Functional characterization and virulence study of ADE8 and GUA1 genes involved in the de novo purine biosynthesis in Candida albicans

Linghuo Jiang1,2, Jingwen Zhao2, Rui Guo2, Jing Li2, Liquan Yu1 & Deming Xu2

1Tianjin Research Center of Basic Medical Sciences, Tianjin Medical University, Tianjin, China; and 2Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, China

Correspondence: Deming Xu, Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Science and Technology, Tianjin 300072, China. Tel./fax: +86 22 2354 2035; e-mail: xu_deming@hotmail.com

Received 12 August 2009; revised 16 November 2009; accepted 6 December 2009. Final version published online 15 January 2010.

DOI:10.1111/j.1567-1364.2009.00600.x

Editor: Richard Calderone

Keywords
Candida albicans; ADE8; GUA1; systemic candidiasis; purine biosynthesis; C1-folate pool.

Abstract
Candida albicans is the principal human fungal pathogen that leads to life-threatening mycoses worldwide. To study its pathobiology, we characterized genes for two enzymes involved in the de novo purine biosynthesis pathway: ADE8 (encoding phosphoribosylglycinamide formyl-transferase) and GUA1 (GMP synthase). Heterozygous and homozygous disruption strains were constructed for both genes. We found that ADE8 and GUA1 are conditionally essential; i.e. can be bypassed in the presence of exogenous adenine and guanine, respectively, and that ADE8 plays an additional role in the C1-folate pool. Furthermore, the heterozygotes of ADE8/ade8 and GUA1/gua1 were hypersensitive to methotrexate (an inhibitor of de novo synthesis of tetrahydrofolate) and 6-azauracil (a known inhibitor of the IMP dehydrogenase involved in GMP biosynthesis), respectively. In a murine model of systemic candidiasis, the virulence of both heterozygous strains was marginally attenuated, while the ade8/ade8 and gua1/gua1 strains were completely avirulent. Our results and those of others indicate that many conditional essential genes involved in different biosynthesis pathways are required for systemic candidiasis, likely due to the host nutritional constraints imposed on the pathogen.

Introduction
Candida species persist as the single most important cause of opportunistic mycoses, and account for ~10% of bloodstream infections in the hospital setting in North America, with a crude mortality rate of ~40% (Pfaller & Diekema, 2007). A recent study revealed that over 10% of patients of liver transplantation in the Chinese hospitals had invasive fungal infection, with an overall mortality rate of ~35% (Shi et al., 2008). Candida albicans is the most dominant fungal pathogen, causing > 50% of all fungal infections (Pfaller & Diekema, 2007; Shi et al., 2008). Therapeutic antifungal drugs commonly used to treat mycoses can be divided into three major classes according to their mechanisms of action: azoles, echinocandins and polyenes. Despite their success in treating invasive candidiasis, long-term exposure to these drugs has raised serious concerns about their impact on epidemiology and drug resistance. For example, a survey on C. albicans from the general population revealed that over 5% of the isolates are resistant to fluconazole (Xu et al., 2008). Evidence from randomized clinical trials also indicated an increased risk of colonization of resistant Candida isolates associated with fluconazole prophylaxis (Brion et al., 2007). As non-albicans Candida spp. are generally less susceptible to antifungal drugs in all three groups (Pfaller & Diekema, 2007; Xu et al., 2008), the narrow mechanistic spectra of antifungal chemotherapy are increasingly being challenged by the emerging fungal epidemiology, compounded by the acquired resistance of Candida isolates to more than one drug (Krogh-Madsen et al., 2006; Laverdiere et al., 2006). There is an urgent need to develop antifungal agents with novel mechanisms of action, broader spectra and improved efficacy and safety. Identifying genes that are essential for systemic candidiasis in animal models will expand the repertoire of potential new antifungal drug targets.
Our understanding of the pathobiology of \textit{C. albicans} and efforts toward developing new technologies that explore suitable antifungal targets have been facilitated considerably by studies in the model yeast \textit{Saccharomyces cerevisiae}. However, the commensal \textit{C. albicans} is pathogenic. This important difference indicates that characterization of any genes (whether as potential antifungal targets or not) should include animal studies for their roles in systemic candidiasis. We are interested in conditional essential genes as most of them are involved in metabolism, their null mutations yield lethality that can be readily rescued by supplementing growth media with the appropriate nutrients. As such, homozygous mutants can be constructed under permissive conditions, their virulence tested directly in a murine model of candidiasis. Although it is conceivable that some of these conditional essential genes are not required for virulence in an animal (Noble & Johnson, 2005), others have been demonstrated experimentally for their essentiality \textit{in vivo}, and thus possible antifungal targets (Rodriguez-Suarez et al., 2007; Xu et al., 2009).

