660 and Afu4g04760 were targeted for entire gene deletion (Figure S2). Possibly due to the large size of the genes (plus the flanking regions), we were not able to complement the deletion strains with the corresponding wild-type genes. Thus, to discard the possibility of the occurrence of likely secondary mutations during the construction of deletion strains, we selected two independent transformants from each deletion experiment to pursue all our phenotypic analyses. First, we investigated whether atmA and atrA null mutations have caused any effect on ploidy and chromosome arrangement (Figure 1 and Figure 2). FACS was used to verify whether wild-type and mutant conidia have different ploidy (Figure 1). Haploid and diploid strains of S. cerevisiae and A. nidulans were used as controls of known cellular DNA content (Figure 1). The Af293 wild-type parental, ΔatmA1 and Δ-a2, and ΔatrA1 and Δ-a2 mutant strains showed a DNA content consistent with a haploid distribution, suggesting that atmA and atrA null mutations have not affected the ploidy (Figure 1).

The haploid genome of A. fumigatus has 29,388,377 bp organized into eight chromosomes (www.aspgd.org). The molecular karyotype of A. fumigatus wild-type and atmA and atrA mutants was defined by PFGE. It comprises five chromosomal bands, whose sizes are slightly different between the wild-type and mutants (Figure 2). The wild-type karyotype has three chromosomal bands of ~ 5.7, 4.9, and 3.9 Mb, and two smaller bands of 2.2 and 1.8 Mb (Figure 2). The mutants differ from the wild-type in the length of most of the chromosomal bands. They have four bands, the first ones with the same size (3.7 Mb) of the wild-type, and three others slightly shorter (4.6, 4.1, and 3.6 Mb) than the wild-type. The 4.1 Mb band could have arisen by a deletion event occurring in the 4.9 Mb band of the wild-type. In the mutants, the 2.2 Mb and 1.8 Mb bands have been replaced by a single band of 2.0 Mb, suggesting the occurrence of deletion and segmental duplication events in the 2.2 and 1.8 Mb bands, respectively. Finally, the shorter chromosomal band (~1.0 Mb) seen in the wild-type and mutants could correspond to minichromosomes, which have been described in strains and progeny of crosses of several fungal species (Zolan 1995).

The fluorescence intensity varied between the bands, indicating that comigrating chromosomes are not necessarily homologous. Based on the chromosome length we tentatively assigned the in silico chromosomes (Chr) to the chromosomal bands of the wild-type: Chr1 (4.92 Mb) to the first band (5.7 Mb), Chr2 (4.84 Mb) to the second band (4.9 Mb), Chr3, Chr4, Chr5, and Chr6 (4.08, 3.92, 3.95 and 3.78 Mb) to the third band (3.9 Mb), Chr7 (2.06 Mb) to the fourth band (2.2 Mb), and Chr8 (1.83 Mb) to fifth band (1.8 Mb). Similarly, Chr3s were assigned to the chromosomal bands of atmA and atrA mutants: Chr1 and Chr2 to the bands of 5.7 and 4.6 Mb, respectively; Chr3, Chr4, and Chr5 to the 4.1 Mb band; Chr6 to the 3.6 Mb band; and Chr7 and Chr8 to a single band of 2.0 Mb. In agreement with the FACS analysis, these results strongly suggest that chromosomal rearrangements found in null mutants did not affect the ploidy.

We tested the influence of several DNA-damaging and oxidative stress agents on the growth of the wild-type and mutant strains (Figure 3 and Figure S3). The ΔatmA and ΔatrA mutants showed different resistance profiles against DNA-damaging agents. In vitro susceptibility testing showed that atrA mutants were 4-NQO and MMS susceptible, and that the growth was completely inhibited at concentrations of 1.0 µg/ml and 0.2% of 4-NQO and MMS, respectively (Figure 3). Interestingly, atrA mutants showed intermediate susceptibility to CPT. For atrA mutants, in vitro resistance against 4-NQO and MMS was found to be within the same range as that for the wild-type. The ΔatmA strains were about twofold more resistant than the wild-type to the quasi-mimetic UV light agent 4-NQO, while the ΔatrA strains (Figure 3A) were as sensitive as the wild-type (Figure 3, A and B). Both the ΔatmA and ΔatrA strains were more sensitive than the wild-type to CPT (Figure 3, E and F). The ΔatrA strains were more sensitive to BLEO than the wild-type strain (Figure 3G). Both the ΔatmA and ΔatrA strains were more sensitive to UV light (Figure 3H). Both
**ΔatmA and ΔatrA** were more resistant than the wild-type strain to paraquat, menadione, and hydrogen peroxide (Figure S2, A–C).

