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OBSERVATION

The Caspofungin Paradoxical Effect is a Tolerant "Eagle Effect" in the Filamentous Fungal Pathogen *Aspergillus fumigatus*

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ABSTRACT Cell responses against antifungals other than resistance have rarely been studied in filamentous fungi, while terms such as tolerance and persistence are well-described for bacteria and increasingly examined in yeast-like organisms. *Aspergillus fumigatus* is a filamentous fungal pathogen that causes a disease named aspergillosis, for which caspofungin (CAS), a fungistatic drug, is used as a second-line therapy. Some *A. fumigatus* clinical isolates can survive and grow in CAS concentrations above the minimum effective concentration (MEC), a phenomenon known as "caspofungin paradoxical effect" (CPE). Here, we evaluated the CPE in 67 *A. fumigatus* clinical isolates by calculating recovery rate (RR) values, where isolates with an RR of $\geq$0.1 were considered CPE$^+$ while isolates with an RR of $<$0.1 were classified as CPE$^-$. Conidia produced by three CPE$^+$ clinical isolates, CEA17 (RR = 0.42), Af293 (0.59), and CM7555 (0.38), all showed the ability to grow in high levels of CAS, while all conidia produced by the CPE$^-$ isolate IFM61407 (RR = 0.00) showed no evidence of paradoxical growth. Given the importance of the calcium/calcineurin/transcription factor-CrzA pathway in CPE regulation, we also demonstrated that all $\Delta crzA^{CEA17}$ (CPE$^+$) conidia exhibited CPE while 100% of $\Delta crzA^{Af293}$ (CPE$^-$) did not exhibit CPE. Because all spores derived from an individual strain were phenotypically indistinct with respect to CPE, it is likely that CPE is a genetically encoded adaptive trait that should be considered an antifungal-tolerant phenotype. Because the RR parameter showed that the strength of the CPE was not uniform between strains, we propose that the mechanisms which govern this phenomenon are multifactorial.

IMPORTANCE The "Eagle effect," initially described for bacterial species, which reflects the capacity of some strains to growth above the minimum inhibitory concentration (MIC) of specific antimicrobial agents, has been known for more than 70 years. However, its underlying mechanism of action in fungi is not fully understood and its connection with other phenomena such as tolerance or persistence is not clear yet. Here, based on the characterization of the "caspofungin paradoxical effect" in several *Aspergillus fumigatus* clinical isolates, we demonstrate that all conidia from *A. fumigatus* CPE$^+$ strains are able to grow in high levels of the drug while all conidia produced by CPE$^-$ strains show no evidence of paradoxical growth. This work fills a gap in the understanding of this multifactorial phenomenon by proposing that CPE in *A. fumigatus* should be considered a tolerant but not persistent phenotype.

KEYWORDS *Aspergillus fumigatus*, caspofungin, tolerance, Eagle effect, drug heterogeneity

The "Eagle effect," a paradoxical reduced killing of bacterial species by specific antimicrobials at concentrations above their minimum inhibitory concentration (MIC), was first described by Eagle in 1948 (1). Since then, this phenomenon has been

