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Mrr1 regulation of methylglyoxal catabolism and methylglyoxal-induced fluconazole resistance in *Candida lusitaniae*

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Abstract

Transcription factor Mrr1, best known for its regulation of *Candida* azole resistance genes such as *MDR1*, regulates other genes that are poorly characterized. Among the other Mrr1-regulated genes are putative methylglyoxal reductases. Methylglyoxal (MG) is a toxic metabolite that is elevated in diabetes, uremia, and sepsis, which are diseases that increase the risk for candidiasis, and MG serves as a regulatory signal in diverse organisms. Our studies in *Clavispora lusitaniae*, also known as *Candida lusitaniae*, showed that Mrr1 regulates expression of two paralogous MG reductases, *MGD1* and *MGD2*, and that both participate in MG resistance and MG catabolism. Exogenous MG increased Mrr1-dependent expression of *MGD1* and *MGD2* as well as expression of *MDR1*, which encodes an efflux pump that exports fluconazole. MG improved growth in the presence of fluconazole and this was largely Mrr1-dependent with contributions from a secondary transcription factor, Cap1. Increased fluconazole resistance was also observed in mutants lacking Glo1, a Mrr1-indedependent MG catabolic enzyme. Isolates from other *Candida* species displayed heterogeneity in MG resistance and MG stimulation of azole resistance. We propose endogenous and host-derived MG can induce *MDR1* and other Mrr1-regulated genes causing increased drug resistance, which may contribute to some instances of fungal treatment failure.

Summary

While it is clear that constitutively active variants of the transcription factor Mrr1 confer resistance to the antifungal fluconazole in *Candida* species, the natural roles of Mrr1 remain poorly understood. Here, we report that in *Clavispora lusitaniae*, also known as *Candida lusitaniae*, two of the genes regulated by Mrr1 encode enzymes that detoxify methylglyoxal, a toxic metabolite and regulatory signal in diverse species. Serum methylglyoxal is elevated in conditions that are also associated with increased risk of infection by *Candida* species, such as diabetes and kidney failure. We discovered that methylglyoxal increased Mrr1-dependent expression of the methylglyoxal metabolic enzymes as well as *MDR1*, a drug efflux pump. Furthermore, methylglyoxal enhanced growth with azole antifungals in strains of *C. lusitaniae* and other *Candida* species. We posit that the induction of azole resistance in response to methylglyoxal may contribute to some instances of fungal treatment failure.

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Keywords

Candida; Fluconazole; Pyruvaldehyde; Candida lusitaniae

Introduction

Candida species are among the most prominent fungal pathogens, with mortality rates for candidemia ranging from 28 to 72% depending on geographic location (reviewed in (Lamoth *et al.*, 2018)), and recent decades have seen a worldwide increase in the overall incidence of candidemia (Arendrup, 2010). Treatment failure of invasive fungal infections remains an important clinical issue (Nucci & Perfect, 2008) due to long-term complications, high mortality rates, and elevated healthcare costs. Perplexingly, treatment may fail even in cases where isolates from a patient have tested as susceptible to a certain antifungal *in vitro*, suggesting that cryptic factors which are not present during *in vitro* testing may influence the outcome of antifungal therapy *in vivo*.

In *Candida* species, one mechanism of azole resistance is overexpression of the gene *MDR1* (Hiller *et al.*, 2006, Jin *et al.*, 2018, Wirsching *et al.*, 2001, Demers *et al.*, 2018), which encodes an efflux pump. Overexpression of *MDR1* is usually caused by gain-of-function mutations in the gene encoding the zinc-cluster transcription factor Mrr1 (Dunkel *et al.*, 2008, Schubert *et al.*, 2008, Morschhauser *et al.*, 2007, Demers *et al.*, 2018). Many studies have focused on the relationship between *Candida* Mrr1 and resistance against clinical, host, and microbially-produced antifungal compounds (Dunkel *et al.*, 2008, Liu & Myers, 2017, Mogavero *et al.*, 2011, Morschhauser *et al.*, 2007, Schubert *et al.*, 2011, Hampe *et al.*, 2017, Demers *et al.*, 2018). However, little is known about other genes that Mrr1 regulates and thus, the natural role of Mrr1 beyond its involvement in drug resistance is not well understood. By studying the biological functions of Mrr1-regulated genes, it is possible to gain insight into important questions such as the evolutionary purpose of Mrr1, drivers of selection for gain-of-function mutations in Mrr1, and other consequences of high Mrr1 activity aside from drug resistance. Independent studies in *C. albicans* (Karababa *et al.*, 2004, Schubert *et al.*, 2011, Hoehamer *et al.*, 2009, Rogers & Barker, 2003, Morschhauser *et al.*, 2007), *Candida parapsilosis* (Silva *et al.*, 2011), and *Clavispora* (*Candida*) *lusitaniae* (Demers *et al.*, 2018, Kannan *et al.*, 2019) have revealed genes that appear coordinately upregulated in fluconazole (FLZ)-resistant isolates with gain-of-function mutations in *MRR1*.

Previously, we demonstrated a link between FLZ resistance and specific single nucleotide polymorphisms in the *MRR1* locus (*CLUG_00542*) among twenty clinical *C. lusitaniae* isolates from a single patient with cystic fibrosis (Demers *et al.*, 2018). We identified multiple *MRR1* alleles containing gain-of-function mutations that correlated with elevated FLZ resistance, though the presence of *MRR1* alleles conferring high FLZ resistance within this population was unexpected, as the patient had no prior history of antifungal use. Thus, we became interested in other potential factors that could have selected for gain-of-function mutations in *MRR1*. An RNA-Seq analysis comparing several isolates with high- or low-activity Mrr1 variants identified nineteen genes that may be regulated by Mrr1 in

C. lusitaniae, including two genes that encoded putative methylglyoxal (MG) reductases (Demers *et al.*, 2018). Although homologs of *CaGRP2*/*MGD1* were known to be more highly expressed in FLZ-resistant *Candida* strains with high Mrr1 activity across multiple species (Karababa *et al.*, 2004, Schubert *et al.*, 2011, Hoehamer *et al.*, 2009, Rogers & Barker, 2003, Silva *et al.*, 2011, Demers *et al.*, 2018, Kannan *et al.*, 2019), the relationship between Mrr1 and MG has not been described. Recently, genome analyses by Kannan, Sanglard, and colleagues (Kannan *et al.*, 2019) found a possible expansion of putative aldehyde reductases including MG reductases in the *C. lusitaniae* genome.

MG is a reactive compound that forms spontaneously during multiple metabolic processes in all known organisms (Fig. 1). Because it is a highly reactive electrophile, MG can irreversibly modify proteins, lipids, and nucleic acids in a nonenzymatic reaction known as glycation, resulting in cellular damage and stress (Zuin *et al.*, 2005, Takatsume *et al.*, 2006). Serum levels of MG are elevated in patients with diabetes (Lu *et al.*, 2011, Wang *et al.*, 2019, McLellan *et al.*, 1994), sepsis (Brenner *et al.*, 2014), and uremia (Mukhopadhyay *et al.*, 2008, Lapolla *et al.*, 2005, Karg *et al.*, 2009, Odani *et al.*, 1998) relative to healthy controls. Additionally, evidence suggests that MG is generated during inflammation as part of the neutrophil respiratory burst (Zhang *et al.*, 2016). In fungi, MG can be formed during metabolism, for example, in *Saccharomyces cerevisiae*, a positive correlation has been shown between rate of glycolysis and MG levels (Stewart *et al.*, 2013). Catabolism of MG can occur through a glutathione-dependent glyoxalase system, Glo1 and Glo2 (Thornalley, 1990), or through NADH- or NADPH-dependent MG reductases (Ray & Ray, 1984) (Fig. 1). MG reductases have been characterized in *S. cerevisiae*, Gre2 (Chen *et al.*, 2003), and *C. albicans*, Grp2 (Kwak *et al.*, 2018).

In the present study, we demonstrated that in *C. lusitaniae*, Mrr1 regulates *MGD1* (*CLUG_01281*) and *MGD2* (*CLUG_04991*), both of which encode proteins important for the detoxification and metabolism of MG. Deletion of one or both genes led to increased sensitivity to high concentrations of exogenous MG and decreased ability to use MG as a sole carbon source. In addition, we demonstrated that MG can induce Mrr1-dependent expression of *MGD1* and *MGD2*, as well as expression of *MDR1* in a partially Mrr1-dependent manner. MG increased growth in FLZ, and this response was largely dependent on *MRR1* and *MDR1*. Furthermore, deletion of *GLO1* increased FLZ resistance, likely due to elevated endogenous levels of MG. Finally, we showed that though MG sensitivity varies across *Candida* species, stimulation of azole resistance by MG is not exclusive to *C. lusitaniae*. Together, these data demonstrate a broader role for Mrr1 in a metabolic process and describe a mechanism by which host or microbial metabolism could increase resistance to azoles *in vivo*.

Results

C. lusitaniae MGD1 and *MGD2* contribute to the detoxification and metabolism of MG

In our previous work, an RNA-seq analysis of clinical *C. lusitaniae* isolates from a chronic lung infection showed that two genes with high sequence identity to each other, *CLUG_01281* and *CLUG_04991*, were significantly upregulated in isolates with gain-of-function mutations in *MRR1* (Demers *et al.*, 2018). The protein sequences encoded by

CLUG_01281 and *CLUG_04991* are 88% percent identical to each other, and both have 59% and 58% identity to *C. albicans* Grp2 and *S. cerevisiae* Gre2, respectively (Kwak *et al.*, 2018, Chen *et al.*, 2003) (Fig. 2A). Based on sequence homology to previously characterized MG reductases and the experimental data shown below, from here forward *CLUG_01281* and *CLUG_04991* are referred to as *MGD1* and *MGD2*, respectively. We further analyzed the relationships between *MGD1, MGD2*, and other putative MG reductases with homology to *C. albicans GRP2* in select *Candida* spp. using FungiDB (Stajich *et al.*, 2012, Basenko *et al.*, 2018) (Fig. 2A). An interesting phylogeny emerged among the homologs with at least 50% amino acid identity to *C. albicans* Grp2. *C. lusitaniae MGD1* and *MGD2* were more similar to each other than to homologs in other *Candida* species, and other *Candida* species, including *Candida auris*, *Candida parapsilosis*, and *Candida tropicalis* also had at least one set of highly similar paralogous putative MG reductases (Fig. 2A). *Candida glabrata* has a pair of related putative MG reductases that are homologous to *S. cerevisiae* Gre2 (Fig. 2A). The phylogeny of Grp2 homologs suggests that a duplication of MG reductase genes has occurred in many *Candida* species, indicating that this function may be biologically important within the natural niches of *Candida*.