Upon DNA damage, the cell cycle is inhibited through the activation of cell cycle checkpoints. HU is an inhibitor of ribonucleotide diphosphate reductase, the rate-limiting enzyme in deoxyribonucleotide (dNTP) biosynthesis. The depletion of dNTPs activates the DNA replication checkpoint, slowing progression through S phase; in addition, the initiation of DNA replication in the presence of high levels of HU causes DSBs (Malavazi et al. 2008). Since HU is an effective inhibitor of DNA synthesis in *A. nidulans* (Bergen and Morris 1983), we investigated the ability of ΔatmA and ΔatrA to replicate DNA during a transient period of growth in the presence of HU. Previously, we have developed a mitosis assay (“DNA replication checkpoint response”; Fagundes et al. 2004) in which mitosis is monitored in the presence of HU for 5–7 hr. The number of nuclei is assessed by Hoescht staining, and if germings have two or more nuclei after HU incubation, they are scored as defective in mitotic arrest (Figure 4). The ΔatrA strains had an intact replication checkpoint while the ΔatmA strains had defects in the intra-S-phase checkpoint (Figure 4).

---

**Figure 5** There is synthetic lethality between *A. fumigatus* atmA and atrA. *A. fumigatus* conidia (10^5) were inoculated on minimal media (MM) + NaNO₃ 0.2 M or MM + 0.2 or 0.4 M NH₄ tartrate in the absence (A) and presence (B) of menadione 0.015 or 0.3 mM. Plates were incubated for 5 d at 37°C. The results were expressed as the average of radial growth of MM + NH₄ tartrate divided by MM + NaNO₃ of three independent experiments ± SD (*P < 0.001, as determined by Student’s t-tests when compared to the control).

---

**Figure 6** *A. fumigatus* ΔatmA and ΔatrA are still virulent. Comparative analysis of wild-type and mutant 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 conidia at a dose of 10^5 conidia. (A) Percent survival of ΔatmA1 and ΔatmA2 mutants compared to the wild-type strain. (B) Percent survival of ΔatrA1 and ΔatrA2 mutants compared to the wild-type strain. (C) Fungal burden quantification, determined by quantitative polymerase chain reaction from lungs obtained 72 hr postinfection. Results are expressed based on quantification of the 18S rRNA gene of *A. fumigatus* divided by the quantification of an intronic region of the mouse GAPDH gene. PBS, phosphate-buffered saline.
We have previously demonstrated that *A. nidulans* AtmA and Uvs-B<sup>ATR</sup> show synthetic lethality (Malavazi et al. 2008). To verify whether there is a genetic interaction between *A. fumigatus* AtmA and AtrA, we further constructed two independent transformants for the conditional double mutant ΔatrA1 niiA::atmA. In this strain, atmA expression is under the control of the nitrite reductase genes promoter (niiA), which is induced by sodium nitrate and repressed by ammonium tartrate (Punt et al. 1991). Indeed, atmA repression, along with ΔatrA deletion, led to an ~50% reduction in *A. fumigatus* growth (Figure 5), indicating that a combination of AtmA and AtrA collaborate for fungal growth and that the double mutant may not be viable. Synthetic lethality was further confirmed by growth on the oxidative stressing agent menadione (Figure 5B). Both ΔatmA and ΔatrA are more resistant to menadione than the wild-type, while the two independent ΔatrA1 niiA::atmA candidates showed increased susceptibility to menadione (Figure 5B and Figure S3).

