Acidic/Alkaline Stress Mediates Responses to Azole Drugs and Oxidative Stress in *Aspergillus fumigatus*

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ABSTRACT A human host exploits stresses such as acidic/alkaline pH, antifungal drugs, and reactive oxygen species to kill microbial pathogens such as the fungus *Aspergillus fumigatus*. However, *A. fumigatus* is resistant to these stresses *in vitro*. Therefore, what accounts for the potent antifungal activity of the human host? In this observation, we show that simultaneous exposure to acidic pH and oxidative stresses is much more potent than the individual stresses themselves and that this combinatorial stress kills *A. fumigatus* synergistically *in vitro*. Interestingly, *A. fumigatus* is resistant to the combination of alkaline pH and oxidative stress. Quantitative real-time PCR analyses showed that acidic/alkaline pH stress can mediate oxidative stress responses in *A. fumigatus* by regulating the expression of catalase-encoding genes. We further show that *A. fumigatus* is sensitive to the combination of acidic/alkaline stress and azole drug stress. Transcriptome analysis revealed that the sensitivity of *A. fumigatus* to azole drugs under acidic/alkaline conditions may be related to changes in genetic stability, sphingolipid metabolism, lipid metabolism, and amino acid metabolism. Collectively, our findings suggest that combinatorial stress represents a powerful fungicidal mechanism employed by hosts against pathogens, which suggests novel approaches to potentiate antifungal therapy.

IMPORTANCE The human host combats fungal infections via phagocytic cells that recognize and kill fungal pathogens. Immune cells combat *Aspergillus fumigatus* infections with a potent mixture of chemicals, including reactive oxygen species, acidic/alkaline stress, and antifungal drugs. However, *A. fumigatus* is relatively resistant to these stresses *in vitro*. In this observation, we show that it is the combination of acidic/alkaline pH and oxidative or azole stress that kills *A. fumigatus* so effectively, and we define the molecular mechanisms that underlie this potency. Our findings suggest that combinatorial stress is a powerful fungicidal mechanism employed by hosts, which suggests novel approaches to potentiate antifungal therapy. This study provides a platform for future studies that will address the combinatorial impacts of various environmental stresses on *A. fumigatus* and other pathogenic microbes.

KEYWORDS acidic/alkaline, *Aspergillus fumigatus*, azole drugs, oxidative stress

The filamentous fungus *Aspergillus fumigatus* is the most prevalent airborne fungal pathogen of humans. It causes severe invasive aspergillosis in immunocompromised patients (1). *A. fumigatus* strains are able to produce an enormous number of conidia (2), and humans are incidentally infected by inhalation of small numbers of spores. The first step in induction of the antifungal innate immune response depends on recognition of conserved fungal structures by front-line immune cells, including neutrophils and macrophages (3). Following recognition of *A. fumigatus* conidia, phagocytes initiate engulfment,

leading to the formation of a phagosome (3). This vesicle then undergoes a series of fissions and fusions, ultimately leading to the formation of the phagolysosome. The degradative and microbicidal microenvironment of this organelle is associated with low pH, the generation of reactive oxygen species, and the presence of defensins and hydrolytic enzymes (3, 4). Moreover, antifungal drugs such as azoles are often used to exert stress on the cell membrane of *A. fumigatus* (5). Thus, human phagocytic cells combat *A. fumigatus* infections by killing this fungus with a potent mixture of these stresses. *In vitro*, *A. fumigatus* exhibits robust adaptation to low-pH stress, oxidative stress, and antifungal drug stress. Therefore, we reasoned that the potency of phagocytic cells might be due to a synergistic combination of these stresses.

One attribute of *A. fumigatus* that makes it such a successful opportunistic pathogen is its ability to adapt to, and proliferate in, a broad range of host environments. One of the most important environmental conditions that fluctuates between niches is the ambient pH (4, 6). *A. fumigatus* is able to grow in media from pH 3.5 to pH 10 (7).

In this report, we describe the effects of the combination of acidic/alkaline pH stress with oxidative stress or azole stress on the viability of *A. fumigatus in vitro*, revealing that this fungus is extremely sensitive to these combined stresses, and then we dissect the mechanistic basis for this extreme sensitivity. To date, the impact of these combinatorial stresses on cell viability has not been described.