To determine if *MGD1* and *MGD2* were involved in MG resistance and gain more insight into the respective roles of these two similar genes, we knocked out each gene independently and in combination in the previously characterized *C. lusitaniae* clinical isolate S18, which contains a constitutively active Mrr1 variant, H467L (referred to as H4) (Demers *et al.*, 2018). We found that although the *mgd1Δ, mgd2Δ*, and *mgd1Δ/mgd2Δ* mutants grew similarly to the S18 parental strain in the absence of MG, they grew significantly worse in the presence of 15 mM MG, with a lower OD_{600} after 36 hours, slower growth rate, and longer lag time (Fig. 2B, 2C and 2D; see Fig. S1A and S1C for representative growth curves). To our surprise, the double mutant did not exhibit a more severe phenotype than either single mutant, suggesting that these genes are not redundant and that both enzymes are required for full function of the cell's NADPH- or NADH-dependent MG reductase machinery. We confirmed these phenotypes in the L17 isolate, which is closely related to S18 and shares the constitutively active Mrr1-H4 variant, and similarly found that the *mgd1*Δ and *mgd2*Δ mutants were more sensitive to MG than the parental strains (Fig. 2E; see Fig. S1D and Fig. S1B for representative growth curves). We were unable to generate an *mgd1Δ*/*mgd2Δ* double mutant in the L17 background for reasons that are not yet known but do not appear to relate to the selectable markers used as each selectable marker can be used singly.

To determine if *MGD1* and *MGD2* also contributed to MG metabolism, we tested whether the *mgd1*Δ, *mgd2*Δ, or *mgd1*Δ/*mgd2*Δ mutants were deficient in utilizing MG as a sole carbon source. In minimal YNB medium with 5 mM glucose, none of the mutants displayed a significant difference in OD_{600} at 36 h relative to the WT (Fig. S2A). With 5 mM MG as the sole carbon source, neither single mutants exhibited a significant defect in growth, but the *mgd1*Δ/*mgd2*Δ displayed a 26.8% reduction in yield ($p < 0.05$) relative to the WT (Fig. S2B). Together, these data suggest that both *MGD1* and *MGD2* play a role in the detoxification and metabolism of MG.

Mrr1 strongly regulates expression of *MGD1*, but *MGD2* is not highly expressed under standard conditions

We have previously reported (Demers *et al.*, 2018) an RNA-seq analysis that showed that clinical isolates with constitutive Mrr1 activity had higher levels of *MGD1* and *MGD2* expression than strains with low basal Mrr1 activity. Furthermore, analyses of *C. albicans*, *C. parapsilosis*, and an independent collection of clinical *C. lusitaniae* isolates also found that gene expression for *MGD1* and *MGD2* and their was elevated in azole-resistant strains with gain-of-function mutations in Mrr1 (Karababa *et al.*, 2004, Schubert *et al.*, 2011, Hoehamer *et al.*, 2009, Rogers & Barker, 2003, Silva *et al.*, 2011, Kannan *et al.*, 2019). In both the S18 and L17 isolate backgrounds, the *mrr1*Δ mutant was significantly more sensitive to 15 mM MG than the WT despite no difference in growth between the isogenic parental and *mrr1*Δ strains in control conditions (Fig. 3A and 3B). Furthermore, we found that S18 *mrr1*Δ had a 32% lower yield relative to the parental strain in MG as a sole carbon source, with no defects in growth on glucose, and that S18 *mrr1*Δ phenocopied the *mgd1*Δ/*mgd2Δ* mutant in this assay (Fig. S2).

To directly assess whether Mrr1 regulates expression of *MGD1* and *MGD2*, we developed a set of isogenic strains that differed only by which *MRR1* allele was present at the native locus. These *MRR1* alleles were complemented into the *mrr1*Δ derivative of U04, which we have previously described (Demers *et al.*, 2018). The naturally-occurring *MRR1* alleles in this set included a high activity variant (Mrr1-Y813C, referred to as Y8) and a low activity variant (Mrr1-L1191H + Q1197* referred to as L1Q1*); the strain with the high activity Mrr1-Y8 variant had a FLZ minimum inhibitory concentration (MIC) that was 64–128-fold higher than the strain with the low activity Mrr1-L1Q1* variant (Table 1). The *mrr1*Δ derivative of U04 had an 8-fold higher FLZ MIC than the strain with a low activity allele, though the mechanism for this is not known (Table 1). As expected, based on results shown in Fig. 2, we found that strains with high Mrr1 activity grew better in medium with MG compared to strains with low or no Mrr1 activity; no growth differences were observed between strains in control conditions (Fig. 3C and 3D). We found that the *mrr1*Δ mutant had significantly lower levels of basal expression of *MGD1* relative to the relative to WT and Y8 revertant, and the strain with low activity Mrr1 variant had even lower *MGD1* expression (Fig. 3E). *MGD2* levels were 10–100-fold lower than *MGD1*, as judged using a standard curve of input DNA with primer sets for *MGD1*, *MGD2*, and *ACT1* (see Methods). Surprisingly, *MGD2* levels were not different across the U04 strains with different Mrr1 variants in YPD medium without MG (Fig. 3F).

Exogenous MG induces Mrr1-regulated genes through Mrr1 with contributions from Cap1

Because of our observations that *MGD1* and *MGD2* are involved in detoxification and metabolism of MG (Fig. 2 and S2B), we tested whether MG induced their expression through Mrr1 in the S18 background. As shown in Fig. 4A and 4B, 5 mM MG significantly induced expression of *MGD1* by 2-fold at 15 minutes and *MGD2* by 16-fold at 30 minutes in the unaltered S18 isolate. Expression of both genes remained elevated after 60 minutes of MG exposure, although they appeared to be trending downward and the difference at 60 min relative to basal expression only reached statistical significance for *MGD1*. MG also induced expression of another Mrr1-regulated gene, *MDR1*, by 6-fold at 15 and 30 minutes, but as

with *MGD1* and *MGD2*, relative *MDR1* levels began trending downward by 60 minutes (Fig. 4C).

As *C. albicans* Mrr1 induces *MDR1* in response to benomyl and hydrogen peroxide (H_2O_2) in conjunction with another transcription factor, Cap1 (Schubert *et al.*, 2011), we hypothesized that Cap1 may similarly contribute to Mrr1 induction of *MDR1* in *C. lusitaniae*. Furthermore, in *S. cerevisiae*, MG directly modifies the Cap1 ortholog Yap1 by reversibly oxidizing cysteines, thereby inducing nuclear translocation (Maeta *et al.*, 2004). To determine whether *C. lusitaniae MRR1* and/or *CAP1* (*CLUG_02670*) were required for the transcriptional response observed in Fig. 4A–C, we used isogenic *mrr1*Δ, *cap1*Δ, and *mrr1*Δ/*cap1*Δ mutants in the S18 background. Consistent with the results in Fig. 4A and 4B, MG induced expression of *MGD1* (Fig. 4D) and *MGD2* (Fig. 4E) by two-fold and 12-fold, respectively in the S18 parental strain, while the *mrr1Δ, cap1*Δ, or *mrr1*Δ/*cap1*Δ derivatives of S18 did not exhibit a significant change in expression of either gene in response to 5 mM MG (Fig. 4D and 4E). These results support the hypothesis that both Mrr1 and Cap1 are necessary for induction of *MGD1* and *MGD2* expression in response to MG. Additionally, the S18 *cap1Δ* mutant was also defective in growth in YPD + 15 mM MG (Fig. S3) providing further evidence that Cap1 plays an important role the upregulation of genes involved in MG detoxification.

Consistent with the transcriptomics evidence that Mrr1 coregulates *MGD1* and *MGD2* with *MDR1*(Demers et al., 2018), that all three genes are induced by MG (Fig. 4A–C), and that MG induction of *MGD1* and *MGD2* depended on Mrr1, we found that Mrr1 also played a role in MG induction of *MDR1*. While there were no differences in *MDR1* levels among the WT, *mrr1*Δ, *cap1*Δ and *mrr1*Δ/*cap1*Δ in control conditions, the S18 *mrr1Δ* and the S18 *mrr1*Δ/*cap1*Δ had significantly lower *MDR1* levels than the WT and *cap1*Δ in medium with MG (Fig. 4F). To confirm these results in strain L17, we repeated our analysis of *MDR1* expression in the original isolate and its *mrr1Δ* and *cap1*Δ derivatives. In agreement with the results in Fig. 4C and 4F, the parental L17 exhibited a significant increase in *MDR1* expression when exposed to MG, and knocking out *MRR1* reduced *MDR1* levels in medium with MG. (Fig S4). In L17, the *cap1*Δ also had significantly lower *MDR1* levels when compared to the WT. Together, it appears that Mrr1 and Cap1 each play a role in MG-dependent *MDR1* induction, though the effects of loss of Cap1 were only significant in strain L17. The weak stimulation of *MDR1* by MG in the S18 *mrr1*Δ/*cap1*Δ background leads us to suggest that there are other factors may also influence the levels of *MDR1* in response to MG as we discuss below.