Taken together, these results suggest that AtmA and AtrA have redundant and separate functions during the DNA damage response in *A. fumigatus*. Furthermore, AtmA is important for the DNA replication checkpoint response, while the lack of one of these genes activates a strong response to agents that induce oxidative stress. We show that atmA is synthetic lethal with atrA, emphasizing the cooperation between both enzymes and their consequent redundancy.

**In vivo analysis of the influence of *A. fumigatus* ΔatmA and ΔatrA mutants on virulence**

In the neutropenic murine model of invasive pulmonary aspergillosis, infection by the wild-type and mutants (ΔatmA1, ΔatmA2, ΔatrA1, and
ΔatrA2) resulted in 50–80% mortality 15 d postinfection, respectively (Figure 6, A and B). Although ΔatrA1 and ΔatrA2 virulence seem to be attenuated when compared to the wild-type, all isolates did not show a statistically significant difference according to the Mantel–Cox and Gehan–Brestow–Wilcoxon tests (Figure 6, A and B). Fungal burden confirmed these results (Figure 6C). Histopathological examination revealed that, at 72 hr postinfection, the lungs of mice infiltrated with PBS showed no signal of inflammation or pathogenesis, while mice infected with the wild-type strain contained multiple foci of invasive hyphal growth, which penetrated the pulmonary epithelium in major airways, while pockets of branched invading hypha originated from the alveoli (Figure 7A). Comparable phenotypes were observed for ΔatmA1 and ΔatmA2 strains (Figure 7B), and ΔatrA1 and ΔatrA2 strains (Figure 7C). These data strongly indicate that, when compared to the wild-type strain, the lack of atmA and atrA did not cause any significant virulence reduction in A. fumigatus.

In another set of experiments, we use the moth G. mellonella as an alternative animal model to compare the virulence between the mutants and wild-type. We verified if sequential mitotic divisions of the wild-type and ΔatmA and ΔatrA mutant strains without selective pressure could have an impact on virulence. First, we established three independent populations for each strain (WT0, WT1, WT2, WT3, ΔATM1-0, ΔATM1-1, ΔATM1-2, ΔATM1-3, ΔATM2-1, ΔATM2-2, ΔATM2-3, ΔATR1-0, ΔATR1-1, ΔATR1-2, ΔATR1-3, ΔATR2-0, ΔATR2-1, ΔATR2-2, and ΔATR2-3). Then, we transferred these populations through 10 conidia passages from each strain, except the parental ones (WT0, ΔATM1-0, ΔATM2-0, ΔATR1-0, and ΔATR2-0), on MM plates and grew them at 37°C without any selective pressure (see Figure 9A, left panel). These evolved strains showed the same phenotypes related to growth, conidiation, and sensitivity to DNA damage agents as the original parental strains (data not shown).

Conidia of the original populations and the last-transferred populations had their virulence compared by inoculating them in G. mellonella larvae. In the G. mellonella model, infection of all the strains resulted in 100% mortality 72–100 hr postinfection (Figure 8). There was no statistical difference between all the strains (Mantel–Cox and Gehan–Brestow–Wilcoxon, P values > 0.05). These results strongly indicate that, in the presence of genetic instability caused by the absence of AtmA and AtrA, A. fumigatus can retain its virulence attributes in the G. mellonella model of infection.

The influence of A. fumigatus ΔatmA and ΔatrA mutants on azole resistance
To investigate if the absence of AtmA and AtrA could impact azole resistance, we determined that the MIC for voriconazole in the populations that were previously transferred 10 times in MM in the absence of voriconazole (Figure 9A). We did not observe significant changes in drug resistance acquisition for the last set of transferred wild-type populations; however, the last set of ΔatmA and ΔatrA populations
Table 1 The erg11A sequence modifications observed in selected A. fumigatus ΔATM1, ΔATM2, ΔATR1, and ΔATR2 voriconazole-evolved populations