In this report, we present the characterization of two genes involved in the \textit{de novo} purine biosynthesis in \textit{C. albicans}: \textit{ADE8} and \textit{GUA1}. Because of the critical role of purine nucleotides as precursors to DNA and RNA, pharmacological inhibition of this pathway has been suggested as a viable approach toward anticancer chemotherapy (Berman & Werbel, 1991). In particular, the enzyme that catalyzes the third step in the pathway, phosphoribosylglycinamidase formyl-transferase [glycinamide ribonucleotide transformylase (GART), Ade8p in \textit{S. cerevisiae} and \textit{C. albicans}], is the target of antifolate compounds (Kaye, 1998). More recently, a small-molecule inhibitor of GMP synthase (fungal Gua1p) was identified as a potent antifungal compound (Rodriguez-Suarez et al., 2007). Prompted by possible chemical inhibition of these two fungal enzymes, we sought to test the hypothesis that the \textit{de novo} purine biosynthesis is essential for systemic candidiasis. We constructed heterozygous and homozygous mutants for both genes, and tested them for virulence in a mouse model of systemic candidiasis. We also determined changes in the susceptibility of the heterozygous mutants to antimetabolites that target the \textit{de novo} purine biosynthesis and other pathways.

\section*{Materials and methods}

\subsection*{Strains, media and chemicals}

The \textit{C. albicans} strain CAI4 (ura3::\lambda. \textit{imm434/ura3::\lambda. \textit{imm434}}) and the \textit{hisG-URA3-hisG} (p5921) cassette were described in Fonzi & Irwin (1993). Synthetic complete (SC) and dropout, and yeast nitrogen base dextrose (YNBD) media supplemented with appropriate nutrients for selection and maintenance were used. Methotrexate, 6-azauracil (6AU) and other chemicals were purchased from Sigma (Beijing, China). Transformation for \textit{C. albicans} cells was carried out as described (Lee et al., 2004).

\section*{Chromosomal disruption of \textit{ADE8} and \textit{GUA1}}

We noted that the two alleles of \textit{ADE8} (orf19.5789 and orf19.13211) are polymorphic in DNA and protein sequences. According to the \textit{Candida} Genome Database, the ORF of orf19.13211 is extended by 51 amino acids at the N-terminus. However, the annotated ORF of orf19.5789 can also be extended by 50 amino acids. The extended portions of both are highly conserved with 49 identical amino acids, and are absent in GARTs from other organisms. To avoid complication, we designed primers from regions that are identical in both alleles to construct the disruption cassette. The 5' genomic fragment was amplified from CAI4 genomic DNA by two primers \textit{ADE8-F1} (5'-CGGGATCCATGG CAAAAGTCTCGTTG, BamHI site underlined) and \textit{ADE8-R1} (5'-GGTCTTAGTTGTACCCG-G). This fragment (~600 bp), containing an internal BamHI site near the region corresponding to \textit{ADE8-R1}, was cloned at the BamHI site in p5921. The 3' fragment (~700 bp, containing an internal KpnI site near the \textit{ADE8-R2} region) was amplified by \textit{ADE8-F2} (5'-GGGGATCTACTACAAAGGAAACCAC, KpnI site underline) and \textit{ADE8-R2} (5'-CAGAATGGTACTTT GAAAGG) primers, and cloned at the KpnI site on the other side of the \textit{hisG-URA3-hisG} cassette. The orientation of both fragments was determined as described previously (Zheng et al., 2007). In the resulting plasmid, the \textit{ADE8/orf19.5789} ORF was deleted from +56 to +186 nt, and replaced with the \textit{hisG-URA3-hisG} cassette. It was linearized by SalI digestion before transformation. Primers \textit{ADE8-F1} and \textit{ADE8-R2} were used to amplify a genomic fragment of \textit{ADE8} (~1.5 kb), which was transformed into the \textit{ade8/ade8} homozygous mutant to reconstruct a heterozygote.

