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PHYSIOLOGY

Fungal Seed Pathogens of Wild Chili Peppers Possess Multiple Mechanisms To Tolerate Capsaicinoids

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ABSTRACT The wild chili pepper *Capsicum chacoense* produces the spicy defense compounds known as capsaicinoids, including capsaicin and dihydrocapsaicin, which are antagonistic to the growth of fungal pathogens. Compared to other microbes, fungi isolated from infected seeds of *C. chacoense* possess much higher levels of tolerance of these spicy compounds, having their growth slowed but not entirely inhibited. Previous research has shown capsaicinoids inhibit microbes by disrupting ATP production by binding NADH dehydrogenase in the electron transport chain (ETC) and, thus, throttling oxidative phosphorylation (OXPHOS). Capsaicinoids may also disrupt cell membranes. Here, we investigate capsaicinoid tolerance in fungal seed pathogens isolated from *C. chacoense*. We selected 16 fungal isolates from four ascomycete genera (*Alternaria*, *Colletotrichum*, *Fusarium*, and *Phomopsis*). Using relative growth rate as a readout for tolerance, fungi were challenged with ETC inhibitors to infer whether fungi possess alternative respiratory enzymes and whether effects on the ETC fully explained inhibition by capsaicinoids. In all isolates, we found evidence for at least one alternative NADH dehydrogenase. In many isolates, we also found evidence for an alternative oxidase. These data suggest that wild-plant pathogens may be a rich source of alternative respiratory enzymes. We further demonstrate that these fungal isolates are capable of the breakdown of capsaicinoids. Finally, we determine that the OXPHOS theory may describe a weak primary mechanism by which dihydrocapsaicin, but not capsaicin, slows fungal growth. Our findings suggest that capsaicinoids likely disrupt membranes, in addition to energy poisoning, with implications for microbiology and human health.

IMPORTANCE Plants make chemical compounds to protect themselves. For example, chili peppers produce the spicy compound capsaicin to inhibit pathogen damage and animal feeding. In humans, capsaicin binds to a membrane channel protein, creating the sensation of heat, while in microbes, capsaicin limits energy production by binding respiratory enzymes. However, some data suggest that capsaicin also disrupts membranes. Here, we studied fungal pathogens (*Alternaria*, *Colletotrichum*, *Fusarium*, and *Phomopsis*) isolated from a wild chili pepper, *Capsicum chacoense*. By measuring growth rates in the presence of antibiotics with known respiratory targets, we inferred that wild-plant pathogens might be rich in alternative respiratory enzymes. A zone of clearance around the colonies, as well as liquid chromatography-mass spectrometry data, further indicated that these fungi can break down capsaicin. Finally, the total inhibitory effect of capsaicin was not fully explained by its effect on respiratory enzymes. Our findings lend credence to studies proposing that capsaicin may disrupt cell membranes, with implications for microbiology, as well as human health.

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Successful seed dispersal is critical to plant fitness (1). Many plants coat seeds in fleshy fruits to attract dispersers, but the high density of sugar and nutrients in fruits also attracts fungal and bacterial pathogens (2). To deter pathogens, plants make a diversity of secondary metabolites, including phenolics and polyphenols, terpenoids, essential oils, alkaloids, lectins, polypeptides, and more (3). Many plants produce these secondary metabolites directly in the fruit to surround the seeds, such as the various amides in *Piper* fruits (4), caffeine in coffee fruits (5), and capsaicinoids in chili peppers.

Capsicum is an anthropologically and economically important plant genus, largely because of its secondary metabolite production (6). The genus is unique in its production of capsaicinoids, the alkaloids that give chili peppers their spicy pungency (7). Early American people began eating chilies over 6,000 years ago and cultivated the plants around 3000 BCE (8). After European discovery of the Americas, pungent chili peppers rapidly became a staple dish in many cuisines around the world (9), at least partially due to the antimicrobial properties of capsaicinoids, such as capsaicin (10).

Plant secondary metabolites, such as capsaicinoids, can protect against novel pathogens (11), but microbes that commonly associate with plants can evolve resistance to defensive compounds (12), the result being a coevolutionary arms race between plants and pathogens (13, 14).

Capsicum plants make capsaicinoids in the placenta of the fruit, which surrounds the seeds (15, 16); this reduces the growth of fungal seed pathogens of a wild chili species, *Capsicum chacoense* (17). Though fungal growth is slowed in the presence of capsaicinoids, these fungi exhibit an extreme tolerance to capsaicinoids relative to the tolerance of other microbes tested (18–20). However, the exact mechanism of capsaicin tolerance in these wild fungi is unknown.

Two lines of research have examined the antimicrobial effects of the primary capsaicinoid, capsaicin. The first body of research has assessed the ability of capsaicin to disrupt cell membranes and has shown that capsaicin can affect the structure and phase organization of model membranes (21). Capsaicin also affects voltage-dependent sodium channels and alters bilayer elasticity in artificial membranes (22), but the effects of capsaicin on microbial cell membranes have not yet, to our knowledge, been tested *in vivo*.

A separate, larger body of work examines the ability of capsaicin to inhibit energy production. In bacteria and fungi, capsaicin binds to complex I of the electron transport chain (ETC), thus inhibiting oxidative phosphorylation (OXPHOS) and subsequent energy (ATP) production (Fig. 1) (23, 24). *Saccharomyces cerevisiae* and many other yeasts are largely insensitive to capsaicin, presumably because they lack complex I, the NADH dehydrogenase (24). Instead, yeasts possess alternative NADH dehydrogenases. These less-efficient alternative respiratory enzymes lack the energy-coupling site to push protons into the mitochondrial intermembrane space but can still transfer electrons from NADH to downstream complexes (Fig. 1) (25).

Other microbes possess alternative NADH dehydrogenase enzymes in addition to complex I; such enzymes have been described in the malaria parasite *Plasmodium yoelii yoelii* (26), in the causal agent of corn smut, *Ustilago maydis*, and in the industrially important filamentous fungus *Aspergillus niger* (27). Many fungi possess genes for alternative respiratory enzymes (28), but the physiological function of these enzymes has only been tested in a few model fungi, such as the yeast *Yarrowia lipolytica* (29) and the filamentous fungus *Neurospora crassa* (30).

Previous work with alternative respiratory enzymes has taken advantage of ETC inhibitors that block standard respiratory enzymes but do not inhibit alternative enzymes. These ETC inhibitors allow researchers to test for the presence of alternative enzymes in nonmodel organisms with relatively intractable genetic systems (Table 1).