| Nucleotide Exchange with Amino Acid Change | Nucleotide Exchange without Amino Acid Change |
|------------------------------------------|-----------------------------------------------|
| Wild-type                                | Wild-type                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| WTV1                                     | WTV1                                         |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| ∆ATMA1V0                                 | ∆ATMA1V0                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| ∆ATMA1VA                                 | ∆ATMA1VA                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| ∆ATMA1VB                                 | ∆ATMA1VB                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| ∆ATMA2V0                                 | ∆ATMA2V0                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| ∆ATMA2VC                                 | ∆ATMA2VC                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| ∆ATRA2V0                                 | ∆ATRA2V0                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| ∆ATRA2VA                                 | ∆ATRA2VA                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| ∆ATRA2VB                                 | ∆ATRA2VB                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| ∆ATRA2VC                                 | ∆ATRA2VC                                     |
| Amino acid position                      | Y46                                          |
| Nucleotide sequence                      | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |
| Y46                                       | V172                                         |
| 136 TAT138                               | T248                                         |
| Y46                                       | E255                                         |
| K427                                      | G89                                          |
| L358                                      | C454                                         |

ND, not different from the erg11A wild-type strain.

and gained and lost 25–50% drug resistance, suggesting that the genetic instability caused by the mutations in the absence of selection does not dramatically affect voriconazole resistance status.

We also evolved three independent populations of the wild-type, Δatma1, Δatma2, Δatra1, and Δatra2 strains on MM plates with a subinhibitory concentration of 0.2 μg/ml of voriconazole. Conidia of these three independent populations for each strain (WTV0, WTV1, WTV2, WTV3, ΔATMA1V0, ΔATMA1V1, ΔATMA1V2, ΔATMA1V3, ΔATMA2V0, ΔATMA2V1, ΔATMA2V2, ΔATMA2V3, ΔATRA1V0, ΔATRA1V1, ΔATRA1V2, ΔATRA1V3, ΔATRA2V0, ΔATRA2V1, ΔATRA2V2, and ΔATRA2V3) were transferred through five passages, except for the parental ones (WTV0, ΔATMAV0, and ΔATRAV0), on MM plates plus 0.2 μg/ml of voriconazole and grown at 37°C (Figure 9B, left panel). After five passages, the three populations of the wild-type strain evolved 3.3-fold greater resistance to voriconazole, while 50% of the Δatma and Δatra populations evolved much greater voriconazole resistance (~5–10-fold resistance, Figure 3B, right panel). The other 50% of the Δatma and Δatra populations evolved voriconazole resistance that was comparable (2.5-fold) or lower than the wild-type populations (for instance, see population ΔATMV2-2, Figure 9B). We also evaluated the occurrence of possible amino acid substitutions of Erg11A in selected mutants (Table 1). In comparison to the corresponding parental strains, the ΔATMA1VA, ΔATMA1VB, ΔATMA2VC, ΔATRA2VA, ΔATRA2VB, and ΔATRA2VC mutants showed a single Y46F amino acid substitution in Erg11A (Table 1A). There is increased erg11A mRNA accumulation (~five- to eightfold) when the WTV0 and selected mutant strains were grown in the presence of.
0.35 μg/ml voriconazole (Figure S4A). In the presence of voriconazole, the erg11A mRNA accumulation is reduced ~30% in the WTV1 and WTV2 mutants when compared to the wild-type strain (Figure S4A). In the absence of voriconazole, the erg11A mRNA accumulation is increased about threefold in ΔATM2V1 when compared to the corresponding parental strain (Figure S4B). Moreover, in the presence of voriconazole, ΔATM1V1, ΔATM2V2, and ΔATM2V1 strains have ~20–30% more erg11A mRNA accumulation than the corresponding parental strain; surprisingly, ΔATM2V1 that has increased mRNA accumulation in the absence of voriconazole showed about threefold less expression than the corresponding parental strain (Figure S4B). In the presence of voriconazole, the ΔATR2V1, ΔATR2V2, and ΔATR2V3 strains showed ~20% less erg11A mRNA accumulation than the corresponding parental strain and, interestingly, ΔATR2V0 showed ~25% more erg11A mRNA accumulation than ΔATR1V0 (Figure S4C). We also investigated if the ABC transporter Cdr1B (Hagiwara et al. 2016) was overexpressed in these strains by using qRT-PCR (Figure S5). As expected, most of the wild-type and mutant strains had increased cdr1B mRNA accumulation in the presence of voriconazole (Figure S5, A–C). However, some of the evolved strains showed decreased cdr1B and discretely increased mRNA accumulation (Figure S5, A–C). These evolved strains have shown the same phenotypes regarding growth, conidiation, and sensitivity to DNA damage agents as the original parental strains (data not shown). The WTV1, ΔATR2V1, and ΔATMA2V1 strains showed the same chromosomal patterns and ploidy of their parental strains (Figure 1 and Figure S6).
genetically interact because the conditional repression of atmA in the ΔatmA background showed synthetic lethality. *Neurospora crassa atmA* and *atra* are involved in the DNA damage response, normal growth, maintenance of chromosome integrity, and the regulation of different pathways; double conditional mutants for these two genes indicate that at least one of the pathways must be functional for survival (Wakabayashi et al., 2008, 2010). These results suggest that AtmA and AtrA have different, separate, and redundant functions in different fungal systems.