The \textit{GUA1} disruption cassette was constructed in a similar manner with genomic fragments (5' portion, ~600 bp; 3' portion ~400 bp) amplified by two pairs of primers, \textit{GUA1-F1} (5'-GTGAAAACCTCACGTGGACG, SalI site underlined) and \textit{GUA1-R1} (5'-GGGGATCCACGG GAAATGTAACAAATC, BamHI site underlined), \textit{GUA1-F2} (5'-GGGGATCTACTACAAAGGAAACCAC, KpnI site underlined) and \textit{GUA1-R2} (5'-CAACCTTAAACATGTTACCCG, KpnI site underlined). In the resulting cassette, a \textit{GUA1} fragment of ~1.5 kb (from −156 to +1351 nt of ORF) was deleted and replaced with the \textit{hisG-URA3-hisG} cassette. The restriction enzymes SacI and HindIII were used to linearize the plasmid. A genomic fragment of \textit{GUA1} (amplified with primers \textit{GUA1-F1} and \textit{GUA1-R2}) was cloned in the \textit{URA3}-marked vector pCR4 (Rocha et al., 2001); the resulting plasmid was used to transform the \textit{gua1/gua1} mutant to generate revertants.
Test of drug susceptibility

For spot tests, overnight cultures of selected strains in SC medium were used to inoculate fresh cultures in YNBD supplemented with 2.5 mM adenine at the initial OD$_{600}$ nm 0.5. After a 6-h growth at 30 °C, each culture was diluted to OD$_{600}$ nm 0.02 with the YNBD, followed by 1/5 and 1/25 dilutions. Aliquots of 3 µL were spotted on solid YNBD media with or without chemical(s) as indicated. Plates were photographed after a 2-day growth at 30 °C. For liquid assay, 1/2 serial dilutions (in 75 µL YNBD) of mycophenolic acid (MPA) and 6AU were performed in 96-well microtiter plates at 2 × final concentrations. Mid-log cultures (YNBD) of selected strains were diluted to OD$_{600}$ nm 0.02, and aliquots of 75 µL were dispensed in wells containing an equal volume of YNBD with or without compounds at 2 × final concentrations. OD$_{600}$ nm was measured following a 20-h growth at 30 °C. The relative growth at each concentration was normalized by the mock treatment, and expressed as % growth of the latter.

Test of virulence in a murine model of candidiasis

Candida albicans strains were streaked out on yeast peptone dextrose (YPD) media supplemented with adenine (2.5 mM) or guanine (0.3 mM). After overnight growth at 30 °C, they were used to inoculate 50 mL liquid YPD with the appropriate nutrients at an initial OD$_{600}$ nm 0.005. Cultures were harvested after a 20-h growth at 30 °C, washed twice with saline and suspended in saline at OD$_{600}$ nm 1. Inbred male BALB/c mice at 8 weeks of age were injected via the lateral tail vein with 100 µL of cell suspension (i.e. ~1.0 × 10$^6$ cells). The remainder of each suspension was diluted and determined for viable cells mL$^{-1}$. The injected mice were kept for 3 weeks and examined daily. On the 21st day, all the surviving animals were sacrificed and the kidneys were ground to determine fungal burden (CFU g$^{-1}$). Animal experiments were performed according to the guidelines established by the Ethics Committee of Tianjin Medical University, Tianjin, China.

Results

We selected two genes encoding enzymes involved in two branches of the de novo purine biosynthesis pathway in C. albicans: ADE8/orf19.5789 and GUA1/orf19.4813 (Fig. 1). While Gua1p, a GMP synthase, is involved exclusively in the final step of GMP synthesis converting XMP to GMP using glutamine as the donor of the amino group, Ade8p is a
10-formyltetrahydrofolate (10-formyl-THF)-dependent phosphoribosylglycinamide formyl-transferase (GART) involved in both an early step of the purine biosynthesis and the C1-folate pool (Fig. 1, and inset). We constructed sequentially heterozygous and homozygous disruption strains for \( \text{ADE8} \) and \( \text{GUA1} \), based on the assumption that both \( \text{ade8/ade8} \) and \( \text{gua1/gua1} \) mutants are auxotrophic.