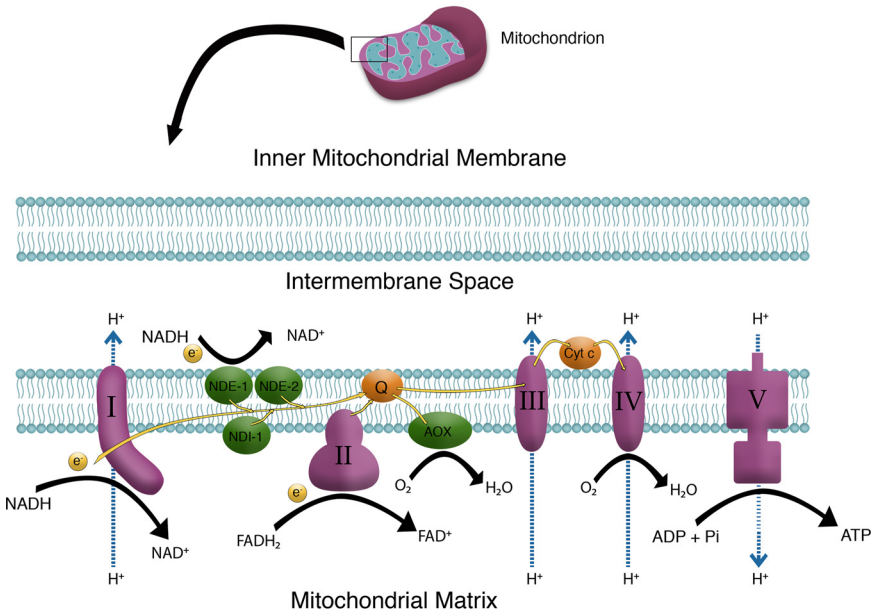

FIG 1 The standard and alternative respiratory enzymes of a fungal electron transport chain. Standard respiratory enzymes are shown in purple. The standard complexes both accept electrons and pump protons to generate proton motive force. The entire standard electron transport chain includes the following: complex I, the NADH dehydrogenase; complex II, the succinate dehydrogenase; membrane-embedded ubiquinone (Q); complex III, the cytochrome bc_1 complex; soluble cytochrome *c* (Cyt c); and complex IV, the cytochrome *c* oxidase. Complex V, the ATP synthase, is the site of electron-linked phosphorylation. Alternative respiratory enzymes named from *Saccharomyces cerevisiae* are shown in green. Three alternative complex I enzymes are known in fungi; these are Nde1 and Nde2, which embed in the external membrane, and Ndi1, in the internal membrane, facing the mitochondrial matrix. These alternative respiratory enzymes accept electrons from NADH but do not pump protons into the intermembrane space. The alternative oxidase (AOX) can directly transfer electrons from ubiquinol to oxygen, reducing oxygen to water, but it bypasses electron transfer through complexes III and IV, reducing overall proton motive force and subsequent ATP production compared to the effects of the standard complexes.

For example, the drug rotenone inhibits complex I, the standard NADH dehydrogenase, but has no effect on alternative NADH dehydrogenases (25, 29, 31). Thus, fungal growth in the presence of this inhibitor indicates the presence of an alternative NADH dehydrogenase.

In addition to alternative respiratory enzymes to bypass the effects of capsaicinoids, fungi that associate with chili peppers may possess degradative enzymes that can decrease the concentrations of the capsaicinoids they encounter. An enzyme that degrades capsaicinoids has been found in several bacteria, but to our knowledge, in only one fungus, *Aspergillus oryzae* (32). Capsaicin-degradative enzymes have been found in *Actinoplanes utahensis* strain NRLL 12052 (echinocandin acylase) (33), *Bacillus* species isolated from Korean kimchi (34), and *Streptomyces mobaraensis* (acylase) (35).

In this study, we measured the growth of fungi isolated from infected seeds of the wild Bolivian chili pepper, *C. chacoense*. For these experiments, we selected fungi from

TABLE 1 ETC inhibitors used in this study and their known targets within the mitochondrion

Inhibitor	Known target(s) in fungi	Concn used in this study
Antimycin	Complex III; does not affect AOX[a]	5 μM
Capsaicin	Complex I; does not affect alternative complex I enzymes	50 μM
Chloramphenicol	Inhibits mitochondrial translation, standard and alternative complex I enzymes, and AOX	7.7638 mM
Dihydrocapsaicin	Complex I; does not affect alternative complex I enzymes	50 μM
Flavone	Nde1, Nde2, and Ndi1; does not target complex I	0.5 mM
Oligomycin	Complex V	5 μM
Rotenone	Complex I; does not affect alternative complex I enzymes	5 μM

[a]AOX, alternative oxidase.