We have observed that ΔatmA and Δatra mutants are haploid and that there is limited chromosomal polymorphism in these mutants. We have not investigated the possible original events that were responsible for this chromosomal polymorphism; however, a striking observation is that these mechanisms are conserved in both ΔatmA and Δatra, since the observed chromosomal polymorphism is identical for all mutants, including those evolved under selective voriconazole pressure. Chromosome size differences between mutants ΔatmA and Δatra and the parental strain were relatively small (< 0.7 Mb), suggesting that chromosome rearrangements might be the result of DNA amplification/deletion events rather than large interchromosomal exchanges. These events could occur at the chromosomal termini in which telomeric and subtelomeric regions are hotspots for recombination events in several unicellular microorganisms. The occurrence of chromosomal breaks in the chromosome ends could be followed by telomerase-mediated healing, which may generate new short telomeres. It is possible that these dramatic rearrangements reflect a role for AtmA and AtrA in telomeric and subtelomeric maintenance. In mammalian cells, ATM and ATR play an important role in telomere shortening and stabilization (Awasthi et al. 2015; Yazinski and Zou 2016). The C. albicans *mecl* (Mec1p is the homolog of AtrA) mutant was also shown to exhibit an increase in genome instability in an assay for chromosome 1 integrity (Legrand et al. 2011).

*A. fumigatus* ΔatmA and Δatra are virulent in both a neutropeic murine model of invasive pulmonary aspergillosis and in *G. mellonella*. We performed sequential growth transfers of the wild-type and mutants, aiming to increase their mitotic instability and verifying if this could affect their virulence. However, we did not observe any virulence decrease in these derived mutant populations. Nevertheless, *C. albicans* mutants that affect homologous recombination or nonhomologous end-joining (NHEJ) repair of DNA DSBs, such as *LIG4* (which is the structural and functional homolog of both yeast and human ligase IV, involved in NHEJ) and *RAD52*, resulted in the attenuation of virulence in a murine model of candidiasis (Andaluz et al. 2001; Chauhan et al. 2005). These results emphasize once more that *A. fumigatus* AtmA and AtrA play redundant roles in DNA stability, growth, and the maintenance of virulence.

We also evaluated the influence of genetic stability on the evolution of voriconazole drug resistance. Upon selective pressure on voriconazole, selected ΔatmA and Δatra evolved strains have increased voriconazole resistance (~4–10-fold more resistant when compared to the wild-type strain). In some selected mutants, we have identified a novel mutation in the Erg11A (Y46F), and/or discrete accumulation of erg11 and/or cdxIB mRNAs upon growth in the presence of voriconazole, as possible causes for resistance. Our results suggest that other mechanisms different from target mutation or increased expression are probably influencing voriconazole resistance in these strains. Previously, we have observed that in vitro evolution of itraconazole resistance in *A. fumigatus* involves multiple mechanisms of resistance, such as the overexpression of drug efflux pumps (da Silva Ferreira et al. 2004). Furthermore, genome sequencing of these mutant strains and SNP comparisons with the parental strains are necessary to understand the mechanisms that are responsible for voriconazole resistance.

In summary, the *A. fumigatus* atmA and atra genes are important for genetic stability and showed strong genetic interaction, but individual mutations do not affect *A. fumigatus* virulence in spite of discrete chromosomal polymorphisms in these mutant strains. However, we have demonstrated that *A. fumigatus* AtmA and AtrA influence the evolution of azole resistance. Additional genomic sequencing studies are necessary to understand the complete array of changes involved in the evolution of voriconazole resistance in these strains.