MG stimulates growth in FLZ in an Mrr1- and Mdr1-dependent manner

Due to the induction of *MDR1* expression by MG (Fig. 4F), we hypothesized that MG could increase *MDR1*-dependent FLZ resistance in *C. lusitaniae*. To test this, we used FLZ at a concentration equal to the MIC (Table 1) and 5 mM MG. While MG alone did not alter the growth of S18 WT (Fig. S5A), it drastically improved growth in the presence of FLZ (Fig. 5A), resulting in a OD_{600} at 16 h that was, on average, 5.2-fold higher than in FLZ alone (Fig. 5). The S18 *mrr1Δ* and *mrr1*Δ/*cap1*Δ mutants exhibited a significantly lower fold increase in yield at 16 h in FLZ upon amendment of the medium with MG compared

to the S18 parental strain, 2.4- and 1.8-fold, respectively and S18 *mdr1*Δ was similar to the *mrr1Δ* and *mrr1*Δ/*cap1*Δ mutants (Fig. 5B). The *cap1*Δ mutant exhibited on average a 4.6-fold increase in growth in FLZ with MG which was not significantly different from the parental S18 in these analyses, but trended lower (Fig. 5B).

We repeated these growth assays in the L17 background with strains lacking *MRR1*, *CAP1*, or *MDR1*. Again, MG did not alter growth for any of the strains relative to the YPD control (Fig. S5B), but it did lead to a robust stimulation of growth in FLZ, with an average fold change in OD_{600} of 8.5 (Fig. S5 C and D). The stimulation of growth in FLZ by MG was partially dependent on Mrr1 as the *mrr1Δ* mutant exhibited a fold change in OD_{600} of 4.2 which was significantly lower than the S18 WT (Fig. S5D). Similar to the S18 background, the L17 *mdr1*Δ mutant exhibited a fold change in OD_{600} at 16h that was significantly lower than the parental isolate (2.7-fold). Consistent with the *MDR1* expression analysis of L17 strains that found that both Mrr1 and Cap1 contributed to the induction of *MDR1* (Fig. S4), both Mrr1 and Cap1 contributed to increased FLZ resistance in the presence of MG (Fig. S5D). The differences between the S18 and L17 backgrounds in the robustness of the *cap1*Δ mutant phenotype, with Cap1 appearing to play a greater role in *MDR1* regulation in L17, suggest that strain-dependent variables may influence the relative importance of the two transcription factors in the MG response.

Strains with constitutively active Mrr1 variants exhibit greater growth with MG in FLZ than strains with low activity Mrr1 variants

Given our discovery of repeated selection for Mrr1 variants with constitutive activity within a chronic *C. lusitaniae* lung infection (Demers *et al.*, 2018), we sought to determine if higher basal Mrr1 activity effected the magnitude of stimulation of FLZ resistance by MG. We compared the effects of a sub-inhibitory concentration of MG on growth in the presence of inhibitory concentrations of FLZ for *C. lusitaniae* strains S18 and L17, which both express the constitutively active Mrr1-H4 variant, to previously published strains U05 and L14, which express the low activity Mrr1-L1Q1* variant. While there were no differences in growth among strains in reference conditions, the combination of FLZ and MG significantly increased the growth of isolates with high Mrr1 activity (S18 and L17) by ~6 fold relative to growth with FLZ alone and isolates with low Mrr1 activity (U05 and L14) showed similar trends, though the differences were not significant (Fig. 6B). These results show strains with highly active Mrr1 variants were able to reach more robust levels of FLZ resistance in response to MG than strains with low Mrr1 activity.

Absence of *GLO1* causes increased sensitivity to MG and increased resistance to FLZ

The experiments above focused on the effects of exogenous MG, but endogenously generated MG is also an important signal that modulates cell behavior (Moraru *et al.*, 2018, Irshad *et al.*, 2019, Nokin *et al.*, 2019, Antognelli *et al.*, 2019). Disruption of the glyoxalase pathway in *S. cerevisiae* has been shown to cause an accumulation of intracellular MG (Maeta *et al.*, 2004, Penninckx *et al.*, 1983) and render cells highly sensitive to exogenous MG (Inoue & Kimura, 1996). The glyoxalase pathway, which consists of the glutathione-dependent enzymes Glo1 and Glo2, is widely recognized as a major mechanism for MG catabolism in eukaryotic cells (see Fig. 1) (Thornalley, 1996). Thus, we were interested

in whether the S18 *glo1*Δ mutant (lacking *CLUG_04105*) was more resistant to FLZ than its parent in the absence of exogenously added MG. We found that S18 *glo1*Δ was highly sensitive to 15 mM MG (Fig. 7A), even more so than the S18 *mgd1*Δ, *mgd2*Δ, and *mgd1*Δ/*mgd2*Δ mutants (Fig. S2C). Although S18 *glo1*Δ had similar growth kinetics in YPD as the S18 WT (Fig. 7A), the *glo1*Δ mutant grew substantially better in FLZ compared to its parent strain (Fig 7B). These data lead us to speculate that the absence of *GLO1* in *C. lusitaniae* leads to an accumulation of intracellular MG, which may influence the activity of Mrr1, causing an increase in FLZ resistance.

C. lusitaniae is more resistant to MG than many other *Candida* species, and some strains of other species exhibit induction of azole resistance by MG

To assess intrinsic MG resistance across multiple *Candida* species, we assessed growth for a panel of isolates representing seven *Candida* species on YPD agar plates in the presence and absence of 15 mM MG. As controls, we included the *C. lusitaniae* S18 isolate and S18 *glo1*Δ, shown above to be highly sensitive to MG (Fig. 7). We found that *C. lusitaniae* and *Candida dubliniensis* strains were only minimally inhibited by 15 mM MG on plates. There was, however, heterogeneity in growth on MG among *C. auris* and *C. albicans* strains, and the tested *Candida guilliermondii*, *C. glabrata*, and *C. parapsilosis* strains were highly sensitive to MG (Fig. 8A). Overall, the results in Fig. 8A, using a limited number of strains, suggest that intrinsic MG resistance varies between *Candida* species and strains.

We used the same strains as in Fig. 8A to determine if the increase in FLZ resistance in the presence of MG was conserved across *Candida* species. Using 3 mM MG, a lower concentration of MG than in Fig. 8A because of high MG sensitivity of some species, we determined resistance to increasing concentrations of either FLZ or voriconazole (VOR) depending on the species. As shown in Fig. 8B, *C. parapsilosis* RC-601 and *C. dubliniensis* CM2 displayed a striking increase of growth on FLZ with MG and *C. glabrata* ATCC 2001 exhibited a striking increase of growth on VOR with MG. *C. auris* CAU-01 demonstrated a more subtle increase in growth with MG (Fig. 8B). Strains that did not demonstrate visible stimulation of growth on FLZ or VOR by MG under the tested conditions are shown in Fig S6. These results suggest that MG stimulation of azole resistance is not exclusive to *C. lusitaniae*, but not every strain within a species can be stimulated under the conditions tested. Future studies are required to determine what factors determine whether a strain is or is not capable of being induced by MG to have higher azole resistance.

Discussion

The findings from this study show that Mrr1 plays an important role in regulating genes other than *MDR1* in ways that impact growth and fitness, thereby adding to the growing appreciation of MG as an important biological signal across the tree of life. Although the serum concentrations of MG reported in humans are lower than those used *in vitro* for this study (Beisswenger *et al.*, 1999, Ogasawara *et al.*, 2016), local MG levels at sites of infection are hard to measure as MG is highly reactive. At the site of a chronic infection, it is likely that microbes are exposed to MG from a variety of exogenous and endogenous sources including the host immune system, other microbes, and the pathogen's

own metabolic activity (see Fig. 1 and reviewed in (Allaman *et al.*, 2015)). Evidence for the generation of MG *in vivo* comes from the fact that group A *Streptococci* require glyoxalase I for resistance to neutrophil killing, suggesting that neutrophils may be a source of MG *in vivo* (Zhang *et al.*, 2016). In addition, *CaGRP2*, along with other stress-response genes, was upregulated in *C. albicans* cells grown in the murine cecum (Rosenbach *et al.*, 2010). Even low levels of exogenous MG may stimulate a transcriptional response if endogenous MG is already high due to basal metabolism or depletion of the reducing agents required for MG detoxification. Production of MG can be affected by the local environment with low carbon or phosphate increasing MG production in mammalian and bacterial cells, respectively (Liu *et al.*, 2011, Masterjohn *et al.*, 2013, Ferguson *et al.*, 1998). In addition, MG reaction with arginine, lysine, and cysteine residues on proteins forms both reversible and irreversible adducts, and thus some effects of MG on transcriptional activation may increase over time upon low level exposure (Zuin *et al.*, 2005, Takatsume *et al.*, 2006). Our demonstration of the induction of azole resistance by MG could be an important step toward understanding and preventing treatment failure in populations who are susceptible to *Candida* infection.

Previous studies of Mrr1 in multiple *Candida* species have focused on the regulation and biological significance of only a small number of Mrr1-regulated genes, primarily the two efflux pumps encoded by *MDR1* (Hiller *et al.*, 2006, Jin *et al.*, 2018, Wirsching *et al.*, 2001, Demers *et al.*, 2018) and *FLU1* (Calabrese *et al.*, 2000, Li *et al.*, 2013, Hampe *et al.*, 2017). Here, we show that isogenic *C. lusitaniae* strains with gain-of-function mutations in Mrr1 led to higher levels of *MGD1* and *MGD2* transcripts, and higher resistance to exogenous MG (Fig. 3D) than strains with low Mrr1 activity. Furthermore, we showed that MG induced Mrr1 activity to increase the expression of not just *MGD1* and *MGD2*, but also *MDR1*. The co-regulation of genes involved in the detoxification of metabolic by-products with efflux pumps may highlight a broad coordination of a stress response that could be important *in vivo*. Future studies will determine whether MG enhances FLZ resistance *in vivo* and if MG exposure can contribute to the selection for high activity Mrr1 variants.

