**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 Candidaazole 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-independent MG catabolic enzyme. Isolates from other Candida species displayed heterogeneity in MG resistance and MG stimulation ofazole 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.

**KEYWORDS**

Candida, Candida lusitaniae, fluconazole, pyruvaldehyde

1 | 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 (Demers et al., 2018; Hiller et al., 2006; Jin et al., 2018; Wirsching et al., 2001), 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 (Demers et al., 2018; Dunkel et al., 2008; Morschhauser et al., 2007; Schubert et al., 2008). Many studies have focused on the relationship between Candida Mrr1 and resistance against clinical,
host, and microbiologically produced antifungal compounds (Demers et al., 2018; Dunkel et al., 2008; Hampe et al., 2017; Liu & Myers, 2017; Mogavero et al., 2011; Morschhauser et al., 2007; Schubert et al., 2011). 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 (Hoehamer et al., 2009; Karababa et al., 2004; Morschhauser et al., 2007; Rogers & Barker, 2003; Schubert et al., 2011), 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 20 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 19 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 (Demers et al., 2018; Hoehamer et al., 2009; Kannan et al., 2019; Karababa et al., 2004; Rogers & Barker, 2003; Schubert et al., 2011; Silva et al., 2011), 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 (Figure 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 (Takatsume et al., 2006; Zuin et al., 2005). Serum levels of MG are elevated in patients with diabetes (Lu et al., 2011; McLellan et al., 1994; Wang et al., 2019), sepsis (Brenner et al., 2014), and uremia (Karg et al., 2009; Lapolla et al., 2005; Mukhopadhyay et al., 2008; 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, 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 [Colour figure can be viewed at wileyonlinelibrary.com]

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.
2.1 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% identical to each other, and both have 59% and 58% identity to C. albicans Grp2 and S. cerevisiae Gre2, respectively (Chen et al., 2003; Kwak et al., 2018) (Figure 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 (Basenko et al., 2018; Stajich et al., 2012) (Figure 2a). An interesting phylogeny emerged among the homologs with at least 50% of 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 (Figure 2a). Candida glabrata has a pair of related putative MG reductases that are homologous to S. cerevisiae Gre2 (Figure 2a). The phylogeny of Grp2 homologs

![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).](image-url)

![Growth of C. lusitaniae S18 WT (black), mgd1Δ (red), mgd2Δ (teal), and mgd1Δ/mgd2Δ (purple) strains in YPD with or without 15 mM MG in terms of OD600 after 36 hr (B), exponential growth rate (C), and lag time (D). Data shown represent the mean ± SD from five independent experiments.](image-url)

![OD600 after 36 hr of strain L17 WT (black), mgd1Δ (red), and mgd2Δ (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 < .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 < .01.](image-url)
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 single mutants exhibited a significant defect in growth, but the WT (Figure S2a). With 5 mM MG as the sole carbon source, neither *mgd1Δ* nor *mgd2Δ* displayed a 26.8% reduction in yield (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 *mrr1Δ* derivative of U04 had an eightfold 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 Figure 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 (Figure 3c,d). We found that the *mrr1Δ* 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 (Figure 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 (Figure 3f).

### 2.3 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 (Figures 2 and S2), we tested whether MG induced their expression through Mrr1 in the S18 background. As shown in Figure 4a,b, 5 mM MG significantly induced expression of MGD1 by twofold at 15 min and MGD2 by 16-fold at 30 min in the unaltered S18 isolate. Expression of both genes remained elevated after 60 min 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 sixfold at 15 and 30 min, but as with MGD1 and MGD2, relative MDR1 levels began trending downward by 60 min (Figure 4c).