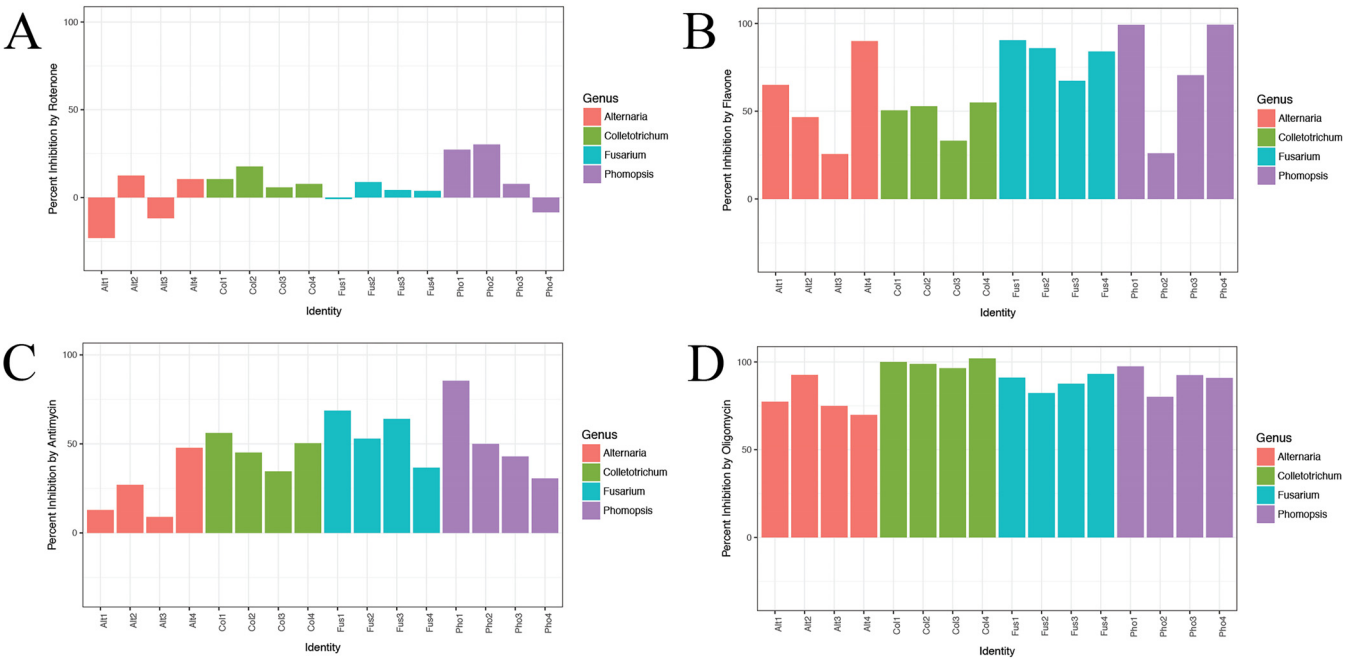

FIG 2 Percentages of inhibition of growth of indicated isolates on glycerol by various drugs. Error bars represent the error propagation standard deviations. (A) Percentages of inhibition on glycerol by rotenone, a complex I inhibitor. Average inhibition across isolates was low at 4.83%, indicating the presence of one or more alternative complex I enzymes. (B) Percentages of inhibition on glycerol by flavone, an inhibitor of multiple targets in the ETC, including alternative NADH dehydrogenases. Average inhibition across isolates was 67.69%. Partial sensitivity confirms the presence of alternative complex I enzyme(s). (C) Percentages of inhibition on glycerol by antimycin, a complex III inhibitor. Average inhibition across isolates was 44.30%, indicating the presence of an alternative oxidase. (D) Percentages of inhibition on glycerol by oligomycin, an ATP synthase inhibitor. Average inhibition across isolates was high at 80.00%, indicating no alternative ATP synthase.

four genera known to be pathogenic on *Capsicum* cultivars in agricultural systems: *Alternaria*, *Colletotrichum*, *Fusarium*, and *Phomopsis*, for a total of 16 isolates—four fungi from each genus. First, we measured fungal growth in the presence or absence of ETC inhibitors to infer whether these fungi possess alternative complex I enzymes and whether they possess alternative complex III enzymes. Using a combination of agar plate-based degradation studies and liquid chromatography-mass spectrometry (LC-MS), we evaluated whether fungal isolates could degrade capsaicin. Finally, we grew fungi on a fermentable (glucose) or a nonfermentable (glycerol) carbon source to investigate whether the inhibitory effect of capsaicinoids on respiratory enzymes wholly explained their effect.

RESULTS

Fungal pathogens of wild chili peppers possess alternative respiratory enzymes. To infer the presence of an alternative complex I enzyme, we tested fungal growth rates when inhibited by the complex I inhibitor rotenone. When grown on nonfermentable glycerol, the fungal isolates showed only 4.83% inhibition by rotenone (Fig. 2A). Conversely, flavone was 62.86% more effective on average than rotenone ($P < 0.0005$), with an average inhibition of 67.69% (Fig. 2B).

Because inhibition by flavone was low in these chili fungi relative to the inhibition rates for microbes used in many other studies (36), we tested inhibition by the complex II inhibitor antimycin. On glycerol, the average percentage of inhibition by antimycin was 44.30%, indicating partial sensitivity (Fig. 2C), implying the presence of an alternative oxidase in many isolates.

To confirm this result, we tested whether the inhibition by antimycin correlated with inhibition by flavone, which inhibits alternative complex I enzymes. Inhibition by antimycin was positively correlated with inhibition by flavone before adjusting for multiple comparisons ($P = 0.045$), but after adjustment, it was not ($P = 0.085$). However, after adjusting for multiple comparisons, its effects correlated positively with those of

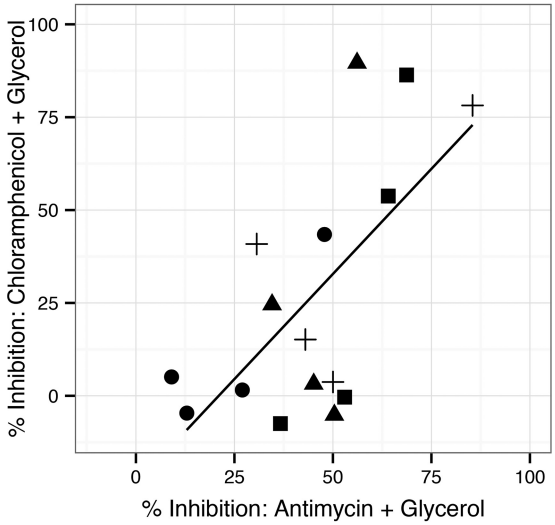

FIG 3 Further evidence of an alternative oxidase. Percentages of inhibition on glycerol by complex III inhibitor antimycin are positively correlated with percentages of inhibition on glycerol by the broad-spectrum ETC inhibitor chloramphenicol, suggesting the presence of an alternative oxidase. Data are denoted by circles for *Alternaria* isolates, by triangles for *Colletotrichum* isolates, by squares for *Fusarium* isolates, and by plus signs for *Phomopsis* isolates. $R^2 = 0.4254$, $P = 0.006176$.

the broad-spectrum ETC inhibitor chloramphenicol ($R^2 = 0.750$, $P = 0.0008$) (Fig. 3), supporting the hypothesis that the fungi possess an alternative oxidase. Correlations of all possible drug combinations were also calculated, and their corresponding R^2 values are shown in Table S1 in the supplemental material.

To rule out the possibility of an alternative ATP synthase, we tested growth in the presence of the ATP synthase inhibitor oligomycin. This drug was the most effective of the drugs administered, with an average inhibition of 80.00% (Fig. 2D), ruling out the presence of an alternative ATP synthase.

In addition to causing the highest overall inhibition, oligomycin was the only drug to cause a significantly higher percentage of inhibition on nonfermentable glycerol than on fermentable glucose (15% higher inhibition on glycerol; one-sided *t* test, $P = 0.03674$) (Fig. 4).