While multiple chemical inducers of Mrr1 activity have been described, including methotrexate, 4-nitroquinoline-N-oxide, *o*-phenanthroline, benomyl, diethyl maleate, diamide, and H_2O_2, (Harry *et al.*, 2005, Mogavero *et al.*, 2011, Schubert *et al.*, 2011), little is known about why or how these inducers activate Mrr1. It has been postulated that many of these compounds many directly or indirectly induce oxidative stress, which then activates Mrr1. MG is especially interesting as a natural inducer of Mrr1 activity because i) it is produced by cells during metabolism and *in vivo* as a antimicrobial agent, ii) Mrr1 regulates enzymes that specifically metabolize and detoxify this compound, and iii) it has similarly been documented to cause oxidative stress like other known inducers of Mrr1 activity. Though the mechanism by which MG activates transcription in *C. lusitaniae* will be the subject of future work, in *S. cerevisiae* MG has been shown to activate the Cap1 homolog Yap1 by reversibly modifying cysteine residues (Maeta *et al.*, 2004). Multiple studies have established that the transcription factors Mrr1 and Cap1, a regulator of oxidative stress, can cooperate to regulate the expression of *MDR1* in *C. albicans* (Schubert *et al.*, 2011, Mogavero *et al.*, 2011) and we found evidence that this can be the case in *C. lusitaniae*. We do not yet know if Mrr1 or Cap1 is directly modified by MG. *C. lusitaniae* Mrr1 contains many cysteine residues near the C-terminal portion that could be react with MG

in a manner similar to *S. cerevisiae* Yap1. Furthermore, the observation that MG slightly induced *MDR1* even in the absence of both *MRR1* and *CAP1* (Fig. 4F) suggests that other transcription regulators may play a role in *MDR1* induction in response to MG. Other known regulators of *MDR1* expression in *C. albicans* include the transcription factors Mcm1, which is required for induction of *MDR1* by benomyl and by hyperactive Mrr1, but not induction by H_2O_2 (Mogavero *et al.*, 2011), and Upc2 (Schubert *et al.*, 2011, Znaidi *et al.*, 2008), as well as the Swi/Snf chromatin remodeling complex (Liu & Myers, 2017).

As MG is elevated in many diseases associated with *Candida* infections, we were struck by the implications of subinhibitory levels of exogenous MG inducing Mrr1 activity and by extension FLZ treatment outcomes. Diabetes (reviewed in (Rodrigues *et al.*, 2019)) and uremia (Pyrgos *et al.*, 2009, Jawale *et al.*, 2018) are considered risk factors for infection by a variety of *Candida* species, and both are associated with higher levels of MG. Our studies with the *C. lusitaniae glo1Δ* mutant suggest that intracellular MG can also influence FLZ resistance (Penninckx *et al.*, 1983, Maeta *et al.*, 2004). The glyoxalase system, utilizing Glo1 and Glo2, requires reduced glutathione (GSH) to function (Fig. 1), so it is possible that oxidants encountered *in vivo* could deplete GSH and cause increased intracellular MG. In fact, GSH levels are lower in chronic infections, such as those associated with cystic fibrosis (Kettle *et al.*, 2014, Dickerhof *et al.*, 2017). It is also worth noting that diethyl maleate, a compound shown to induce *MDR1* expression in *C. albicans* (Harry *et al.*, 2005), is commonly used in laboratory studies to deplete GSH (Yamauchi *et al.*, 2011, Urban *et al.*, 2017, Enkvetchakul & Bottje, 1995, Mitchell *et al.*, 1983, Zheng *et al.*, 2018).

Importantly, we found that MG induction of azole resistance was not specific to *C. lusitaniae* but more broadly applicable to other *Candida* species though with clear strain-to-strain differences in MG sensitivity (Fig. 8B). Interestingly, several species of bacteria exhibit an increase of drug resistance-related genes in response to MG; for example, MG induces expression of the MexEF-OprN multidrug efflux system in *Pseudomonas aerguinosa* (Juarez *et al.*, 2017), and derepresses *Escherichia coli* TetR family repressor NemR (Lee *et al.*, 2013). Clearly, MG is an important stimulus and stressor that many microbes encounter and understanding how MG affects microbial physiology and drug resistance can open doors to novel means of modulating pathogenic and/or commensal microbes for better health outcomes. For example, it would be interesting to investigate whether supplementation with carnosine, a known scavenger of MG (Hipkiss & Chana, 1998) that is readily available as a dietary supplement, could improve the efficacy when treating infection by *Candida* species, particularly in patients who are predisposed to elevated serum MG.

Methods

Generation of MG reductase phylogenetic tree

Orthologs of CaGrp2 from *S. cerevisiae* and multiple *Candida* species were identified in FungiDB (https://fungidb.org) (Stajich *et al.*, 2012, Basenko *et al.*, 2018) and selected for a protein Clustal Omega multiple sequence alignment (Sievers *et al.*, 2011). The resulting alignment was then used to generate a phylogenetic tree using the Interactive Tree of Life (ITOL) tool (https://itol.embl.de) (Letunic & Bork, 2007).

Strains, media, and growth conditions

The sources of all strains used in this study are listed in Table S1. All strains were stored long term in a final concentration of 25% glycerol at −80°C and freshly streaked onto yeast extract peptone dextrose (YPD) agar (10 g/L yeast extract, 20 g/L peptone, 2% glucose, 1.5% agar) once every seven days and maintained at room temperature. Cells were grown in YPD, yeast nitrogen base (YNB) (0.67% yeast nitrogen base medium with ammonium sulfate (RPI Corp)) supplemented with either 5 mM dextrose or 5 mM MG (Sigma-Aldrich, 5.55 M), or RPMI-1640 (Sigma, containing L-glutamine, 165 mM MOPS, 2% glucose at pH 7) liquid as noted. Media was supplemented with FLZ (Sigma-Aldrich, stock 4 mg mL^{-1} in DMSO) or 3 mM, 5 mM or 15 mM MG as noted. Unless otherwise noted, all overnight cultures were grown in 5 mL YPD liquid medium (10 g/L yeast extract, 20 g/L peptone, 2% glucose) on a rotary wheel at 30°C. *E. coli* strains were grown in LB with either 100 μg mL^{-1} carbenicillin (carb) or 15 μg mL^{-1} gentamycin (gent) as necessary.

Plasmids for complementation of *MRR1*

We amplified i) the *MRR1* gene and terminator with ~1150 bp upstream for homology from the appropriate strain's genomic DNA, ii) the selective marker, *HygB* from pYM70 (Basso *et al.*, 2010), and iii) ~950 bp downstream of *MRR1* for homology from genomic U05 (identical sequence for all relevant strains using primers listed in Table S2. PCR products were cleaned up using the Zymo DNA Clean & Concentrator kit (Zymo Research) and assembled using the *S. cerevisiae* recombination technique previously described (Shanks *et al.*, 2006). Plasmids created in *S. cerevisiae* were isolated using a yeast plasmid miniprep kit (Zymo Research) and transformed into High Efficiency NEB®5-alpha competent *E. coli* (New England BioLabs). *E. coli* containing pMQ30 derived plasmids were selected for on LB containing 15 μg mL^{-1} gentamycin. Plasmids from *E. coli* were isolated using a Zyppy Plasmid Miniprep kit (Zymo Research) and subsequently verified by Sanger sequencing with the Dartmouth College Genomics and Molecular Biology Shared Resources Core. *MRR1* complementation plasmids were linearized with Not1-HF (New England BioLabs), cleaned up the Zymo DNA Clean & Concentrator kit (Zymo Research) and eluted in molecular biology grade water (Corning) before transformation of 2 μg into *C. lusitaniae* strain U04 *mrr1*Δ as described below.

All plasmids for complementing *MRR1* were constructed using the *S. cerevisiae* recombination technique previously described (Shanks *et al.*, 2006) and primers listed in Table S2. To create the precursor plasmid pMQ30$^{MRR1\text{-}L1191H+Q1197*}$-*URA3*, *MRR1* with ~1150 bp upstream of *MRR1* and separately ~950 bp downstream of *MRR1* were amplified from genomic U05 (containing $MRR1^{L1191H+Q1197*}$) DNA and *C. albicans URA3* under the controls of a TEF1 promoter was amplified from pTEF1-*URA3*. This construct did not restore growth of 5-FOA resistant *C. lusitaniae* strains on uracil deplete medium, so we replaced *URA3* with a different selectable marker. Linearized pMQ30$^{MRR1\text{-}L1191H+Q1197*}$-*URA3* (using XbaI) and PCR amplified HygB, the hygromycin B resistance gene from pYM70 (Basso *et al.*, 2010), were combined using the *S. cerevisiae* recombination technique to create pMQ30$^{MRR1\text{-}L1191H+Q1197*}$-*HygB*. To create the pMQ30$^{MRR1\text{-}Y813C}$-*HygB* plasmid, pMQ30$^{MRR1\text{-}L1191H+Q1197*}$-*HygB* was linearized with XbaI and NotI to

remove *MRR1* and the upstream sequence. Replacement sequence including *MRR1* with ~1150 bp upstream of *MRR1* were amplified from U04 (*MRR1*^{Y813C}) gDNA.

Plasmids created in *S. cerevisiae* were isolated using a yeast plasmid miniprep kit (Zymo Research) and transformed into High Efficiency NEB®5-alpha competent *E. coli* (New England BioLabs). *E. coli* containing pMQ30 derived plasmids were selected for on LB containing 15 μg mL^{-1} gentamycin. Plasmids from *E. coli* were isolated using a Zyppy Plasmid Miniprep kit (Zymo Research) and subsequently verified by Sanger sequencing with the Dartmouth College Genomics and Molecular Biology Shared Resources Core. All restriction enzymes were purchased from New England BioLabs and used as recommended by the manufacturer.