As *C. albicans* Mrr1 induces MDR1 in response to benomyl and hydrogen peroxide (H$_2$O$_2$) 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 Figure 4a-c, we used isogenic mrr1Δ, cap1Δ, and mrr1Δ/cap1Δ mutants in the S18 background. Consistent with the results in Figure 4a,b, MG induced expression of *MGD1* (Figure 4d) and *MGD2* (Figure 4e) by two-fold and 12-fold, respectively, in the S18 parental strain, while the mrr1Δ, cap1Δ, or mrr1Δ/cap1Δ derivatives of S18 did not exhibit a significant change in expression of either gene in response to 5 mM MG (Figure 4d,e). 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 (Figure 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 (Figure 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, mrr1Δ, cap1Δ, and mrr1Δ/cap1Δ mutants in control conditions, the S18 mrr1Δ and the S18 mrr1Δ/cap1Δ had significantly lower MDR1 levels than the WT and cap1Δ in medium with MG (Figure 4f). To confirm these results in strain L17, we repeated our analysis of MDR1 expression in the original isolate and its mrr1Δ and cap1Δ derivatives. In agreement with the results in Figure 4c,f, 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 (Figure S4). In L17, the cap1Δ 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
TABLE 1  FLZ MIC and relative Mrr1 activity of C. lusitaniae strains used in this paper

| Strain          | FLZ MIC (µg/ml) | Relative Mrr1 activity |
|-----------------|-----------------|------------------------|
| S18             | 8               | High                   |
| S18 mgd1Δ       | 8               | High                   |
| S18 mgd2Δ       | 8               | High                   |
| S18 mgd1Δ/mgd2Δ | 8               | High                   |
| S18 mrr1Δ       | 4               | N/A                    |
| S18 cap1Δ       | 8               | High                   |
| S18 mrr1Δ/cap1Δ | 4               | N/A                    |
| S18 mdr1Δ       | 2               | High                   |
| S18 glo1Δ       | 8               | High                   |
| L17             | 8               | High                   |
| L17 mgd1Δ       | 8               | High                   |
| L17 mgd2Δ       | 8               | High                   |
| L17 mrr1Δ       | 4               | N/A                    |
| L17 cap1Δ       | 8               | High                   |
| L17 mdr1Δ       | 2               | High                   |
| U04             | 32              | High                   |
| U04 mrr1Δ       | 4-8             | N/A                    |
| U04 mrr1Δ + MRR1-Y8 | 32         | High                   |
| U04 mrr1Δ + MRR1-L1Q1* | 0.25–0.5 | Low                    |
| U05             | 0.5–1           | Low                    |
| L14             | 0.5–1           | Low                    |

in the S18 mrr1Δ/cap1Δ 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.

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

Due to the induction of MDR1 expression by MG (Figure 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 (Figure S5a), it drastically improved growth in the presence of FLZ (Figure 5a), resulting in an OD_{600} at 16 hr that was, on average, 5.2-fold higher than in FLZ alone (Figure 5). The S18 mrr1Δ and mrr1Δ/cap1Δ mutants exhibited a significantly lower fold increase in yield at 16 hr in FLZ upon amendment of the medium with MG compared to the S18 parental strain, 2.4- and 1.8-fold, respectively, and S18 mdr1Δ was similar to the mrr1Δ and mrr1Δ/cap1Δ mutants (Figure 5b). The cap1Δ 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 (Figure 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 (Figure S5b), but it did lead to a robust stimulation of growth in FLZ, with an average fold change in OD_{600} of 8.5 (Figure S5c,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 (Figure S5d). Similar to the S18 background, the L17 mdr1Δ mutant exhibited a fold change in OD_{600} at 16 hr 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 (Figure S4), both Mrr1 and Cap1 contributed to increased FLZ resistance in the presence of MG (Figure S5d). The differences between the S18 and L17 backgrounds in the robustness of the cap1Δ 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.

2.5 | 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 affected 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 (Figure 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.