**ACKNOWLEDGMENTS**

We are grateful for the comments and suggestions of William Nierman, Sinem Beyhan, Giulia Rancati, Norman Pavelka, and the anonymous reviewers. We thank the Fundação de Amparo a Pesquisa do Estado de São Paulo and the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil, for financial support. F.R. and B.H.F. were supported by the Northern Portugal Regional Operational Program (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (NORTE-01-0145-FEDER-000013).

**LITERATURE CITED**

Almeida, A. J., D. R. Matute, J. A. Carmona, M. Martins, I. Torres et al., 2007 Genome size and ploidy of *Paracoccidioides brasiliensis* reveals a haploid DNA content: flow cytometry and GP43 sequence analysis. *Fungal Genet. Biol.* 44:25–31.

Andaluz, E., R. Calderone, G. Reyes, and G. Larriba, 2001 Phenotypic analysis and virulence of *Candida albicans* LIG4 mutants. *Infec. Immun.* 69:137–147.

Awasthi, P., M. Foiani, and A. Kumar, 2015 ATM and ATR signaling at a glance. *J. Cell Sci.* 128:4255–4262.

Bakkenist, C. J., and M. B. Kastan, 2004 Initiating cellular stress responses. *Cell* 118:9–17.

Bennett, R. J., and B. G. Turgeon, 2016 Fungal sex: the Ascomycota. *Microbiol. Spectr.* 4: FUNK-0005-2016.

Bennett, R. J., A. Forche, and J. Berman, 2014 Rapid mechanisms for generating genome diversity: whole ploidy shifts, aneuploidy, and loss of heterozygosity. Cold Spring Harb. Perspect. Med. 4: a019604.

Bergen, L. G., and N. R. Morris, 1983 Kinetics of the nuclear division cycle of *Aspergillus nidulans*. *J. Bacteriol.* 156:155–160.

Berman, J., 2010 Evolutionary genomics: when abnormality is beneficial. *Nature* 468:183–184.

Boyce, K. J., Y. Wang, S. Verma, V. P. S. Shakya, C. Xue et al., 2017 Mismatch repair of DNA replication errors contributes to microevolution in the pathogenic fungus *Cryptococcus neoformans*. *MBio* 8: e00595-17.

Branzei, D., and M. Foiani, 2008 Regulation of DNA repair throughout the cell cycle. *Nat. Rev. Mol. Cell Biol.* 9:297–308.

Chauhan, N., T. Ciudad, A. Rodríguez-Alejandre, G. Larriba, R. Calderone et al., 2005 Virulence and karyotype analyses of *Candida albicans* regeneration of a truncated chromosome of a reintegrant strain (rad52/RAD52) in the host. *Infect. Immun.* 73:8069–8078.

Chow, E. W., C. A. Morrow, J. T. Djordjevic, I. A. Wood, and J. A. Fraser, 2014 Evolutionary genomics: when abnormality is beneficial. *Clinical and Laboratory Standards Institute*, 2008 *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi*, Ed. 2. Available at: https://clsi.org/standards/products/microbiology/documents/ m38/A. Accessed: December 1, 2016.

Colabardini, A. C., N. A. Brown, M. Savoldi, M. H. Goldman, and G. H. Goldman, 2013 Functional characterization of Aspergillus nidulans ypkA, a homologue of the mammalian kinase SGK. *PLoS* One. 8: e57630.
Color, H. V., G. Park, G. E. Turner, C. Ringelberg, C. M. Crew et al., 2006. A high-throughput gene knockout procedure for Neurospora reveals functions for multiple transcription factors. Proc. Natl. Acad. Sci. 103: 10352–10357.

da Silva Ferreira, M. E., J. L. Capellaro, E. dos Reis Marques, I. Malavazi, and D. Perlin, 2004. In vitro evolution of itraconazole resistance in Aspergillus fumigatus involves multiple mechanisms of resistance. Antimicrob. Agents Chemother. 48: 4405–4413.