Mutant construction

Mutants were generated using an expression-free CRISPR-Cas9 method, as previously described (Grahl *et al.*, 2017), with the exception of the *mgd1Δ/mgd2Δ* double mutant, as detailed below. In brief, cultures were grown to exponential phase in 50 mL YPD on a shaker at 150 rpm, then washed and incubated in TE buffer and 0.1 M lithium acetate at 30°C for one hour. Dithiothreitol was added to a final concentration of 100 mM and cultures were incubated for an additional 30 minutes at 30°C. Cells were washed and resuspended in 1 M sorbitol before being transferred to electroporation cuvettes. To each cuvette was added 1.5 μg of DNA for the knockout or *MRR1* complementation construct and Cas9 ribonucleoprotein containing crRNA specific to the target gene. Following electroporation, cells were allowed to recover in YPD at 30°C for four to six hours. Cells were then plated on YPD agar supplemented with 200 μg mL^{-1} nourseothricin (NAT) or 600 μg mL^{-1} hygromycin B (HYG) and incubated at 30°C for two days. The *mgd1Δ/mgd2Δ* double mutant was generated from the S18 *mgd1Δ* single mutant using the microhomology repair method (Al Abdallah *et al.*, 2017). In brief, the knockout construct containing 50 bp homology to the flanking regions of *MGD2* was transformed alongside Cas9 complexed with two crRNA, targeting the 5' and 3' region immediately adjacent to *MGD2*. PCR with primers inside the *NAT1* or *HygB* cassette and in the flanking regions of the genes outside of each construct were used to confirm all mutants. Primers (IDT) used to create knockout constructs and verify mutants are listed in Table S2.

Minimum Inhibitory Concentration (MIC) Assay

MIC assays for FLZ were performed as described in (Demers *et al.*, 2018) using the broth microdilution method. In brief, overnight cultures were diluted to an OD_{600} of 0.1 in 200 μL dH_2O and 60 μL of each dilution were added to 5 mL RPMI-1640 medium. FLZ was serially diluted across a clear, flat-bottom 96-well plate (Falcon) from 128 μg mL^{-1} down to 0.25 μg mL^{-1} in RPMI-1640. To each well was added 100 μL of cell suspension in RPMI-1640. Upon addition of cells, the final concentration of FLZ ranged from 64 μg mL^{-1} to 0.125 μg mL^{-1}. Plates were incubated at 35°C and scored for growth at 24 hours; the results are summarized in Table 1. The MIC was defined as the drug concentration that abolished visible growth compared to a drug-free control.

Growth Kinetics

C. lusitaniae cultures were grown overnight, diluted 1:50 into 5 mL fresh YPD, and grown for four to six hours at 30°C. After washing, the cultures were diluted to OD_{600} of 1 in 200 μL dH_2O. Each inoculum was prepared by pipetting 60 μL of the OD_{600} of 1 suspension into 5 mL YPD. Clear 96-well flat-bottom plates (Falcon) were prepared by adding 100 μl per well YPD or YPD with MG and/or FLZ at twice the desired final concentrations. 100 μL of inoculum was added to each row of the plate. Each plate was set up in technical triplicate for each strain and condition. The plates were incubated in a Synergy Neo2 Microplate Reader (BioTek, USA) to generate a kinetic curve. The plate reader protocol was as follows: heat to 37°C, start kinetic, read OD_{600} every 60 minutes for 16 or 36 hours, end kinetic.

Spot Assays

Candida cultures were grown overnight, diluted 1:50 into 5 mL fresh YPD, and grown for four to six hours at 30°C. Cultures were diluted to OD_{600} of 1 in 200 μL dH_2O. Each strain was then serially diluted by 1:10 down to an OD_{600} of approximately 1×10^{-6}. 5 μL of each dilution was spotted onto YPD alone or YPD containing the specified concentrations of MG, FLZ or VOR (Cayman Chemical Company, stock 1 mg mL^{-1} in DMSO). Plates were incubated at 37°C for two days before imaging.

Quantitative Real-Time PCR

C. lusitaniae cultures were grown overnight, diluted 1:50 into 5 mL fresh YPD, and grown for four hours at 30°C. Control cultures were harvested at this point and MG was added to a final concentration of 5 mM to all other cultures, which were returned to 30°C on a roller drum. Cultures were then harvested after 15, 30, or 60 minutes. To harvest, 2 mL of culture was spun in a tabletop centrifuge at 13.2 × *g* for 5 min and supernatant was discarded. RNA isolation, gDNA removal, cDNA synthesis, and quantitative real-time PCR were performed as previously described (Demers *et al.*, 2018). Transcripts were normalized to *ACT1* expression. Primers are listed in Table S2.

Statistical Analysis and Figure preparation

All graphs were prepared with GraphPad Prism 8.3.0 (GraphPad Software). One- and two-way analysis of variance (ANOVA) tests were performed in Prism; details on each test are described in the corresponding figure legends. All p values were two-tailed and $p < 0.05$ were considered to be significant for all analyses performed and are indicated with asterisks or letters in the text: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The graphical abstract was prepared using BioRender (biorender.com).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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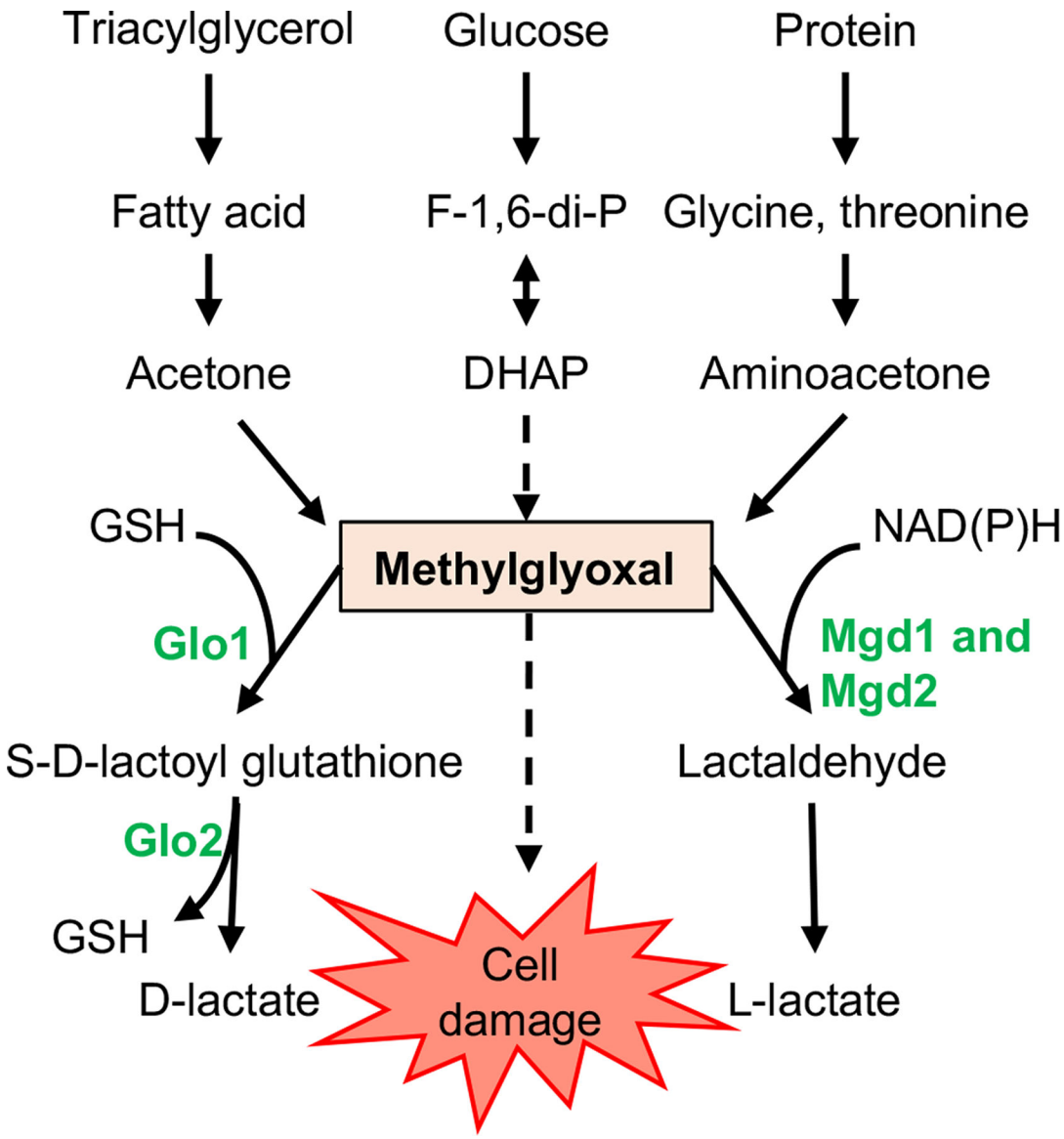

Fig. 1. Schematic of Methylglyoxal (MG) metabolism and catabolism.

MG is a highly reactive, toxic product that forms spontaneously during the catabolism of sugars, fatty acids, and proteins. It can be detoxified to D-lactate via the GSH-dependent glyoxalase system, consisting of Glo1 and Glo2, or to lactaldehyde through NAD(P)H-dependent MG reductases such as Mgd1 and Mgd2, which are homologs of *C. albicans* Grp2. F-1,6-di-P, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GSH, reduced glutathione. Solid arrows represent enzymatic processes; dashed arrows represent nonenzymatic processes.