2.6 | 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 (Antognelli et al., 2019; Irshad et al., 2019; Moraru et al., 2018; Nokin 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 Figure 1) (Thornalley, 1996). Thus, we were interested in whether the S18 glo1Δ mutant (lacking CLUG_04105) was more resistant to FLZ than its parent in the absence of exogenously added MG. We found that S18 glo1Δ was highly sensitive to 15 mM MG (Figure 7a), even more so than the S18 mgd1Δ, mgd2Δ, and mgd1Δ/mgd2Δ mutants (Figure 2c). Although S18 glo1Δ had similar growth kinetics in YPD as the S18 WT (Figure 7a), the glo1Δ mutant grew substantially better in FLZ.

**FIGURE 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 < .05, **p < .01, ns not significant. (D-F) C. lusitaniae S18 wild type (black) and mrr1Δ (orange), cap1Δ (green), and mrr1Δ/cap1Δ (yellow) mutants were grown to exponential phase at 30°C and treated with 5 mM MG for 15 min 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 < .05 [Colour figure can be viewed at wileyonlinelibrary.com]

**FIGURE 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 hr 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 < .05 [Colour figure can be viewed at wileyonlinelibrary.com]
compared to its parent strain (Figure 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.

2.7 | 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 gloΔ, shown above to be highly sensitive to MG (Figure 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 (Figure 8a). Overall, the results in Figure 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 Figure 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 Figure 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 Figure 8b. C. parapsilosis strains were more sensitive to MG than C. glabrata ATCC 2001, exhibiting a striking increase of growth on VOR with MG. C. auris CAU-01 demonstrated a more subtle increase in growth with MG (Figure 8b).
Strains that did not demonstrate visible stimulation of growth on FLZ or VOR by MG under the tested conditions are shown in Figure 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.

3 | 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 Figure 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,
CoGRP2, 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 (Ferguson et al., 1998; Liu et al., 2011; Masterjohn et al., 2013). 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 (Takatsume et al., 2006; Zuin et al., 2005). 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 Mr1 in multiple *Candida* species have focused on the regulation and biological significance of only a small number of Mr1-regulated genes, primarily the two efflux pumps encoded by *MDR1* (Demers et al., 2018; Hiller et al., 2006; Jin et al., 2018; Wirsching et al., 2001) and FLU1 (Calabrese et al., 2000; Hampe et al., 2017; Li et al., 2013). Here, we show that isogenic *C. lusitaniae* strains with gain-of-function mutations in Mr1 led to higher levels of MGD1 and MGD2 transcripts, and higher resistance to exogenous MG (Figure 3d) than strains with low Mr1 activity. Furthermore, we showed that MG induced Mr1 activity to increase the expression of not just MGD1 and MGD2, but also MDR1. The coregulation 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 Mr1 variants.

While multiple chemical inducers of Mr1 activity have been described, including methotrexate, 4-nitroquinoline-N-oxide, o-phenanthroline, benomyl, diethyl maleate, diamide, and H$_2$O$_2$ (Harry et al., 2005; Mogavero et al., 2011; Schubert et al., 2011), little is known about why or how these inducers activate Mr1. It has been postulated that many of these compounds many directly or indirectly induce oxidative stress, which then activates Mr1. MG is especially interesting as a natural inducer of Mr1 activity because (a) it is produced by cells during metabolism and in vivo as an antimicrobial agent, (b) Mr1 regulates enzymes that specifically metabolize and detoxify this compound, and (c) it has similarly been documented to cause oxidative stress like other known inducers of Mr1 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 Mr1 and Cap1, a regulator of oxidative stress, can cooperate to regulate the expression of MDR1 in *C. albicans* (Mogavero et al., 2011; Schubert et al., 2011) and we found evidence that this can be the case in *C. lusitaniae*. We do not yet know if Mr1 or Cap1 is directly modified by MG. *C. lusitaniae* Mr1 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 (Figure 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 Mr1, but not induction by H$_2$O$_2$ (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 Mr1 activity and by extension FLZ treatment outcomes. Diabetes (reviewed in Rodrigues et al., 2019) and uremia (Jawale et al., 2018; Pyrgos et al., 2009) 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 (Maeta et al., 2004; Penninckx et al., 1983). The glyoxalase system, utilizing Glol and Glo2, requires reduced glutathione (GSH) to function (Figure 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 (Dickerhof et al., 2017; Kettle et al., 2014). 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 (Enkvetchakul & Bottje, 1995; Mitchell et al., 1983; Urban et al., 2017; Yamauchi et al., 2011; 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 (Figure 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 aeruginosa* (Juarez et al., 2017), and inhibits *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.