First, we tested the levels of killing by a combination of acidic/alkaline pH and oxidative stresses in complete medium (yeast extract-agar-glucose [YAG] medium). As shown in Fig. 1A, *A. fumigatus* cells were killed synergistically by the combination of oxidative stress plus acidic stress. Interestingly, *A. fumigatus* was resistant to the combination of alkaline pH and oxidative stress. Furthermore, the addition of menadione to the medium at pH 8.5 or 9.5 had different inhibitory effects. When the alkalinity in the medium was stronger, the resistance of *A. fumigatus* to menadione was greater. Next, we carried out the experiment in minimal medium (MM), and our results showed that the phenotype in MM was consistent with that in YAG medium (see Fig. S1A in the supplemental material). Taken together, these results indicate that the oxidative stress resistance of *A. fumigatus* might be alkaline dependent. Moreover, like in YAG medium, *A. fumigatus* cells were killed synergistically by the combination of oxidative stress plus acidic stress in MM (see Fig. S1B), suggesting that *A. fumigatus* has the same phenotype on YAG medium and MM with specific pH values. Superoxide dismutase (SOD) and catalase (CAT) are enzymes that help cells resist oxidative damage (8–11); SOD converts superoxide anion free radicals into hydrogen peroxide (H_2O_2) and oxygen, while CAT detoxifies H_2O_2 into oxygen and water. To investigate the mechanistic basis of the effects of the combined stresses, we analyzed and compared the transcription levels of the genes *catA*, *catB*, *catC*, *sodA*, *sodB*, and *sodC* by real-time-PCR, using tubulin as the reference gene. As shown in Fig. 1B, under oxidative stress the transcription levels of *catA*, *catB*, and *catC* significantly decreased in *A. fumigatus* treated with acid (pH 4.0), compared with *A. fumigatus* at normal pH (pH 6.5). Interestingly, the transcription levels of *catA*, *catB*, and *catC* significantly increased in *A. fumigatus* treated with alkali (pH 8.5), compared with *A. fumigatus* at normal pH (Fig. 1C). Taken together, these results suggest that acidic/alkaline pH stress mediates oxidative stress responses in *A. fumigatus* by regulating the expression of *catA/B/C*.