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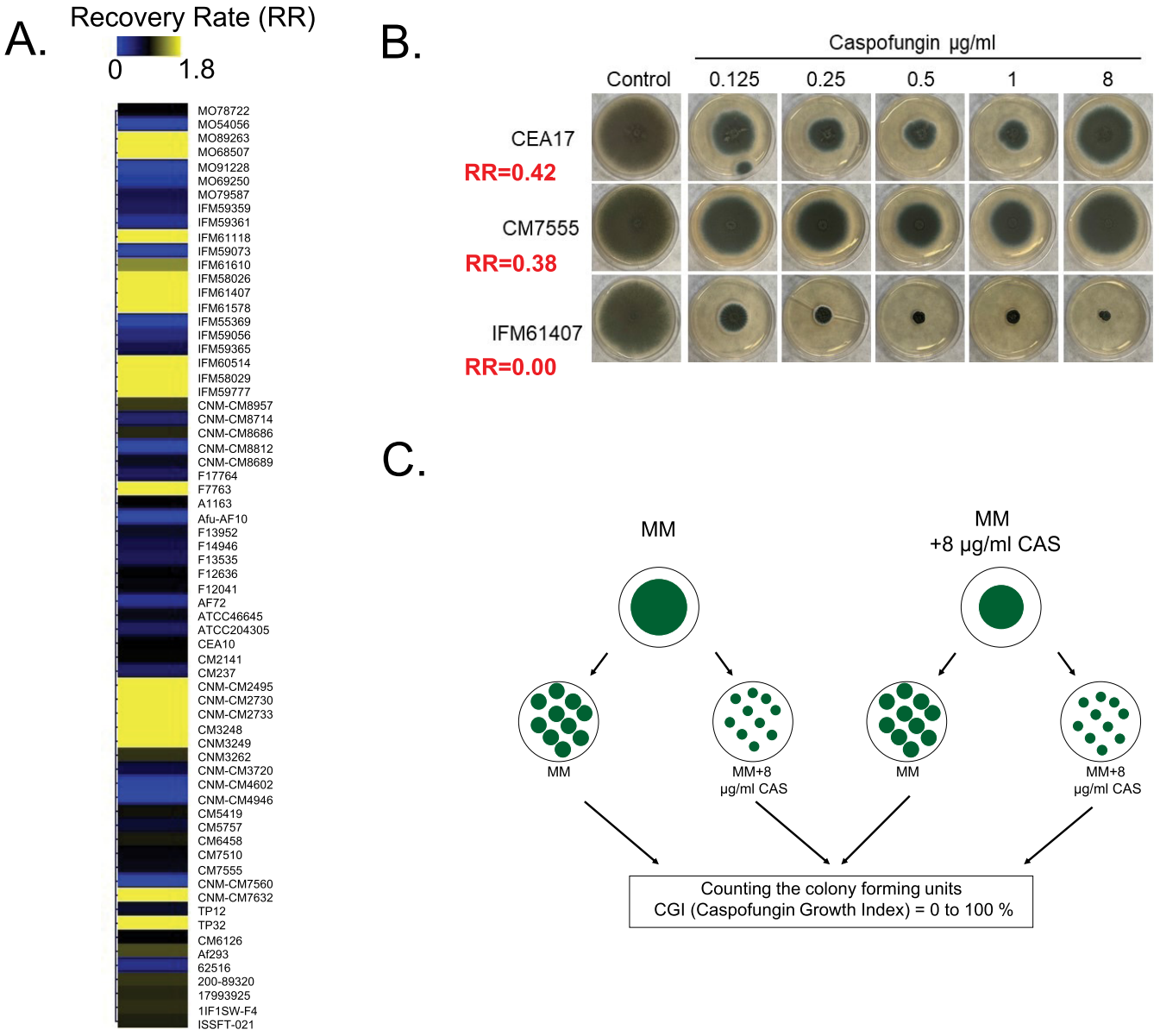

FIG 1 Distribution of *A. fumigatus* CAS tolerance in 67 clinical isolates, recovery rate values (RR) and definition of CAS growth index (CGI). (A) Heat map depicting recovery rate (RR) according to the following formula: colony diameter (8 μg/mL CAS) − minimum colony diameter/colony diameter (MM) − minimum colony diameter, where RR $\geq$ 0.1 isolates are CPE$^+$ and RR $<$ 0.1 isolates are CPE$^-$. Heat map scale and gene identities are indicated. Hierarchical clustering was performed in MeV (http://mev.tm4.org/) using Pearson correlation with complete linkage clustering. (B) Growth of *A. fumigatus* CEA17, CM7555, and IFM61407 clinical isolates on MM and MM + CAS (increasing concentrations). Strains were grown for 5 days at 37°C. (C) Scheme showing how the CGI was calculated. *A. fumigatus* isolates were grown on MM or MM + 8 μg/mL CAS for 5 days at 37°C. Conidia were harvested in phosphate-buffered saline (PBS)-Tween 0.1%, filtered, and diluted to 10^3 sp/mL, and 100 μL was plated in MM or MM + 8 μg/mL CAS and incubated for 2 or 3 days at 37°C. The number of colonies was counted in both treatments and CGI was determined as follows: CGI (%) = (number of colonies with radial diameter of $\geq$0.5 cm on MM + 8 μg/mL CAS/number of colonies radial diameter of $\geq$0.5 cm on MM) $\times$ 100.