4 | METHODS

4.1 | Generation of MG reductase phylogenetic tree

Orthologs of CaGrp2 from *S. cerevisiae* and multiple *Candida* species were identified in FungiDB (https://fungidb.org) (Basenko et al.,...
2018; Stajich et al., 2012) 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).

### 4.2 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, and 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 in DMSO) or 3, 5, 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, and 2% glucose) on a rotary wheel at 30°C. E. coli strains were grown in LB with either 100 µg/ml carbenicillin (carb) or 15 µg/ml gentamycin (gent) as necessary.

### 4.3 Plasmids for complementation of MRR1

We amplified (a) the MRR1 gene and terminator with −1,150 bp upstream for homology from the appropriate strain's genomic DNA, (b) the selective marker, HygB from pYM70 (Basso et al., 2010), and (c) −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 NEX®-5-alpha competent E. coli (New England BioLabs). E. coli containing pMQ30 derived plasmids were selected for on LB containing 15 µg/ml gentamycin. Plasmids from E. coli were isolated using a Zippy 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.

### 4.4 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 min 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 nourseothricin (NAT) or 600 µg/ml 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 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 precursor plasmid pMQ30\textsuperscript{MRR1-L1191H+Q1197*-URA3, MRR1 with \textsuperscript{-1.150 bp upstream of MRR1 and separately –950 bp downstream of MRR1 were amplified from genomic U05 (containing MRR1\textsuperscript{L1191H+Q1197*}) DNA and C. albicans URA3 under the controls of the 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\textsuperscript{MRR1-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\textsuperscript{MRR1-L1191H+Q1197*-HygB. To create the pMQ30\textsuperscript{MRR1-Y813C-HygB plasmid, pMQ30\textsuperscript{MRR1-L1191H+Q1197*-HygB was linearized with XbaI and NotI to remove MRR1 and the upstream sequence. Replacement sequence including MRR1 with –1.150 bp upstream of MRR1 were amplified from U04 (MRR1\textsuperscript{Y813C}) gDNA. Plasmids created in S. cerevisiae were isolated using a yeast plasmid miniprep kit (Zymo Research) and transformed into High Efficiency NEX®-5-alpha competent E. coli (New England BioLabs). E. coli containing pMQ30 derived plasmids were selected for on LB containing 15 µg/ml gentamycin. Plasmids from E. coli were isolated using a Zippy 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.
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.

4.5 | 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$_2$O 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 down to 0.25 µg/ml 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 to 0.125 µg/ml. Plates were incubated at 35°C and scored for growth at 24 hr; 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.

4.6 | 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$_2$O. 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. A 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 min for 16 or 36 hr, end kinetic.

4.7 | 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$_2$O. Each strain was then serially diluted by 1:10 down to an OD$_{600}$ of approximately $1 \times 10^{-6}$. 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 in DMSO). Plates were incubated at 37°C for two days before imaging.

4.8 | 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 min. To harvest, 2 ml of culture was spun in a tabletop centrifuge at 13.2 x 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.

4.9 | 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 < .05 were considered to be significant for all analyses performed and are indicated with asterisks or letters in the text: *p < .05, **p < .01, ***p < .001, ****p < .0001. The graphical abstract was prepared using BioRender (biorender.com).

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CONFLICT OF INTERESTS

The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS

ARB, EGD, and DAH conceived and designed the experiments and wrote the paper. ARB and EGD performed the experiments. ARB, EGD, and DAH analyzed the data.

DATA AVAILABILITY STATEMENT

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

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.