Second, we tested the levels of killing resulting from a combination of acidic/alkaline pH (4.0 or 8.5) and azole drug (1 μg/mL) stresses in YAG medium. As shown in Fig. 2, *A. fumigatus* was more susceptible to itraconazole (ITZ) treatment at acidic pH than at normal pH. Interestingly, our results showed that *A. fumigatus* was hypersensitive to the combination of alkaline pH and ITZ stresses. Moreover, *A. fumigatus* was also hypersensitive to ITZ treatment at alkaline pH in MM (see Fig. S1C). Thus, simultaneous exposure to a combination of acidic/alkaline pH and azole drug stress killed wild-type *A. fumigatus* cells much more effectively than the corresponding individual stresses, indicating that these stresses might act synergistically. Previously, some results demonstrated that antifungal compounds trigger the intracellular accumulation
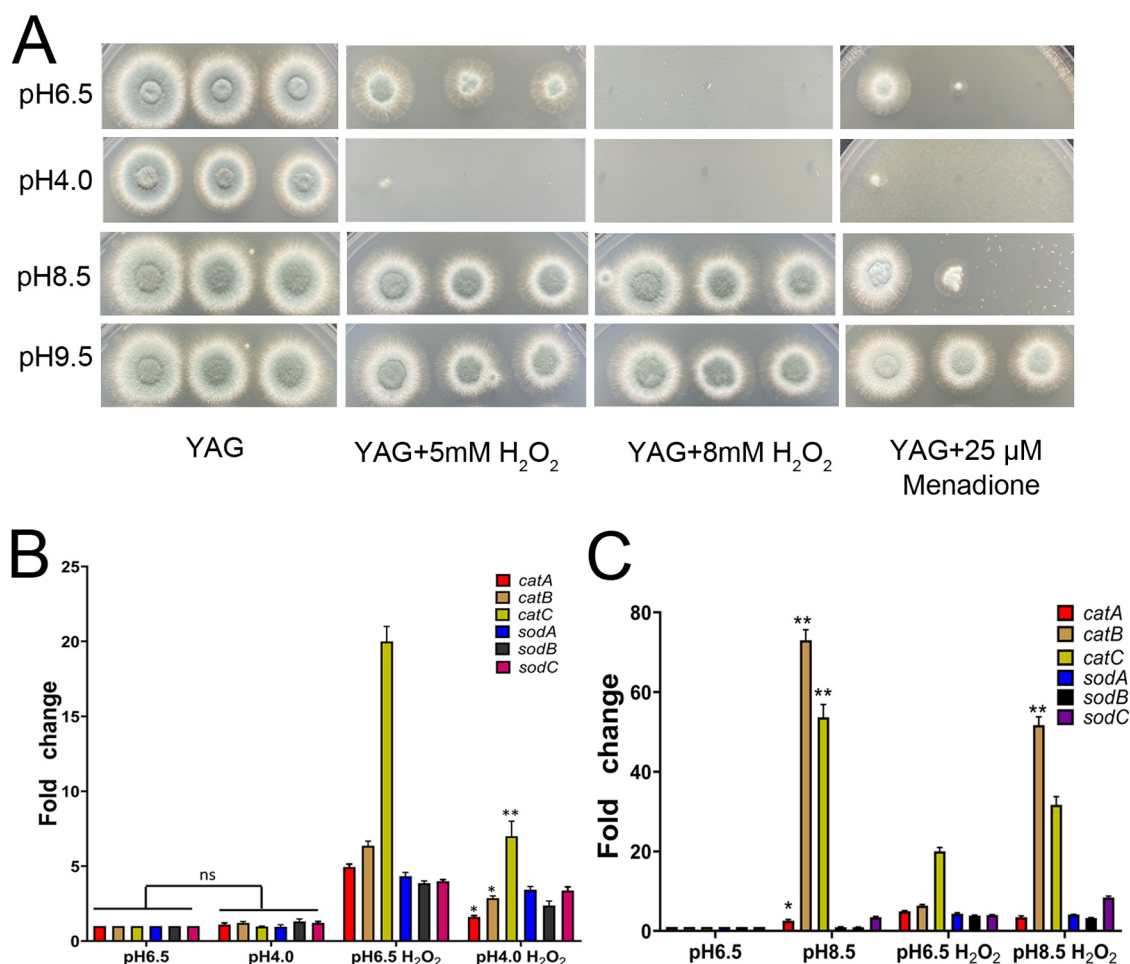
FIG 1 Acidic/alkaline pH stress affects the sensitivity of *A. fumigatus* to oxidative stress. (A) *A. fumigatus* strains were inoculated as a series of 3-μL 10-fold dilutions derived from a starting suspension of 10^7 conidia per mL onto solid YAG medium (pH 6.5, pH 4.0, pH 8.5, or pH 9.5) with or without H_2O_2 or menadione and were cultured at 37°C for 2 days. (B) Analysis of the acidic pH stress impact on transcript levels of genes involved in ROS detoxification. Quantitative real-time reverse transcription (RT)-PCR of *catA/B/C* and *sodA/B/C* transcript levels in *A. fumigatus* strains under the combination of acidic pH stress and H_2O_2 stress was performed. (C) Analysis of the alkaline pH stress impact on transcript levels of genes involved in ROS detoxification. Quantitative real-time RT-PCR of *catA/B/C* and *sodA/B/C* transcript levels in *A. fumigatus* strains under the combination of alkaline pH stress and H_2O_2 stress was performed. Gene expression was normalized to the endogenous reference gene *tubA*. Experiments were carried out in triplicate. Values are reported as the means $\pm$ standard errors of the means (SEMs). Statistical significance was calculated using the unpaired two-tailed *t* test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

of ROS in fungal pathogens and the induction of ROS production contributes to the ability of antifungal compounds to inhibit fungal growth (12). Thus, we measured the endogenous ROS levels in *A. fumigatus* in pH ranges of 4 to 8.5 with ITZ treatment. Our results showed that the ROS production in *A. fumigatus* treated with ITZ at acidic pH was significantly higher than that in *A. fumigatus* treated with ITZ at normal pH (see Fig. S2). Since intracellular ROS accumulation can lead to oxidative damage and lipid peroxidation in *A. fumigatus*, we speculated that lipid peroxidation induced by the elevated ROS accumulation might be the reason why *A. fumigatus* was sensitive to the combination of acidic pH stress and ITZ stress. However, the ROS production in *A. fumigatus* at alkaline pH was lower than that in *A. fumigatus* at normal pH (see Fig. S2). Additionally, under ITZ stress, the transcription levels of *catA*, *catB*, and *catC* significantly increased in *A. fumigatus* treated with alkaline pH, compared with *A. fumigatus* at normal pH (pH 6.5) (see Fig. S3). This result explains why ROS levels in *A. fumigatus* decreased during ITZ treatment at alkaline pH. Taken together, these results indicated that the hypersensitivity of *A. fumigatus* to the combination of alkaline pH stress and
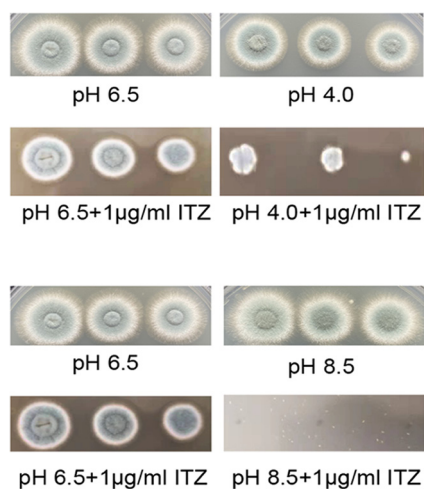
FIG 2 Acidic/alkaline pH stress affects the sensitivity of *A. fumigatus* to azole drugs. *A. fumigatus* is exquisitely sensitive to the combination of acidic/alkaline pH and azole drugs. *A. fumigatus* strains were inoculated as a series of 3-μL 10-fold dilutions derived from a starting suspension of 10^7 conidia per mL onto solid YAG medium (pH 6.5, pH 4.0, or pH 8.5) with or without ITZ and were cultured at 37°C for 2 days.