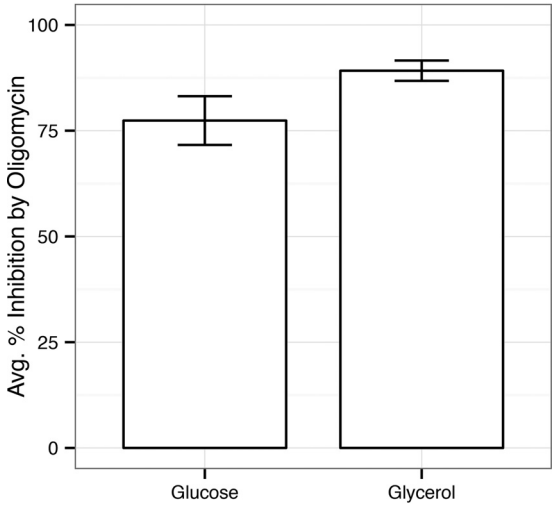

FIG 4 Effects of the ATP synthase inhibitor oligomycin are carbon dependent. Inhibition by oligomycin was greater on glycerol than on glucose, and percentages of inhibition were 15% higher on glycerol, the carbon source that cannot be fermented (one-sided *t* test, $P = 0.03674$; error bars show standard deviations).

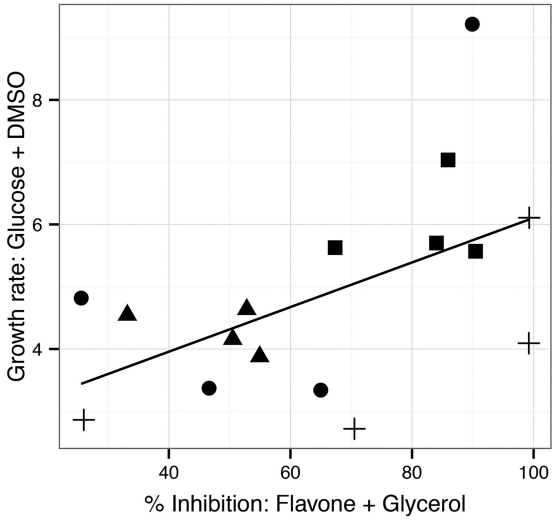

FIG 5 The cost of tolerance to flavone. The lower the inhibitory effects of flavone on an isolate, the more slowly that isolate is able to grow on glucose in the absence of drugs (controlling for effects of the drug solvent DMSO). Data are denoted by circles for *Alternaria* isolates, by triangles for *Colletotrichum* isolates, by squares for *Fusarium* isolates, and by plus signs for *Phomopsis* isolates ($R^2 = 0.2826$, $P = 0.03406$).

Presence of alternative respiratory enzymes comes at a fitness cost to basal growth rate. Because the alternative respiratory enzymes are less efficient than the conventional ETC complexes at energy production, we next tested whether tolerance to any drug came at a fitness cost to basal growth rate. To do so, we compared the percentage of inhibition for each drug on both glycerol (nonfermentable) and glucose (fermentable) without drugs. A positive correlation between these two values would indicate that the enzymes used to confer tolerance in the presence of a drug also retard basal growth rate in the absence of drugs.

The only drug that showed a significantly positive correlation was the broad-spectrum ETC inhibitor flavone ($R^2 = 0.2826$, $P = 0.034$; values were adjusted for multiple comparisons) (Fig. 5), indicating that tolerance to flavone comes at a cost to basal growth rate.

Fungal pathogens of wild chili peppers are capable of capsaicinoid breakdown. In addition to alternative respiratory enzymes, fungi may possess enzymes that can break down capsaicinoids, reducing their concentration. The addition of capsaicinoids to minimal medium renders the medium cloudy (Fig. S1A) and precipitates millimeter-long flavone crystals. These effects allow the observance of potential zones of clearance of the capsaicin, dihydrocapsaicin, and flavone treatments.

To validate the results of the plate-based assays, we also measured the total concentrations of capsaicin in plates with fungal growth via LC-MS. After 10 days of growth, over 99% of the capsaicin had disappeared relative to the concentrations in uninoculated controls (Fig. 6). Previous work has reported potential routes of capsaicin catabolism (37, 38), but current LC–time of flight (TOF)-MS analysis of spent medium could not detect specific masses consistent with predicted degradation products, such as vanillylamine, vanillin, or vanillate (Fig. S2A). In fungal capsaicin plates, a large peak appeared at retention time 6.5 min that was unique to that condition (Fig. S2B) and contained a compound with an observed *m/z* of 137.0610. A search of chemicals compatible with this *m/z* revealed the compound 2-methoxy-4-methylenecyclohexa-2,5-dien-1-one (predicted mass of 137.0597), which may represent a novel metabolite formed in capsaicin degradation.

OXPHOS inhibition alone does not explain the inhibitory effects of capsaicinoids. Finally, we tested whether the capsaicinoids' inhibition of components of the ETC explained the overall inhibitory effects of either drug. To do this, we first determined how the fungi primarily obtain energy. Because glycerol can only be used in OXPHOS and glucose can be used in either OXPHOS or fermentation, a positive

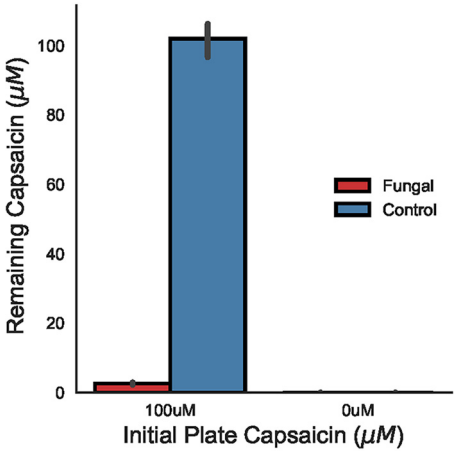

FIG 6 LC-MS analysis of capsaicin degradation. LC-MS analysis confirmed that capsaicin was degraded in fungal plates but not in controls (two-sided *t* test, $P \leq 0.001$, $n = 3$; error bars show 95% confidence intervals).

correlation between growth rates on the two carbon sources would indicate that, given the choice between OXPHOS and fermentation, the fungi primarily use OXPHOS.