**Chromosomal disruption of ADE8**

The \( \Delta \text{ade8::hisG-URA3-hisG} \) disruption cassette was transformed into the \( \text{C. albicans} \) strain CAI4 to generate \( \text{ade8/ADE8} \) heterozygotes. The transformants, selected on SC-URA medium, were determined by PCR for correct disruption of one allele of \( \text{ADE8} \) via homologous recombination (data not shown). Two independent mutants of \( \Delta \text{ade8::hisG-URA3-hisG/ADE8} \) (8a1 and 8a2 in Fig. 2a) were counter-selected on SC medium containing 1 mg mL\(^{-1}\) 5-FOA and 2.5 mM uridine. The resulting resistant cells (\( \alpha' \) in Fig. 2a) of both isolates were then transformed with the same cassette to generate \( \text{ade8/ade8} \) homozygotes. None of the \( \Delta \text{ade8::hisG-URA3-hisG/ade8::hisG} \) mutants, confirmed by PCR, grew on medium without adenine (data not shown). Two independent \( \text{ade8/ade8} \) mutants were chosen for each heterozygote (8b1-1, -2 and 8b2-1, -2 in Fig. 2a). They were transformed with a genomic fragment (~1.5 kb) of \( \text{ADE8} \), and selected on SC-ADE medium for revertants (8c1-1, -2 and 8c2-1, -2 in Fig. 2a), whose genotype was confirmed by PCR (data not shown).

**Characterization of ade8/ADE8 and ade8/ade8 mutants**

We characterized all heterozygous and homozygous mutants for adenine auxotrophy and growth defects. As there was no difference between isolates of heterozygote or homozygote (data not shown), representative results are shown here (Fig. 2b). No phenotypic defects were observed with the \( \Delta \text{ade8::hisG-URA3-hisG/ade8::hisG} \) mutants, confirmed by PCR, grown on medium without adenine (data not shown). Two independent \( \text{ade8/ade8} \) mutants were selected for each heterozygote (8b1-1, -2 and 8b2-1, -2 in Fig. 2a). They were transformed with a genomic fragment (~1.5 kb) of \( \text{ADE8} \), and selected on SC-ADE medium for revertants (8c1-1, -2 and 8c2-1, -2 in Fig. 2a), whose genotype was confirmed by PCR (data not shown).
that ADE8/orf19.5789 is involved in adenine biosynthesis. However, the ade8/ade8 mutant displayed a phenotype of slower growth on YNBD (+adenine) than SC media (Fig. 2b).

As depicted in Fig. 1, GART (Ade8p) is involved in not only adenine biosynthesis but also the C1-folate pool, the latter of which is the reservoir of derivatives of one-carbon (C1) units, such as methyl (–CH3), methylene (–CH2–), formyl (–CH = O), formimino (–CH = HN) and methenyl (–CH =), attached to 5,6,7,8-tetrahydrofolate (THF) at the 5 and/or the 10 position(s). The interconversion of THF with different C1 units is coupled with various metabolic reactions, in particular, those in the amino acid biosynthesis and the de novo purine pathway (see KEGG Pathway Database for details). The growth defects associated with ade8/ade8 on YNBD medium containing adenine (Fig. 2b) are likely due to impaired amino acid biosynthesis caused by perturbation of the C1-folate pool, as they were not observed on medium containing amino acids (i.e. SC) (Fig. 2b). The adenine auxotrophy and the growth defects on YNBD medium were due to disruption of ADE8, because both were rescued fully by the reintroduction of one allele of ADE8 into the ade8/ade8 strain (Fig. 2b).

We sought to test the hypothesis that the slow growth phenotype of ade8/ade8 on YNBD medium (supplemented with adenine) reflects the role of Ade8p in the C1-folate pool. The reaction catalyzed by GART generates THF (Fig. 1, inset). Other enzymes also convert 10-formyl-THF into THF. However, we reasoned that Ade8p plays a more prominent role in this conversion (see Discussion). By impairing other sources of THF, it is possible to exacerbate the aforementioned growth defects. Conversely, an exogenous supply of tetrahydrofolate could reverse the growth defects. Methotrexate is a known inhibitor of the dihydrofolate reductase involved in the biosynthesis of THF. Although methotrexate is not a potent antiproliferative compound against C. albicans on solid media, at 200 μg mL−1, it had minimal inhibitory effects on the control strain SC5314 (and other heterozygote controls, data not shown) on YNBD medium with or without adenine; the ade8/ade8 heterozygote was preferentially hypersensitive to the compound on the adenine-containing medium (Fig. 3b). This hypersensitivity was further enhanced in the absence of adenine (Fig. 3b), even though the heterozygote displayed no growth defects otherwise (Fig. 3a). As expected, the growth defects of the ade8/ade8 mutant in the presence of adenine were exacerbated by methotrexate (Fig. 3a and b). On the other hand, exogenous folinic acid (5-formyl-THF) and adenine fully rescued the homozygote. However, by itself, folinic acid failed to suppress ade8/ade8 (Fig. 3c). These results further confirmed the essential role of Ade8p in adenine biosynthesis, and its additional, but prominent function in the C1-folate pool (see Discussion for details).