observed in a wide range of microorganisms with different drugs. However, its underlying mechanism of action in fungi is not fully understood, and has been related to tolerance, persistence, and treatment failure (2). Drug tolerance has been extensively studied in bacterial pathogens, where it is defined as the ability of all cells of an isogenic strain to survive and even grow at low rates in the presence of drug concentrations that are greater than the MIC. The term "persistence" describes a phenomenon where only a subpopulation of cells within an isogenic strain are drug-tolerant (3).

Aspergillus fumigatus is the most important agent of fungal pulmonary infection and causes a a wide range of conditions, including chronic and allergic lung disease (chronic pulmonary and allergic bronchopulmonary aspergillosis), which affects around

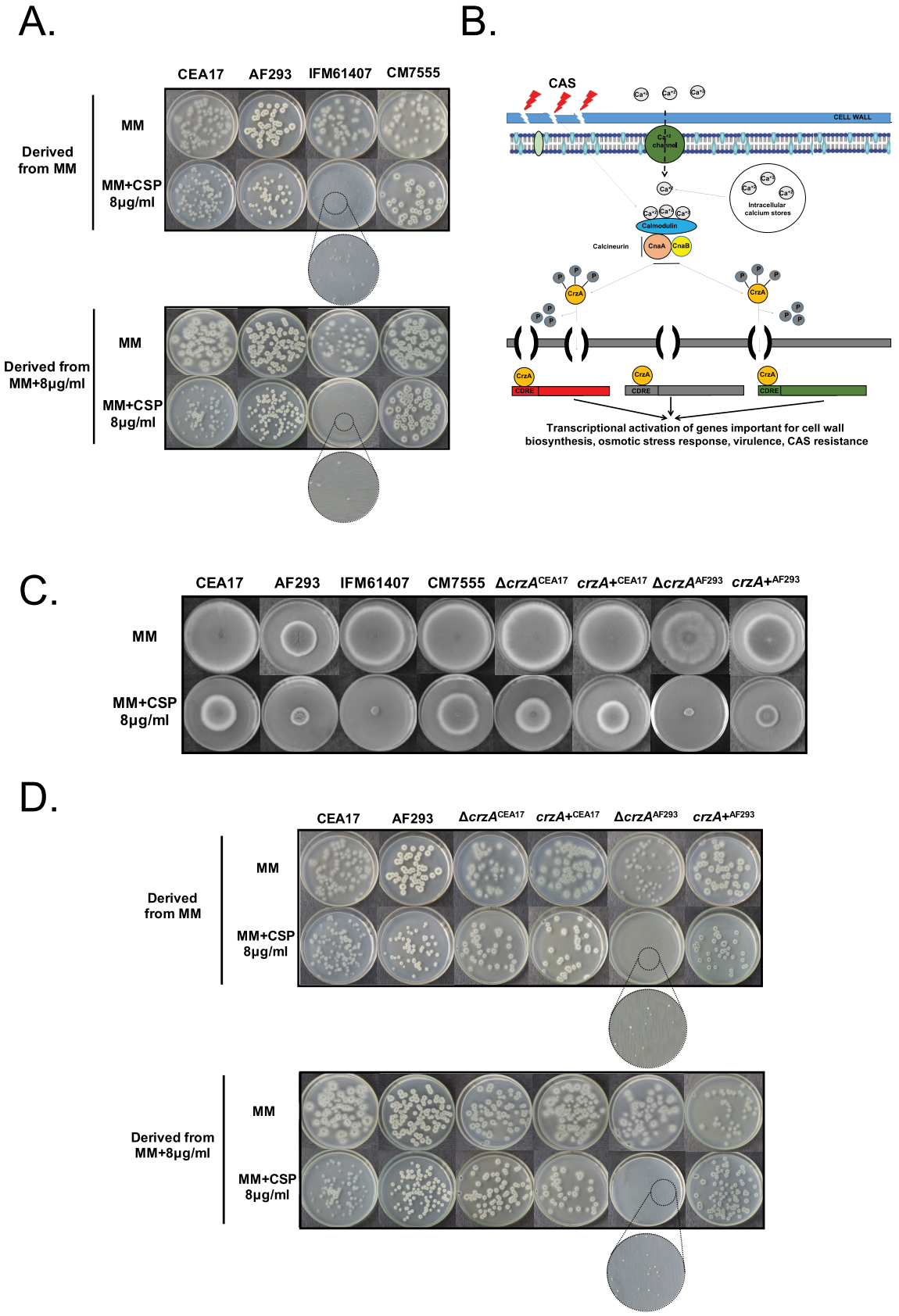

FIG 2 CAS growth index for A. fumigatus clinical isolates. (A) CEA17, Af293, CM7555, and IFM61407 clinical isolates were grown on MM and MM + 8 μg/mL CAS for 5 days at 37°C. Conidia were harvested in PBS-Tween 0.1%, filtered, and diluted to 10^3 sp/mL, and

(Continued on next page)

8 million people worldwide, and life-threatening systemic infections (invasive aspergillosis) with more than 300,000 cases per year (4). Few antifungal agents, such as the fungicidal azoles (first-line therapy, itraconazole, posaconazole, voriconazole, and isavuconazole), amphotericin B, and the fungistatic echinocandins (caspofungin, CAS, second-line therapy) are available to treat aspergillosis while, worryingly, clinical azole resistance has been increasingly reported (5–7). While azoles inhibit the ergosterol biosynthesis pathway by directly targeting the eburicol-14-demethylase (Cyp51A/ERG11) (8), CAS acts by noncompetitively inhibiting the fungal β-1,3-glucan synthase (Fks1), which is essential for the biosynthesis of β-1,3-glucan in the fungal cell wall (9). In patients suffering from invasive aspergillosis, strains resistant to azoles are often shown to have been acquired from the environment; however, in those suffering from chronic forms of aspergillosis, resistance typically occurs during the course of infection (10). CAS resistance has been increasingly observed in *Candida* spp. and, although infrequently described, there are reports of *A. fumigatus* CAS resistance from patients with chronic aspergillosis (11, 12).