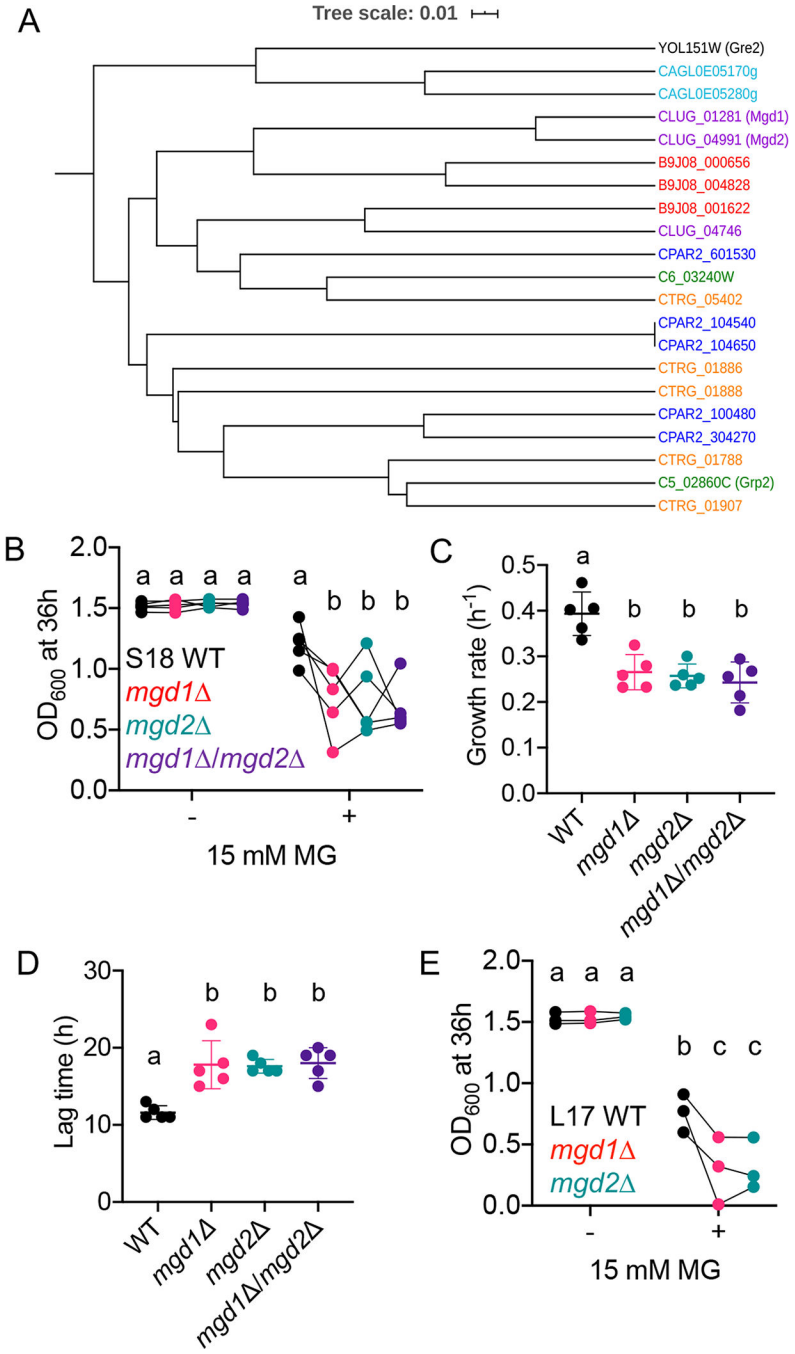

Fig. 2. *MGD1* and *MGD2* are required for fitness in the presence of high MG.

(A) Phylogeny of known and putative MG reductase based on amino acid sequences with homology to *C. albicans* Grp2, *S. cerevisiae* Gre2, and *C. lusitaniae* Mgd1 and Mgd2. *Candida* species is denoted by color: *C. lusitaniae* (purple); *C. auris* (red); *C. tropicalis* (orange); *C. parapsilosis* (blue); *C. glabrata* (teal) and *C. albicans* (green). **(B-D)** Growth of *C. lusitaniae* S18 WT (black), *mgd1*Δ (red), *mgd2*Δ (teal), and *mgd1*Δ/*mgd2Δ* (purple) strains in YPD with or without 15 mM MG in terms of OD_{600} after 36 h **(B)**, exponential growth rate **(C)**, and lag time **(D)**. Data shown represent the mean ± SD from five

independent experiments. **(E)** OD_{600} after 36 h of strain L17 WT (black), *mgd1*Δ (red), and *mgd2*Δ (teal) in YPD with or without 15 mM MG. Data shown represent the mean ± SD from three independent experiments. Ordinary two-way ANOVA with Tukey's multiple comparison test was used for statistical evaluation in **(B)** and **(E)**; a-b, a-c, b-c, $p < 0.05$. Data points connected by line in **(B)** and **(E)** are from the same experiment. Ordinary one-way ANOVA with Tukey's multiple comparison test was used for statistical evaluation in **(C)** and **(D)**; a-b, $p < 0.01$.

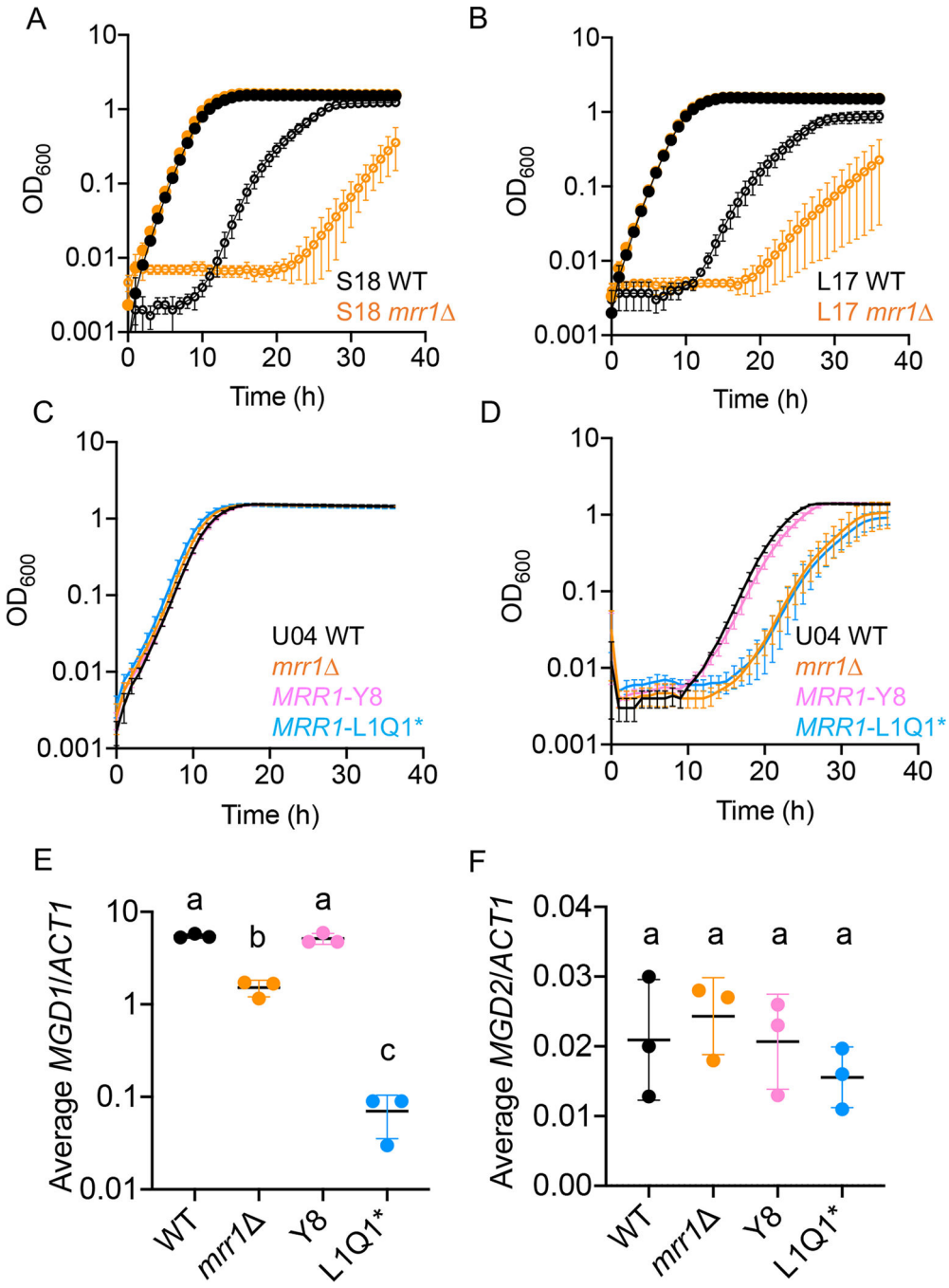

Fig. 3. Mrr1 regulates MG resistance and basal expression of *MGD1* but not *MGD2*.

(A-B) Growth curves of *C. lusitaniae* S18 **(A)** and L17 **(B)** wild type (black) and *mrr1*Δ (orange) in YPD alone (closed circles) or with 15 mM MG (open circles). One representative experiment out of three independent experiments is shown; error bars represent the standard deviation of technical replicates within the experiment. **(C-D)** Growth curves of *C. lusitaniae* U04 (black), U04 *mrr1*Δ (orange), U04 *mrr1*Δ + $MRR1^{Y8}$ (pink) and U04 *mrr1*Δ + $MRR1^{L1Q1*}$ (light blue) in YPD alone **(C)** or with 15 mM MG **(D)**. One representative experiment out of three independent experiments is shown; error

bars represent the standard deviation of technical replicates within the experiment. **(E-F)** Expression of *MGD1* **(E)** and *MGD2* **(F)** in *C. lusitaniae* U04 WT (black), U04 *mrr1*Δ (orange), U04 *mrr1*Δ + $MRR1^{Y8}$ (pink) and U04 *mrr1*Δ + $MRR1^{L1Q1*}$ (light blue). Data shown represent the mean ± SD from three independent experiments. Ordinary one-way ANOVA with Tukey's multiple comparison test was used for statistical evaluation in **(E-F)**; a-b and a-c, $p < 0.0001$; b-c, $p < 0.01$.