**Chromosomal disruption of GUA1**

The gua1/GUA1 and gua1/gua1 mutants were constructed in a manner similar to the ADE8 counterparts using the Δgua1::hisG-URA3-hisG cassette, with the appropriate selective conditions (with or without 0.3 mM guanine and/or uridine) on SC media (Fig. 4a). As expected, the gua1/GUA1 mutant displayed no detectable growth defects under the standard conditions (SC, YPD and YNBD media), with or without exogenous guanine (Fig. 4b, and data not shown). The homozygous disruption of GUA1

---

**Fig. 3.** Hypersensitivity of the Δade8/ΔADE8 mutants to methotrexate and full rescue of the Δade8/Δade8 mutant by adenine and folinic acid. Spot tests were used to determine the growth of selected strains on YNBD media with (+) or without (–) exogenous adenine (2.5 mM) containing methotrexate (b) and folinic acid (c) at the concentrations indicated. The mock treatment is shown in (a). Strain 8c1-1 was the revertant of strain 8b1-1 in which ADE8 was reintroduced (see Fig. 4a). Note that both Δade8/ΔADE8 mutants were hypersensitive to methotrexate (b), and that the slow growth of the Δade8/Δade8 mutant (in the presence of adenine) was exacerbated by methotrexate (b), but rescued by folinic acid (c). The results of a 2-day growth at 30 °C are shown.
conferred a guanine auxotrophy that was fully rescued in media supplemented with guanine (Fig. 4b, and data not shown). To rescue the homozygous mutant genetically, we introduced a 2.6-kb genomic fragment of GUA1 carried on a UR3-marked plasmid into a gua1/gua1 strain in which the UR3 marker was excised (b in Fig. 4a). This plasmid, not the vector alone, reversed the guanine auxotrophy fully (Fig. 4b), indicating that the observed phenotype is associated with the disruption of GUA1. The C. albicans GUA1 gene and its requirement in systemic candidiasis were characterized in an independent study (Rodriguez-Suarez et al., 2007). One of our original objectives of selecting this gene was to explore its potential as an alternative marker for the construction of deletion strains. However, our gua1/gua1 strain could not survive storage at 4 °C for more than a week.

Characterization of the gua1/GUA1 mutant

The GMP synthase (Gua1p) synthesizes GMP by transferring the amino group from glutamate to XMP. The enzyme responsible for the synthesis of XMP, IMP dehydrogenase (IMPDH), is usually rate-limiting in the de novo synthesis of guanine nucleotides. It is also a medically important target for antimetabolite, antimicrobial, anticancer and immuno-suppressive drugs (Shu & Nair, 2008), such as mycophenolic acid (MPA), 6-azauracil (6AU), tiazofurin and ribavirin. We tested the heterozygous mutants (a and a' in Fig. 5a) against MPA and 6AU. As reported (Rodriguez-Suarez et al., 2007), neither heterozygote displayed any changes in susceptibility to MPA as compared with the wild-type and other heterozygous strains (Fig. 5a and data not shown). Because uridine suppresses the activity of 6AU (unpublished data), we tested...
In the first set of experiments of a virulence study in the murine model of candidiasis, we tested the conditional essentiality of two genes, ADE8 and GUA1, involved in the de novo purine biosynthesis in C. albicans in relation to their essentiality for growth and virulence in the murine model of systemic infection. Homozygous disruption of both genes conferred auxotrophy for adenine and guanine, respectively (Figs 2 and 4). Neither disruption resulted in lethal candidiasis in mice (Fig. 6). We provided evidence for the dual functions of ADE8 in adenine metabolism and maintenance of the C1-folate pool (Fig. 3). We also demonstrated that chemical perturbation of the relevant metabolic pathways could impair preferentially the growth of the heterozygous mutants of both ADE8 and GUA1 (Figs 3 and 5) and that the virulence of both ade8/ADE8 and gua1/GUA1 mutants was attenuated in vivo, albeit marginally (Fig. 6).