To examine the roles of the capsaicinoids on the ETC, we compared the percentage of inhibition by each drug on glycerol to the percentage of inhibition on glucose. Because these fungi generate a majority of their energy via OXPHOS (Fig. 7), a positive correlation between percentages of inhibition by a drug on glycerol and on glucose indicates that the drug inhibits OXPHOS but does not noticeably affect other aspects of metabolism. A lack of correlation indicates that the drug has significant effects on more components than the electron transport chain or that the fungus is unable to ferment.

We found a weakly positive correlation between percentages of inhibition on glucose and glycerol for dihydrocapsaicin ($R^2 = 0.455$, $P = 0.0225$) (Fig. 8A) but did not find a significant correlation between percentages of inhibition on the different carbon sources for capsaicin ($R^2 = 0.01105$, $P = 0.2981$) (Fig. 8B). From this, we conclude that

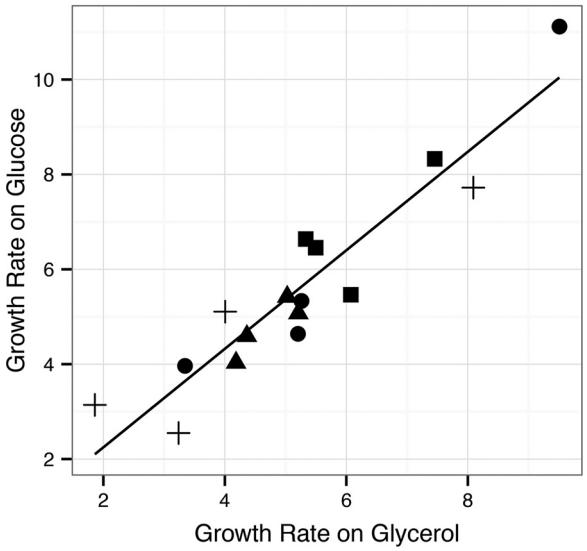

FIG 7 Fungal growth rates in the absence of drugs. Growth rates on glucose correlate strongly with growth rates on glycerol. Under normal conditions, these fungi use oxidative phosphorylation to produce the majority of their energy. Data are denoted by circles for *Alternaria* isolates, by triangles for *Colletotrichum* isolates, by squares for *Fusarium* isolates, and by plus signs for *Phomopsis* isolates ($R^2 = 0.8736$, $P \leq 0.001$).

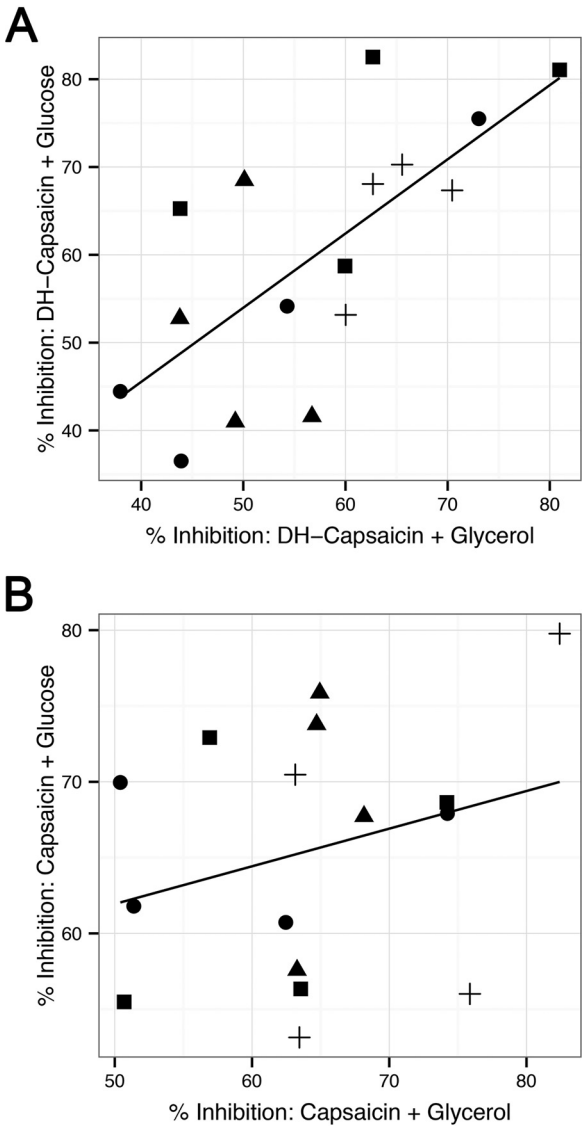

FIG 8 The effects of capsaicinoids outside the ETC. (A) OXPHOS inhibition alone explains the inhibitory effects of dihydrocapsaicin (DH-Capsaicin) ($R^2 = 0.45$, $P = 0.0025$). (B) OXPHOS inhibition alone does not explain the inhibitory effects of capsaicin ($R^2 = 0.07698$, $P = 0.2981$).

dihydrocapsaicin likely solely affects the ETC, while capsaicin exerts additional effects on the cell.

These results are corroborated by the presence of alternative respiratory enzymes in several species within the genera tested. Homologs of the *N. crassa* alternative NADH dehydrogenase genes *nde-1* and *nde-2* were also present in multiple species of *Fusarium*, *Alternaria*, and *Colletotrichum* (Fig. S3A and S3B), while homologs of the alternative NADH dehydrogenase gene *ndi-1* were only found in *Fusarium* and *Colletotrichum* (Fig. S3C). Homologs of the *Neurospora crassa* alternative oxidase gene *aox* were found in multiple species within *Fusarium*, *Alternaria*, and *Colletotrichum* (Fig. S3D). No homologs of any of the alternative respiratory enzymes were identified within *Phomopsis* species, likely due to the low number of sequenced genomes currently available.

DISCUSSION

We discovered that fungal pathogens of wild chili peppers, which have evolved in the presence of capsaicinoids, possess multiple means to tolerate capsaicinoids.

First, all fungal isolates tested were insensitive to the complex I inhibitor rotenone, indicating that all isolates possess at least one alternative NADH dehydrogenase (Fig. 2A) (25, 29, 31). The fungal species used include three fungal genera in which alternative complex I enzymes can be inferred from published genomic data, i.e., *Alternaria*, *Colletotrichum*, and *Fusarium*, in addition to a genus not previously described as possessing respiratory alternatives, *Phomopsis* (Fig. S3 in the supplemental material).