To date, the description of tolerance in fungi has focused almost exclusively on yeast-like fungi, where tolerance is frequently observed to occur in subpopulations within an isogenic strain, detected in some reports as a "fraction of growth" (13–15). Although there are scarce reports defining drug tolerance and persistence in filamentous fungi, one adaptive phenomenon has been reported regularly in *A. fumigatus*. It is known as the "caspofungin paradoxical effect" (CPE) and relies on the capacity of some clinical isolates to grow and tolerate CAS concentrations above the minimum effective concentration (MEC). Despite several existing mechanisms having already been described for *A. fumigatus* CPE (16, 17), there is little understanding of whether CPE occurs as a result of phenotypic heterogeneity within an isogenic population. Here, based on the characterization of CPE presence in a series of *A. fumigatus* clinical isolates, we demonstrate that conidia from *A. fumigatus* CAS-tolerant strains do not exhibit CAS heterogeneity and hence, that CPE should be considered a tolerant but not persistent phenotype.

We investigated CPE in 67 *A. fumigatus* clinical isolates (Tables S1 and S2 in the supplemental material at 10.6084/m9.figshare.19178888; S. Zhao et. al., unpublished data) by calculating the recovery rate (RR) parameter as follows: colony diameter (8 μg/mL CAS) – minimum colony diameter/colony diameter (control condition) – minimum colony diameter, where isolates with an RR of $\geq$0.1 were considered CPE$^+$ while isolates with an RR of $<$0.1 were classified as CPE$^-$. Figure 1A shows a heat map representing the RR values of 67 *A. fumigatus* clinical isolates grown for 4 days at 37°C on minimal medium (MM) with 0.125 to 8 μg/mL of CAS. Radial growth in the presence of CAS is exemplified for three clinical isolates: CEA17/A1163 (RR = 0.42), CM7555 (RR = 0.38), and IFM61407 (RR = 0.00) (Fig. 1B).