ITZ stress might not be related to ROS accumulation, and other molecular mechanisms might be involved in this process.

Next, we explored the molecular basis of the sensitivity of *A. fumigatus* to azole drugs at acidic/alkaline pH by genome-wide expression profiling. Wild-type *A. fumigatus* cells were exposed to acidic pH (pH 4.0) or alkaline pH (pH 8.5) for 1 h, harvested, and subjected to microarray analysis. Gene set enrichment analysis was performed to identify the pathways most affected by acidic/alkaline pH stress. Because of the large number of differentially expressed transcripts identified in this analysis, we performed a modified Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using FungiDB (https://fungidb.org/fungidb/app) and genes with a ≥2-fold change in transcript abundance in *A. fumigatus* treated with acidic/alkaline pH, compared with *A. fumigatus* at normal pH. Genes repressed in *A. fumigatus* treated at acidic pH are involved in biological processes including sphingolipid metabolism, glycerolipid metabolism, glycosphingolipid biosynthesis, fatty acid degradation, glycolysis/gluconeogenesis, and amino acid metabolism. Of the 26 genes that were downregulated in response to acidic pH stress, 7 were involved in lipid metabolism and 6 in amino acid metabolism (see Fig. S4A). These results indicate that acidic treatment of *A. fumigatus* caused the processes of lipid metabolism and amino acid metabolism to be greatly reduced. Furthermore, genes that were induced in acid-treated *A. fumigatus* were involved in glycosphingolipid biosynthesis, steroid biosynthesis, and fatty acid degradation. As shown in Table S1 in the supplemental material, microarray experiments revealed significant numbers of genes related to lipid metabolism and amino acid metabolism. Previous studies reported that amino acid metabolism and lipid metabolism, such as sphingolipid metabolism, are closely related to the regulation of azole sensitivity (5, 13–18). Therefore, we speculate that the increased sensitivity of *A. fumigatus* to azole drugs under acidic conditions may be related to a change of cell membrane lipid composition.

In exploring the mechanisms by which alkaline pH stress enhanced the susceptibility of *A. fumigatus* to azole drugs, our KEGG analysis showed that categories of downregulated genes in alkaline-treated *A. fumigatus* included sphingolipid metabolism, DNA repair, DNA replication, meiosis, cell cycle, ribosome, ribosome biogenesis, and amino acid metabolism (see Fig. S4B). These results indicate that alkaline treatment of *A. fumigatus* caused translation and processes related to genetic stability to be greatly reduced. As shown in Table S2, microarray experiments revealed 54 downregulated genes related

to gene stability and lipid biosynthesis. Genetic stability is essential for the survival and maintenance of living organisms, and a very complex set of genes has evolved to repair damaged DNA. Recent studies showed that evolution of fungal azole drug resistance is associated with genetic stability (19–21). Additionally, previous studies reported that sphingolipid metabolism is closely related to the regulation of azole sensitivity (5, 22). Taken as a whole, the sensitivity of *A. fumigatus* to azole drugs under alkaline conditions may be related to a change of genetic stability and cell membrane lipid composition. Of course, it is necessary to further study how these related genes regulate azole resistance under alkaline conditions in the future.

In this study, we have addressed the impact of combined acidic/alkaline pH stress plus oxidative or azole drug stress on *A. fumigatus*. However, we predict that additional environmental cues, such as cationic stress or nutrient availability, might also influence combinatorial stress outputs in this fungus. This study provides a platform for future studies that will address the combined impacts of various environmental conditions and additional stresses on the adaptive responses of *A. fumigatus* and other pathogenic microbes.

Data availability. The RNA sequencing data have been deposited in the NCBI BioProject database under accession numbers PRJNA789093 and PRJNA789032.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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