The phylogenetic breadth of these alternative complex I enzymes in these fungi suggests that alternative NADH dehydrogenases may be more widespread in fungal phytopathogens than previously appreciated. Plant fruits are often rich in secondary metabolites that target the electron transport chain. Recognition of fungal alternative NADH dehydrogenases has already resulted in the creation of novel drugs that specifically target them (39). Mammals do not possess alternative NADH enzymes, making them excellent targets for treating opportunistic fungal infections in humans, pets, and livestock.

In other genetically intractable fungi, the presence of an alternative NADH has been inferred by the lack of sensitivity to rotenone coupled with the presence of sensitivity to flavone (26, 40). Though the fungal isolates tested here were highly tolerant to rotenone (Fig. 2A), they showed an unanticipated range of tolerance to flavone (Fig. 2B). The range of levels of tolerance to the complex III inhibitor antimycin (Fig. 2C), combined with the tolerance to flavone, suggests many isolates (e.g., isolates Alt1, Alt3, Col3, Fus4, and Pho4) possess an alternative cytochrome oxidase as well (41, 42). When genome data of a microbe of interest are not available, drug tolerance can be a useful screen for the presence of alternative respiratory enzymes.

The effects of other inhibitors bolster support for the presence of an alternative oxidase in some isolates. The inhibition of antimycin correlated significantly with inhibition by chloramphenicol, a broad-spectrum ETC inhibitor that targets multiple standard and alternative enzymes, such as the alternative oxidase, and decreases total mitochondrial translation ($R^2 = 0.43$, $P = 0.006$) (Table 1; Fig. 3) (41). In particular, chloramphenicol disrupts translation of mitochondrial complex III. As Descheneau et al. observe, previous researchers have misinterpreted chloramphenicol resistance as general drug resistance, when the reason for the resistance is the presence of a specific alternative oxidase (41).

Alternative oxidases have been described in the filamentous fungi *Neurospora crassa* (41), *Aspergillus fumigatus* (40), *Fusarium oxysporum* (43), and others. In fungi such as *Ustilago maydis*, the alternative oxidase is not necessary for normal growth but confers a fitness advantage in the presence of respiratory stress (44). Fungi that associate with spicy chili pepper fruits experience a substantial degree of respiratory stress induced by capsaicinoids, but that stress may be alleviated by an alternative oxidase.

We found evidence for alternative oxidases in all four genera tested (Fig. S2). Much like alternative NADH dehydrogenases, alternative oxidases may be more prevalent among wild-plant pathogens than previously believed. However, while this work is strong in its ability to detect ecologically relevant patterns, it is weak in terms of molecular precision. Much work remains to isolate the proteins inferred here and to perform knockout studies to test how much each enzyme contributes to capsaicinoid tolerance. Further work with the fungal isolates will aim to elucidate the structures and functions of these enzymes.

Here, the variation in percentages of inhibition by flavone (Fig. 2B) may be partially due to flavone's action on other enzymes in the cell. In humans, flavonoids bind to a broad range of enzymes, including human topoisomerase I (45), cyclin-dependent kinases (CDKs) (46), phosphatidylinositol 3-kinase alpha (47), and xanthine oxidase (48). In isolated rat liver mitochondria, the flavonoids quercetin and galangin interact with the mitochondrial membrane (49). Dorta et al. attribute some of the compounds' activity to the double bond between the ether and ketone of the middle C ring, which is also present in flavone (49). Flavone's ability to affect a variety of cell

components could help explain why inhibition by antimycin did not correlate with inhibition by flavone but did correlate with that of chloramphenicol; the effects of chloramphenicol are more targeted to mitochondrial protein synthesis (31) and the ETC (50, 51).

Tolerance to drugs can come at a cost to basal growth rate. Here, we observed such a trade-off when examining tolerance to flavone (Fig. 5): the lower the inhibitory effects of flavone on an isolate, the more slowly that isolate grew on glucose in the absence of ETC-inhibiting drugs. Flavone binds to multiple ETC complexes and can bind to both the external (31) and internal (36) NADH enzymes. To use OXPHOS in the presence of flavone, fungi must engage both the alternative NADH dehydrogenase(s) and the alternative oxidase, which transfer electrons down the ETC but do not pump protons into the intermembrane space of the mitochondria, lessening the proton motive force (25). For fungi that associate with plants that produce high titers of electron transport chain disruptors, such as chili peppers, ETC disruptors may exact a higher selective pressure than the slowing of basal growth. More research interest in the prevalence and action of alternative respiratory enzymes in fungi will inform our understanding of their evolutionary and ecological significance. This knowledge can be leveraged in direct applications relevant to plant pathology and food crop contamination and also to aid medical efforts to treat opportunistic fungal infections (52).

Oligomycin, the ATP synthase inhibitor, had the highest percentage of inhibition of any drug, as well as the lowest standard deviation (Fig. 2D). It is likely that this is because the ATP synthase is the largest complex related to the ETC: it is a supercomplex weighing in at over 500 kDa, composed of two separate rotary motors (53). Because the ATPase consists of so many separate proteins, the synthase would be especially difficult for natural selection to duplicate. In a recent analysis of fungal genomes, there were no duplications of the ATP synthase genes (28). Targeting the ATP synthase may be an effective strategy for slowing fungal pathogens that possess multiple alternative enzymes.

Furthermore, oligomycin caused 15% more inhibition on the carbon source glycerol, which cannot be fermented, than on the fermentable sugar glucose (Fig. 4). We propose that drugs that strongly inhibit OXPHOS in phytopathogenic fungi can cause different effect sizes depending on the carbon source used. This effect was found in human fibroblasts (54) but has not, to our knowledge, been shown in fungal plant pathogens. We advocate for mindful selection of carbon sources when testing the efficacy of drugs on plant pathogens *in vitro*. Moreover, knowledge of which modes of energy production fungi favor can inform applied research with plant pathogens *in vivo* as well. For example, the observation that cancerous cells prefer to ferment (55) has led to novel treatments that target glycolysis in tumor cells (56). In agriculture, applying combinations of fungicides to target both fermentation and OXPHOS may be an effective strategy to limit crop loss and improve yield.

The present results also show that tolerance to capsaicin may involve mechanisms outside the ETC. Further resistance to capsaicinoids may be due to these fungal isolates' ability to degrade capsaicinoids, as was inferred by a clear zone around many of the fungal plugs (Fig. S1A) and confirmed by LC-MS (Fig. 6). While the degradation of capsaicinoids has been studied in other organisms, in those previous studies (32–34), the capsaicinoids are always broken into vanillylamine, which we did not specifically identify (Fig. S2A). Here, the fungi are either immediately modifying vanillylamine into the detected products (Fig. S2B) or they are breaking down capsaicinoids using a novel, hitherto undescribed pathway. Future work will explore the structures and functions of the enzymes involved in this pathway and possible applications, such as treating accidental applications of mace or despicing food.