A. fumigatus sexual and asexual spores are the single developmental "cell-like" structures with a single nucleus in the fungus. Germlings and mycelia are syncytia with several nuclei present in a common cytoplasm. Is the CPE present in a "fraction" of the conidial population or in every single conidium in a single CPE$^+$ clinical isolate? To address this question, we grew two *A. fumigatus* reference isolates, CEA17/A1163 (RR = 0.42) and Af293 (CPE = 0.59), in MM in the presence or absence of CAS 8 μg/mL for 4 days at 37°C. Then, conidia were harvested in phosphate-buffered saline (PBS)-Tween 0.1%, filtered, and diluted to 10^3 conidia/mL, and 100 μL was plated on MM

FIG 2 Legend (Continued)
100 μL was plated in MM or MM + 8 μg/mL CAS and incubated for 2 or 3 days at 37°C. The number of colonies was counted in both treatments and the CGI was determined. (B) Scheme showing the calcium/calcineurin/CrzA pathway. Upon cell wall damage by CAS, calcium concentrations increase in the cytoplasm by calcium transport or mobilization of endogenous calcium deposits. Calcium binds to calmodulin, activating calcineurin, which directly dephosphorylates CrzA, resulting in its translocation to the nucleus. CrzA binds to calcineurin-dependent response element promoters, activating the transcriptional programs that promote stress tolerance. (C) CEA17, Af293, $\Delta crzA^{CEA17}$, and $\Delta crzA^{Af293}$ strains were grown on MM and MM + CAS for 5 days at 37°C. (D) $\Delta crzA^{CEA17}$ and $\Delta crzA^{Af293}$ strains were grown on MM and MM + CAS for 5 days at 37°C. Conidia were harvested in PBS-Tween 0.1%, filtered, and diluted to 10^3 sp/mL, and 100 μL was plated on MM or MM + 8.0 μg/mL CAS and incubated for 2 or 3 days at 37°C. The number of colonies was counted in both treatments and the CGI was determined.

(control) or on MM + 8 μg/mL CAS (CPE concentration) (Fig. 1C). After 48 h (MM) or 72 h (MM + CAS) of growth at 37°C, the number of colonies with a radial diameter of ≥0.5 cm was counted (Fig. 1C) and the CAS growth index (CGI) was determined according to the following formula: % = (number of colonies with radial diameter of ≥0.5 cm on MM + 8.0 μg/mL CAS/number of colonies with radial diameter of ≥0.5 cm on MM) × 100 (Fig. 1C). When CEA17, Af293, and CM7555 clinical isolates were grown on either MM or MM + 8.0 μg/mL CAS, we observed a CGI of 100% (Fig. 2A, see Table S3 at 10.6084/m9.figshare.19178888). We did not observe radial diameter size heterogeneity in any of the colonies grown on MM or MM + 8 μg/mL CAS (all were > 0.5 cm radial diameter; Fig. 2A). These results indicate that every single conidium in *A. fumigatus* CPE$^+$ strains was intrinsically able to grow at CPE CAS concentrations. We then evaluated the CGI for the clinical isolate IFM61407 (CPE$^-$) (Fig. 1C). IFM61407 conidia derived from MM or MM + 8.0 μg/mL CAS both showed a CGI of 0% (Fig. 2A, Table S3 at 10.6084/m9.figshare.19178888).