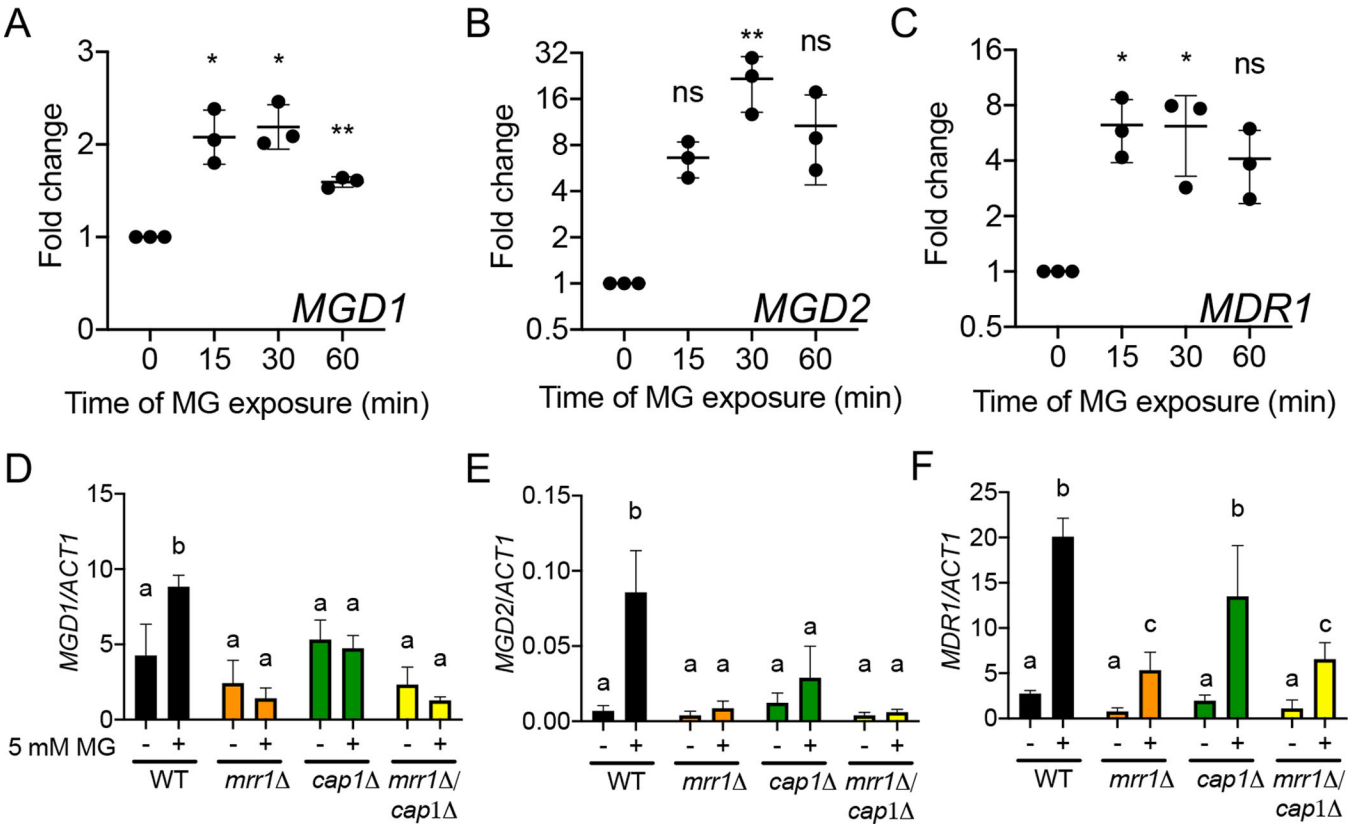

Fig. 4. Levels of *MGD1*, *MGD2*, and *MDR1* transcripts were increased in response to MG in a partially Mrr1- and Cap1-dependent manner.

(A-C) *C. lusitaniae* isolate S18 was grown to exponential phase at 30°C and treated with 5 mM MG for the time indicated prior to analysis of *MGD1* **(A)**, *MGD2* **(B)**, and *MDR1* **(C)** transcript levels by qRT-PCR. Transcript levels are normalized to levels of *ACT1* and presented as ratio at each time point relative to 0 min for three independent experiments. Error bars represent the standard deviation across the three independent experiments. Ordinary one-way ANOVA with Dunnett's multiple comparison test was used for statistical evaluation of each time point compared to t=0; * $p < 0.05$, ** $p < 0.01$, ns not significant. **(D-F)** *C. lusitaniae* S18 wild type (black) and *mrr1*Δ (orange), *cap1*Δ (green), and *mrr1*Δ/*cap1Δ* (yellow) mutants were grown to exponential phase at 30°C and treated with 5 mM MG for 15 minutes prior to analysis of *MGD1* **(D)**, *MGD2* **(E)**, and *MDR1* **(F)** transcript levels by qRT-PCR. Transcript levels are normalized to *ACT1*. Data shown represent the mean ± SD for three independent experiments. Ordinary two-way ANOVA with Tukey's multiple comparison test was used for statistical evaluation; a-b, a-c, and b-c $p < 0.05$.

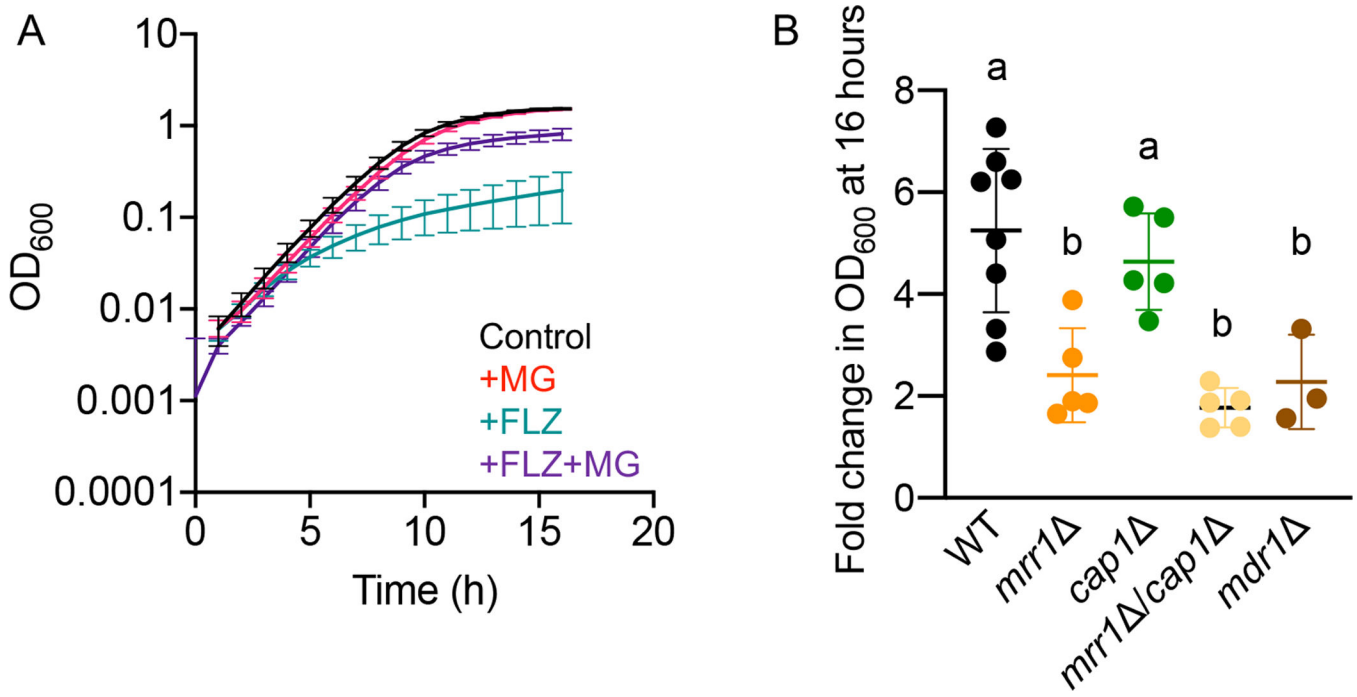

Fig. 5. MG increases FLZ resistance via *MRR1* and *MDR1*.

(A) *C. lusitaniae* isolate S18 was grown at 37°C in YPD alone (black), or with 5 mM MG (red), FLZ (equal to the MIC) (teal), or FLZ + 5 mM MG (purple). Data shown represent the mean ± SD for eight independent experiments. **(B)** Fold change in OD_{600} after 16 hours of growth for each indicated strain at 37°C in FLZ versus FLZ + 5 mM MG. Data shown represent the mean ± SD from at least three independent experiments. Ordinary one-way ANOVA with Tukey's multiple comparison test was used for statistical evaluation; a-b, $p < 0.05$.

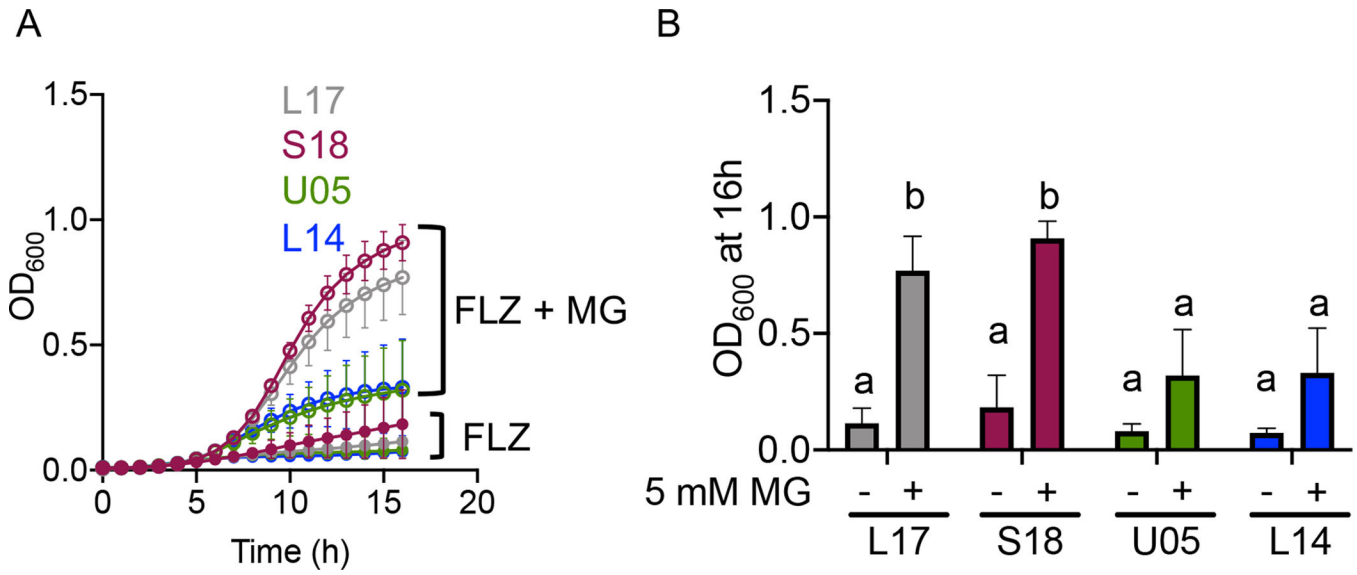

Fig. 6. Strains with a constitutively active Mrr1 variant show a greater increase in growth with FLZ by MG than strains with low activity Mrr1 variants.