We designed an assay to test whether we could rule out the small number of studies purporting to show that capsaicin disrupts cell membranes. After confirming that our isolates generate the majority of their energy via OXPHOS (Fig. 7), we tested the inhibition by the two primary capsaicinoids on the two carbon sources. We found that the effect of capsaicin on OXPHOS did not explain the total inhibition by capsaicin (Fig.

8B). The effect on OXPHOS, however, did weakly explain the inhibition by dihydrocapsaicin, the second-most-common capsaicinoid in chili peppers (Fig. 8A). The two compounds are each roughly the length of half a phospholipid bilayer, and the only structural difference between them is that capsaicin has a double bond in the hydrophobic tail (Fig. S2B and C). This nonrotating double bond makes capsaicin more rigid than dihydrocapsaicin, which, in a cell, might cause more membrane disruption and subsequent cell content leakage.

Determining the potential effect of capsaicin on cell membranes is important not only for quantifying the total effect of capsaicin on microbes but also for human medicine. Capsaicin is used in a number of medical applications, ranging from topical creams to treat human ailments such as diabetic neuropathy, psoriasis, and rheumatoid arthritis (16) to treatment of various types of cancer, such as skin cancers, colon cancer, and lung cancer (57). At the same time, research has hinted that capsaicin itself can be a carcinogen (58), and researchers have yet to reach a conclusion on whether this compound is more often beneficial or harmful to human health (59). While the binding of capsaicin to human TRPV receptors has been studied in detail (60), the ability of capsaicin to disrupt human cell and mitochondrial membranes has been largely ignored and demands immediate research.

In summary, our data suggest that fungal seed pathogens of wild chili peppers possess alternative complex I enzymes that allow them to produce energy in the presence of capsaicinoids. We also found evidence for an alternative oxidase, suggesting that plant pathogens may possess more alternative respiratory enzymes than were previously known and may have evolved these enzymes in response to secondary metabolite production by plants. Fungal growth inhibition by dihydrocapsaicin is explained through dihydrocapsaicin's effects on OXPHOS, but OXPHOS inhibition alone does not explain the total effects of capsaicin. Our results suggest that the separate bodies of work regarding capsaicin's effects on microbes are actually complementary: capsaicin likely affects both respiratory enzymes and cell membranes. To tolerate capsaicin, fungi employ a variety of strategies, including multiple alternative respiratory enzymes and capsaicin degradation. This work offers practical applications for the protection of *Capsicum* and other crops against seed pathogens, presents implications for human medicine, and contributes to the evolutionary and ecological theories of plant-pathogen evolution.

MATERIALS AND METHODS

Collection sites and fungal isolation. All fungi were isolated from fruits collected from previously established study sites in the Gran Chaco region, Bolivia (17).

We isolated fungi from infected seeds by transferring one seed per fruit to agar plates that contained dilute potato-dextrose agar (PDA) medium with a 0.2% solution of the antibiotics tetracycline, ampicillin, and streptomycin. We incubated seeds in the dark at 25°C and checked them daily for fungal growth. Isolates were subcultured until they were axenic, transferred onto agar slants for storage, and maintained at 6°C until use.

We selected a total of 16 isolates that could be assigned to one of four different genera: *Fusarium* (*Sordariomycetes*: *Hypocreales*), *Phomopsis* (*Sordariomycetes*: *Diaporthales*), *Colletotrichum* (*Sordariomycetes*: *Glomeralleles*), and *Alternaria* (*Dothidiomycetes*: *Pleosporales*). Within each fungal genus studied, we selected four fungal isolates that had the highest levels of capsaicin tolerance when grown on false fruit medium (data not shown). Aside from isolate Alt1, which was contaminated and could not be reisolated, the isolates used in this study will be deposited in the Fungal Genetics Stock Center, and subcultures of all isolates in the collection can be supplied upon request.

DNA extraction and sequencing. To determine the identities of fungal isolates, we grew all the fungi in our 200-plus culture collection on potato-dextrose agar prior to DNA extraction and PCR of the internal transcribed spacer 1 (ITS1) region. We amplified the ITS1-5.8S-ITS2 region of nuclear ribosomal DNA with 1 μl each (25 μM) of ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and NI4 (5′-GGTCCGTGTTTC AAGACGG-3′). PCR protocol details are described elsewhere (70). We submitted samples for automatic sequencing in both directions to the Penn State Genomic Core Facility, University Park, PA, on an ABI 3739XL instrument (Applied Biosystems, Foster City, CA). We edited sequence data and assembled consensus sequences using Sequencher (Gene Codes Corporation, Ann Arbor, MI).

We estimated taxonomic placement at the genus level for each isolate by BLAST searching the NCBI GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/BLAST/). To assign a genus, we used the highest GenBank sequence similarity scores available and assigned a genus name if the sequence similarity score was 90% or higher and the expect value (E value) was 0.0.

Tested compounds. We used the following drugs, purchased from the manufacturers listed: the complex I inhibitors capsaicin {(*E*)-*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamide; Sigma-Aldrich}, dihydrocapsaicin {*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methylnonanamide; Sigma-Aldrich}, and rotenone {(2*R*,6a*S*,12a*S*)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-b] furo(2,3-h)chromen-6-one; Sigma-Aldrich}, the complex II inhibitor antimycin {[(2*R*,3*S*,6*S*,7*R*,8*R*)-3-[(3-formamido-2-hydroxybenzoyl)amino]-8-hexyl-2,6-dimethyl-4,9-dioxo-1,5-dioxonan-7-yl] 3-methylbutanoate; Sigma-Aldrich}, the complex V inhibitor oligomycin {(1*S*,4*E*,5′*R*,6*R*,6′*R*,7*S*,8*R*,10*S*,11*S*,12*R*,14*S*,15*R*,16*S*,18*E*,20*E*,22*S*,25*R*,27*S*,28*R*,29*S*)-22-ethyl-7,11,14,15-tetrahydroxy-6′-[(2*S*)-2-hydroxypropyl]-5′,6,8,10,12,14,16,28,29-nonamethylspiro[2,26-dioxabicyclo[23.3.1]nonacosa-4,18,20-triene-27,2′-oxane]-3,9,13-trione; Sigma-Aldrich}, the mitochondrial protein synthesis inhibitor chloramphenicol {2,2-dichloro-*N*-[1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl]acetamide; CalBioChem}, and the alternative NADH dehydrogenase inhibitor flavone (2-phenyl-4*H*-1-benzopyran-4-one, 2-phenylchromone; Sigma-Aldrich) (52). The targets of the drugs are listed in Table 1.