Calcium homeostasis has been reported to play a central role in the CPE cellular response in *A. fumigatus* (18, 19) (Fig. 2B). CAS increases the intracellular calcium (Ca^{2+}) concentration, activating the calcineurin-CrzA pathway (20). CrzA regulates the activation of several stress responses and cell-wall modifications (19, 21). Interestingly, *crzA* deletion in the clinical strain Af293 results in CPE loss (22) (Fig. 2C), while *crzA* deletion in the CEA17 background results in CPE maintaining (19, Fig. 2C), demonstrating intraspecies differences or CPE heterogeneity. Unlike the Δ*crzA*^{CEA17} mutant, the Δ*crzA*^{Af293} mutant cannot activate cell-wall remodeling genes upon CAS exposure, affecting its CPE (23). The CGIs for the Δ*crzA*^{CEA17} and Δ*crzA*^{Af293} mutant strains are 100 and 0%, respectively, when the strains were grown on MM + 8.0 μg/mL CAS independently if the conidia were derived from MM or MM + 8.0 μg/mL CAS (Fig. 2D, Table S3 at 10.6084/m9.figshare.19178888). Taken together, these results indicate that the transcription factor CrzA, whose deletion results in heterogeneity in the response of the CEA17 and Af293 strains to CAS, does not show CPE heterogeneity, since all the conidia from the CPE$^-$ Δ*crzA*^{Af293} strain were CPE$^-$, while all the conidia from the CPE$^+$ Δ*crzA*^{CEA17} strain were CPE$^+$ (Fig. 2D).

Our results emphasize the view that every single conidium in an *A. fumigatus* CPE$^+$ strain is able to grow and tolerate CPE CAS concentrations. In contrast, conidia from *A. fumigatus* strains which lacked CPE showed no evidence of paradoxical growth, strongly suggesting that there are no *A. fumigatus* CAS-tolerant subpopulations. As a conclusion, *A. fumigatus* CPE is a homogeneous trait within an isogenic population and should be considered an antifungal-tolerant phenotype, while CPE heterogeneity exists between strains, indicating a multifactorial origin.

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REFERENCES

1. Eagle H. 1948. A paradoxical zone phenomenon in the bactericidalaction of penicillin *in vitro*. Science 107:44–45. https://doi.org/10.1126/science.107.2767.44.
2. Prasetyoputri A, Jarrad AM, Cooper MA, Blaskovich MAT. 2019. The Eagle effect and antibiotic-induced persistence: two sides of the same coin? Trends Microbiol 27:339–354. https://doi.org/10.1016/j.tim.2018.10.007.
3. Brauner A, Fridman O, Gefen O, Balaban NQ. 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 14:320–330. https://doi.org/10.1038/nrmicro.2016.34.
4. Bongomin F, Gago S, Oladele RO, Denning DW. 2017. Global and multinational prevalence of fungal diseases: estimate precision. J Fungi (Basel) 18:57. https://doi.org/10.3390/jof3040057.
5. Ostrosky-Zeichner L, Al-Obaidi M. 2017. Invasive fungal infections in the intensive care unit. Infect Dis Clin North Am 31:475–487. https://doi.org/10.1016/j.idc.2017.05.005.