C. lusitaniae isolates with low activity Mrr1 variants, U05 (green) and L14 (blue), or with constitutively active Mrr1 variants, L17 (grey) and S18 (dark red), were grown at 37°C in YPD with FLZ in the presence or absence of 5 mM MG. **(A)** Growth kinetics of isolates U05, L14, S18, and L17 in FLZ with (open circles) or without (closed circles) 5 mM MG. Data shown represent the mean ± SD from three independent experiments. **(B)** OD_{600} after 16 hours of growth for each indicated strain at 37°C in FLZ with or without 5 mM MG as indicated. Data shown represent the mean ± SD from three independent experiments. Ordinary two-way ANOVA Tukey's multiple comparison test was used for statistical analysis; a-b, $p < 0.05$.

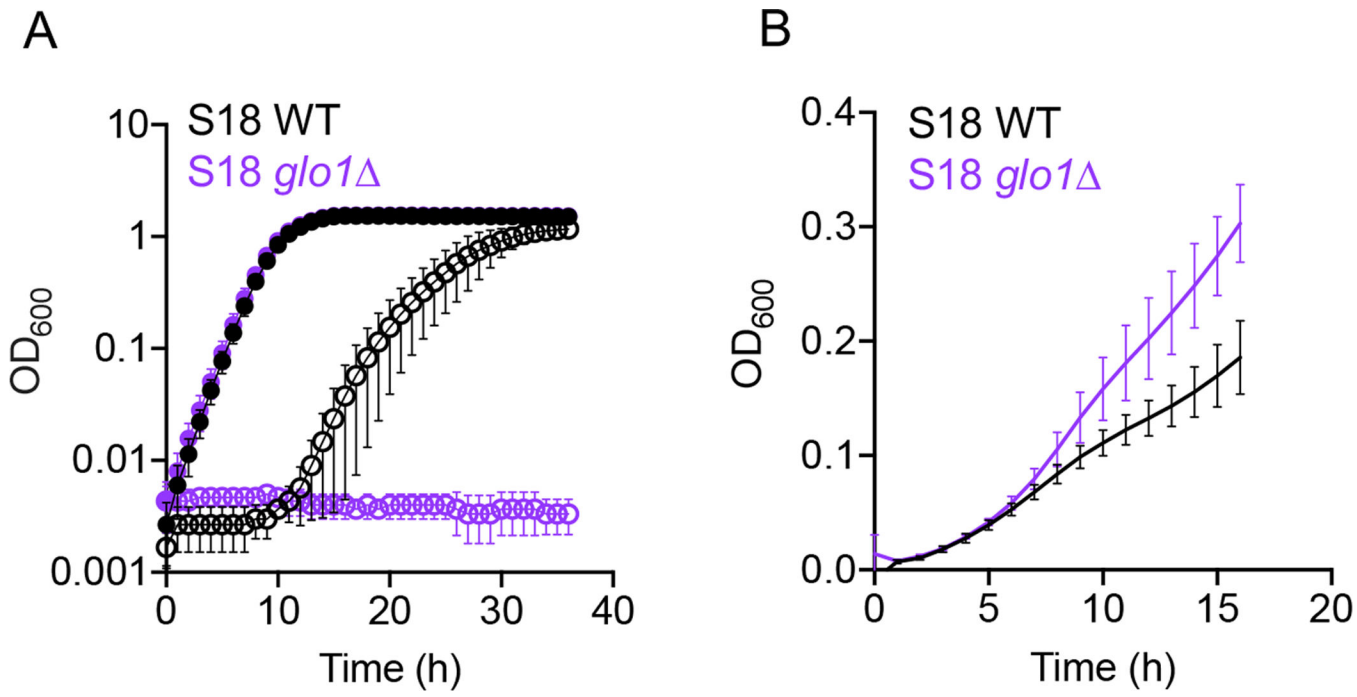

Fig 7. The absence of *GLO1*, which encodes a MG catabolizing enzyme, leads to increased sensitivity to MG and increased resistance to FLZ.

(A) *C. lusitaniae* S18 wild type (WT, black) and *glo1*Δ derivative (purple) were grown in YPD with (open circles) or without (closed circles) 15 mM MG. **(B)** Growth of S18 wild type (WT, black) and *glo1*Δ (purple) derivative in YPD with 8 μg mL^{-1} FLZ. Data shown represent the mean ± SD from three independent experiments.

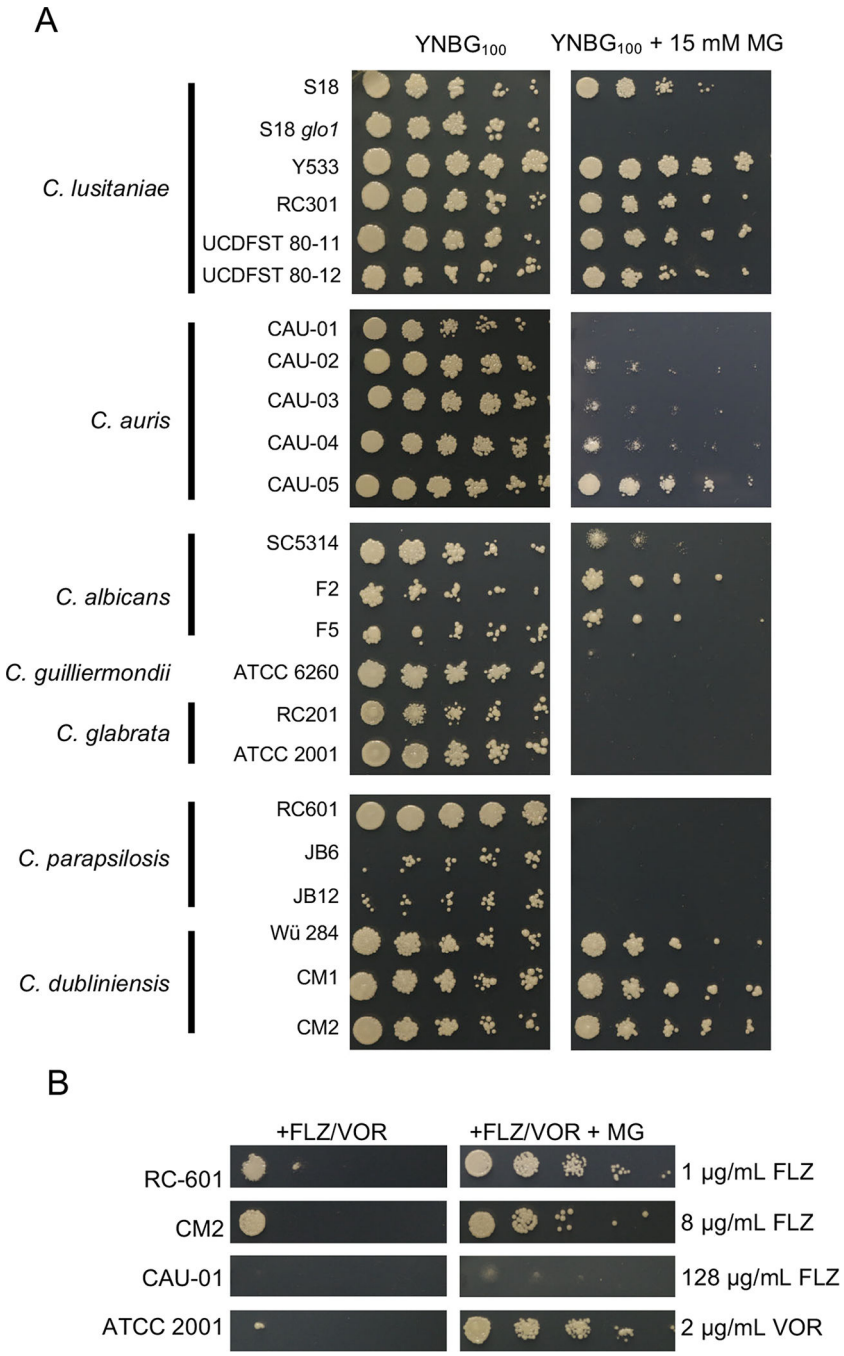

Fig 8. MG sensitivity and MG stimulation of azole resistance varies among *Candida* species and strains.

(A) Serial 1:10 dilutions of each *Candida* strain were spotted onto $YNBG_{100}$ plates without or with 15 mM MG, then grown at 37°C for two days. One representative out of three independent experiments is shown. **(B)** Serial 1:10 dilutions of *Candida* strains were spotted onto $YNBG_{100}$ plates containing the indicated concentration of FLZ or VOR without or with 3 mM MG. Only strains that demonstrated improved growth with the presence of 3 mM MG

are shown, the other strains are shown in Fig. S6. One representative experiment out of two independent experiments is shown.

Table 1.

FLZ MIC and relative Mrr1 activity of *C. lusitaniae* strains used in this paper

Strain	FLZ MIC (μg mL^{-1})	Relative Mrr1 activity
S18	8	High
S18 *mgd1*Δ	8	High
S18 *mgd2*Δ	8	High
S18 *mgd1*Δ/*mgd2*Δ	8	High
S18 *mrr1*Δ	4	N/A
S18 *cap1*Δ	8	High
S18 *mrr1*Δ/*cap1*Δ	4	N/A
S18 *mdr1*Δ	2	High
S18 *glo1*Δ	8	High
L17	8	High
L17 *mgd1*Δ	8	High
L17 *mgd2*Δ	8	High
L17 *mrr1*Δ	4	N/A
L17 *cap1*Δ	8	High
L17 *mdr1*Δ	2	High
U04	32	High
U04 *mrr1*Δ	4–8	N/A
U04 *mrr1*Δ + *MRR1*-Y8	32	High
U04 *mrr1*Δ + *MRR1*-L1Q1*	0.25 – 0.5	Low
U05	0.5 – 1	Low
L14	0.5 – 1	Low