Tests of antifungal activities. (i) Carbon sources. To pinpoint the effects of each drug on OXPHOS via the ETC, we used two different carbon sources. Glucose can be fermented and used in glycolysis, while glycerol, which cannot be fermented, is only used in OXPHOS (61).

(ii) Preparation of the ETC inhibitors. All the test compounds (except chloramphenicol; see below) were dissolved in dimethyl sulfoxide (DMSO), sterilized by filtration using a 0.2-μm VWR sterile syringe filter (catalog number 28145-501), and kept as stock solutions.

The targets of all the drugs and the concentrations of the drugs used are listed in Table 1. To determine a relevant concentration of capsaicin and dihydrocapsaicin to use in experiments, we referred to previous work with high-performance liquid chromatography (HPLC) assays of fruits of wild *Capsicum chacoense* (62). Pungent fruits possess capsaicinoids in amounts of roughly 0.5 mg/g dry weight. This concentration moderately inhibits fungal growth without causing complete inhibition (17). Thus, to mimic this ecologically relevant concentration, we added capsaicinoids at a concentration of 0.25 mg/ml, or 50 μM (Table 1).

Antimycin, oligomycin, and rotenone were each added to the medium at a concentration of 5 μM. Flavone was added at a concentration of 0.5 mM, as described in reference 26.

A subset of fungi showed growth on glycerol at every concentration of chloramphenicol tested, so we selected the highest concentration at which chloramphenicol is soluble in water (7.7368 mM). To avoid confounding effects of high volumes of DMSO solvent, unsterilized chloramphenicol powder was added directly to the autoclaved liquid medium. We observed no contamination.

(iii) Fungal growth assays. For all treatments, we dispensed 5 ml of each growth medium into 60-mm petri dishes. We grew fungi on minimal medium with 1.5% agar and 0.0555 molarity of carbon from either glucose or glycerol. To standardize the size of the initial inoculum, we used sterile 5-mm Pasteur pipettes to excise and transfer plugs onto each plate. On each carbon type, each isolate was plated on a set of experimental drug plates, as well as solvent and plain-medium control plates. Solvent control plates contained 2 ml of the drug solvent DMSO per 750 ml of medium and were used to separate the effects of drugs from possible toxic effects of the drug solvent. To quantify any possible effects of DMSO, we also made negative controls with no DMSO added (plain medium). Plain-medium controls lacked all drugs and solvents.

Precultures were grown on plates with 20 ml of PDA. We replicated each drug treatment three times for each of the 16 isolates, with solvent and carbon controls, for a total of 832 plates. Plates were kept in the dark at 25°C. At 48 h after inoculation, we measured two perpendicular colony diameters and noted the time of measurement. We used the mean colony diameter for each isolate and calculated colony diameter increase in units of mm/h.

(iv) Confirmation of degradation with LC-MS. To confirm whether the observed zone of clearance on capsaicinoid plates (Fig. S1A) was truly indicative of capsaicin degradation, isolate Fus3 was selected for further investigation. Plates were made with either 100 μM capsaicin or 0 μM capsaicin and inoculated with fungus (fungal plate) or not (control plate) in triplicate. After 10 days of growth, one 5-mm plug was excised from the edge of fungal growth (or equivalent) and transferred to a 2-ml Eppendorf tube. Two volumes of ice-cold methanol were then added to the sample and stored at −20°C until analysis.

LC-UV-MS analysis was performed on an Agilent 6120 single-quadrupole LC-MS instrument equipped with an Agilent Eclipse plus C_{18} column (4.6 by 100 mm). A linear gradient of 2 to 98% CH_3CN with 0.1% formic acid (vol/vol) over 30 min in H_2O with 0.1% formic acid (vol/vol) at a flow rate of 0.5 ml/min was used. Samples were monitored at 280 nm using a coupled Agilent 1260 Infinity diode array detector (DAD). Extracted ion chromatograms were integrated, and peak areas were used to construct a standard curve using an authentic capsaicin standard. Concentrations of capsaicin within samples were interpolated from this curve.

Bioinformatics. To identify alternative respiratory enzymes in the genera *Phomopsis*, *Fusarium*, *Alternaria*, and *Colletotrichum*, we queried sequences in the NCBI nonredundant nucleotide database that had at least 50% amino acid identity to the *Neurospora crassa* alternative respiration enzymes encoded by *aox* (UniProt no. Q01355), *nde-1* (UniProt no. Q7RVX4), *nde-2* (UniProt no. Q7S2Y9), and *ndi-1* (UniProt no. Q7RXQ5) using BLAST (63). Multiple sequence alignments were created using MAFFT (64), and phylogenetic trees were made with FastTree2 (65). Phylogenetic trees were visualized with iTOL (66).

Data analysis. We considered the effect of each drug when fungi were grown on either fermentable glucose or nonfermentable glycerol. On each carbon type, we calculated the percentage of inhibition of each drug by subtracting the average growth rate on solvent control plates from the average

growth rate on treatment plates and then dividing by the average growth rate on solvent control plates. For chloramphenicol, which was not dissolved in DMSO, we compared growth to that of medium controls.

Statistical analyses were conducted using the R language, version 3.0.2 (67). We calculated growth rate with the lubridate package (68), and plots were generated with the ggplot2 package (69). Propagation of error was used to construct the error bars in Fig. 5. For correlation analyses, we used linear models, and we used one-sided *t* tests to compare between averages, except in the case of the data in Fig. 6, for which we used a two-sided *t* test.

Data availability. The ITS sequences for each isolate are available through GenBank under the following accession numbers: isolate Alt1, MN626665; Alt2, MN626671; Alt3, MN626666; Alt4, MN626670; Col1, MN626667; Col2, MN626669; Col3, MN626678; Col4, MN626680; Fus1, MN626668; Fus2, MN626675; Fus3, MN626679; Fus4, MN626672; Pho1, MN626676; Pho2, MN626673; Pho3, MN626677; and Pho4, MN626674.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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