6. Perfect JR. 2017. The antifungal pipeline: a reality check. Nat Rev Drug Discov 16:603–616. https://doi.org/10.1038/nrd.2017.46.
7. Robbins N, Caplan T, Cowen LE. 2017. Molecular evolution of antifungal drug resistance. Annu Rev Microbiol 71:753–775. https://doi.org/10.1146/annurev-micro-030117-020345.
8. Warrilow AG, Parker JE, Price CL, Nes WD, Kelly SL, Kelly DE. 2015. *In vitro* biochemical study of CYP51-mediated azole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother 59:7771–7778. https://doi.org/10.1128/AAC.01806-15.
9. Perlin DS. 2015. Mechanisms of echinocandin antifungal drug resistance. Ann N Y Acad Sci 1354:1–11. https://doi.org/10.1111/nyas.12831.
10. Garcia-Rubio R, Cuenca-Estrella M, Mellado E. 2017. Triazole resistance in *Aspergillus* species: an emerging problem. Drugs 77:599–613. https://doi.org/10.1007/s40265-017-0714-4.
11. Shields RK, Nguyen MH, Clancy CJ. 2015. Clinical perspectives on echinocandin resistance among *Candida* species. Curr Opin Infect Dis 28:514–522. https://doi.org/10.1097/QCO.0000000000000215.
12. Jiménez-Ortigosa C, Moore C, Denning DW, Perlin DS. 2017. Emergence of echinocandin resistance due to a point mutation in the *fsk1* gene of *Aspergillus fumigatus* in a patient with chronic pulmonary aspergillosis. Antimicrob Agents Chemother 61:e01277-17. https://doi.org/10.1128/AAC.01277-17.
13. Delarze E, Sanglard D. 2015. Defining the frontiers between antifungal resistance, tolerance and the concept of persistence. Drug Resist Updat 23:12–19. https://doi.org/10.1016/j.drup.2015.10.001.
14. Berman J, Krysan DJ. 2020. Drug resistance and tolerance in fungi. Nat Rev Microbiol 18:319–331. https://doi.org/10.1038/s41579-019-0322-2.
15. Ben-Ami R, Kontoyiannis DP. 2021. Resistance to antifungal drugs. Infect Dis Clin North Am 35:279–311. https://doi.org/10.1016/j.idc.2021.03.003.
16. Aruanno M, Glampedakis E, Lamoth F. 2019. Echinocandins for the treatment of invasive aspergillosis: from laboratory to bedside. Antimicrob Agents Chemother 63:e00399-19. https://doi.org/10.1128/AAC.00399-19.
17. Papon N, Morio F, Sanglard D. 2020. Signaling pathways governing the caspofungin paradoxical effect in *Aspergillus fumigatus*. mBio 11:e01816-20. https://doi.org/10.1128/mBio.01816-20.
18. Juvvadi PR, Munoz A, Lamoth F, Soderblom EJ, Moseley MA, Read ND, Steinbach WJ. 2015. Calcium-mediated induction of paradoxical growth following caspofungin treatment is associated with calcineurin activation and phosphorylation in *Aspergillus fumigatus*. Antimicrob Agents Chemother 59:4946–4955. https://doi.org/10.1128/AAC.00263-15.
19. Ries LNA, Rocha MC, de Castro PA, Silva-Rocha R, Silva RN, Freitas FZ, de Assis LJ, Bertolini MC, Malavazi I, Goldman GH. 2017. The *Aspergillus fumigatus* CrzA transcription factor activates chitin synthase gene expression during the caspofungin paradoxical effect. mBio 8:e00705-17. https://doi.org/10.1128/mBio.00705-17.
20. Park HS, Lee SC, Cardenas ME, Heitman J. 2019. Calcium-calmodulin-calcineurin signaling: a globally conserved virulence cascade in eukaryotic microbial pathogens. Cell Host Microbe 26:453–462. https://doi.org/10.1016/j.chom.2019.08.004.
21. Soriani FM, Malavazi I, da Silva Ferreira ME, Savoldi M, Von Zeska Kress MR, de Souza Goldman MH, Loss O, Bignell E, Goldman GH. 2008. Functional characterization of the *Aspergillus fumigatus CRZ1* homologue, CrzA. Mol Microbiol 67:1274–1291. https://doi.org/10.1111/j.1365-2958.2008.06122.x.
22. Fortwendel JR, Juvvadi PR, Perfect BZ, Rogg LE, Perfect JR, Steinbach WJ. 2010. Transcriptional regulation of chitin synthases by calcineurin controls paradoxical growth of *Aspergillus fumigatus* in response to caspofungin. Antimicrob Agents Chemother 54:1555–1563. https://doi.org/10.1128/AAC.00854-09.
23. Colabardini AC, Wang F, Dong Z, Pardeshi L, Rocha MC, Costa JH, Dos Reis TF, Brown A, Jaber QZ, Fridman M, Fill T, Rokas A, Malavazi I, Wong KH, Goldman GH. 2022. Heterogeneity in the transcriptional response of the human pathogen *Aspergillus fumigatus* to the antifungal agent caspofungin. Genetics 220:iyab183. https://doi.org/10.1093/genetics/iyab183.