Dinamarco, T. M., R. S. Almeida, P. A. de Castro, N. A. Brown, T. F dos Reis et al., 2012. Molecular characterization of the putative transcription factor SeB involved in virulence in Aspergillus fumigatus. Eukaryot Cell. 11: 518–531.

Doksani, Y., R. Bermejo, S. Fiorani, J. E. Haber, and M. Foiani, 2009. Replicon dynamics, dormant origin firing, and terminal fork integrity after double-strand break formation. Cell 137: 247–258.

Drummond, R. A., S. L. Gaffen, A. G. Hise, and G. D. Brown, 2014. Innate defense against fungal pathogens. Cold Spring Harb. Perspect. Med. 5: a019620.

Ene, I. V., and R. J. Bennett, 2014. The cryptic sexual strategies of human fungal pathogens. Nat. Rev. Microbiol. 12: 239–251.

Fuchs, B. R., E. O’Brien, J. B. Khoury, and E. Mylonakis, 2010. Methods for using Galleria mellonella as a model host to study fungal pathogenesis. Virulence 1: 475–482.

Garcia-Rubio, R., M. Cuenca-Estrella, and E. Mellado, 2017. Triazole resistance in Aspergillus species: an emerging problem. Drugs 77: 599–613.

Goldman, G. H., and R. Morris, 1995. Aspergillus nidulans as a model system for cell and molecular biology studies. Methods in Molecular Genetics. 1995:6:48–65.

Hagivara, D., A. Watanabe, K. Kamei, and G. H. Goldman, 2016. Epidemiological and genomic landscape of azole resistance mechanisms in Aspergillus fungi. Front. Microbiol. 7: 1382.

Healey, K. R., C. Jimenez Ortigosa, E. Shor, and D. S. Perlin, 2016a. Genetic drivers of multidrug resistance in Candida glabrata. Front. Microbiol. 7: 1995.

Healey, K. R., Y. Zhao, W. B. Perez, S. R. Lockhart, J. D. Sobel et al., 2016b. Prevalent mutator genotype identified in fungal pathogen Candida glabrata promotes multi-drug resistance. Nat. Commun. 7: 11128.

Jazayeri, A., J. Fulck, C. Lukas, J. Bartek, G. C. M. Smith et al., 2006. ATM and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat. Cell Biol. 8: 37–45.

Legrand, M., C. L. Chan, P. A. Jauert, and D. T. Kirkpatrick, 2011. The contribution of the S-phase checkpoint genes MEC1 and SGS1 to genome stability maintenance in Candida albicans. Fungal Genet. Biol. 48: 823–830.

Liu, O. W., C. D. Chun, E. D. Chow, C. Chen, H. D. Madhani et al., 2008. Systematic genetic analysis of virulence in the human fungal pathogen Cryptococcus neoformans. Cell 135: 174–188.

Malavazi, I., M. Savoldi, S. M. Di Mauro, C. F. Menck, S. D. Harris et al., 2006. Transcriptome analysis of Cryptococcus neoformans overcomes stress of azole drugs by formation of disomy in specific multiple chromosomes. PLoS Pathog. 6: e1000848.

Shiloh, Y., 2001. ATM and ATR: networking cellular responses to DNA damage. Curr. Opin. Genet. Dev. 11: 71–77.

Shiloh, Y., 2003. ATM and related protein kinases: safeguarding genome integrity. Nat. Rev. Cancer 3: 155–168.

Sionov, E., Y. C. Chan, and K. J. Kwon-Chung, 2013. Azole heteroresistance of Cryptococcus neoformans overcomes stress of azole drugs by formation of disomy in mitochondrial DNA. Fungal Genet. Biol. 49: 122–130.

Semighini, C. P., A. F. Averette, J. R. Perfect, and J. Heitman, 2011. Deletion of Cryptococcus neoformans AIF ortholog promotes chromosomal aneuploidy and fluconazole resistance in a metacaspase-independent manner. PLoS Pathog. 7: e1002364.

Tashiro, M., and K. Izumikawa, 2016. The current status of drug-resistant Aspergillus. Med. Mycol. J. 57: J103–J112.