**Discussion**

In this study, we characterized two genes, ADE8 and GUA1, involved in the de novo purine biosynthesis in C. albicans in relation to their essentiality for growth and virulence in the murine model of systemic infection. Homozygous disruption of both genes conferred auxotrophy for adenine and guanine, respectively (Figs 2 and 4). Neither disruption resulted in lethal candidiasis in mice (Fig. 6). We provided evidence for the dual functions of ADE8 in adenine metabolism and maintenance of the C1-folate pool (Fig. 3). We also demonstrated that chemical perturbation of the relevant metabolic pathways could impair preferentially the growth of the heterozygous mutants of both ADE8 and GUA1 (Figs 3 and 5) and that the virulence of both ade8/ADE8 and gua1/GUA1 mutants was attenuated in vivo, albeit marginally (Fig. 6).

**In vitro essentiality vs. in vivo essentiality**

Genes for metabolic enzymes (amino acids, nucleotides, fatty acids, unsaturated fatty acids, etc.) usually show conditional essentiality. Their requirements can be readily bypassed by supplementing the appropriate nutrients in media (Zhao et al., 1997; Roemer et al., 2003; Noble & Johnson, 2005; Rodriguez-Suarez et al., 2007; Xu et al.,
2007). Nevertheless, not all conditional essential genes are indispensable for systemic candidiasis in vivo. The best example is the URA3 gene involved in pyrimidine biosynthesis. Not only is it required for virulence but also the level of its expression (at different chromosomal locations) could affect the outcome of systemic candidiasis (Brand et al., 2004). Other genes in this group include ADE8 (this study), GUAI (this study; Rodriguez-Suarez et al., 2007) and others (unpublished data) involved in purine biosynthesis; FAS1 and FAS2 (fatty acid synthase complex, Zhao et al., 1997; Xu et al., 2009); OLE1 (fatty acid desaturase, Xu et al., 2009). In the case of OLE1, increases in dietary monounsaturated fatty acids failed to reverse, even partially, the avirulence of its genetic inactivation in hematogenously disseminated candidiasis (Xu et al., 2009). It is most likely that the nutritional conditions in a mammalian host are such that only the levels of (certain) amino acids are compatible with the requirements of auxotrophic fungal cells. The in vivo essentiality of other conditional essential genes clearly demonstrates the nutritional constraints that the commensal C. albicans has to overcome in order to establish and maintain a systemic infection. The glyoxylate cycle serves as another example that illustrates the host nutritional constraints imposed on the pathogen (Lorenz & Fink, 2001). These metabolic enzymes confer alternative antifungal targets based on their essentiality in vivo.

**Metabolic perturbation and haploinsufficiency**

We noted that both ade8/ADE8 and gua1/GUA1 strains were attenuated in their virulence (Fig. 6). Although it cannot be ruled out that this was caused by the positioning effect of the URA3 marker, similar to the observations by Brand et al. (2004), the two strains were hypersensitive to methotrexate and 6AU, respectively (Figs 3b and 5b). Methotrexate inhibits the dihydrofolate reductase involved in the last step of the de novo biosynthesis of THF. There are two 10-formyl-THF-dependent transformylases in the purine pathway: Ade8p (third step) and Ade17p (the last two steps in the synthesis of IMP) (Fig. 1). However, only the ade8/ADE8 mutant was hypersensitive to methotrexate (Fig. 3b, and unpublished data). The two alleles of ADE8 are polymorphic in DNA and protein sequences (see Materials and methods for details). Because of minor differences annotated in the database, we could not distinguish which allele was disrupted in the heterozygotes. However, because all the biosynthesis are not required in vivo. This was clearly demonstrated by the wild-type or the nearly wild-type virulence of C. albicans strains in which genes for the amino acid biosynthesis (ARG4, HIS1 and LEU2) were deleted individually or in combination (as many as three) (Noble & Johnson, 2005).

On the other hand, genes for other metabolic enzymes that have been characterized are without exception indispensable for systemic candidiasis in vivo. The case for ADE8 involves in pyrimidine biosynthesis. Not only is it required for virulence but also the level of its expression (at different chromosomal locations) could affect the outcome of systemic candidiasis (Brand et al., 2004).

Other genes in this group include ADE8 (this study), GUAI (this study; Rodriguez-Suarez et al., 2007) and others (unpublished data) involved in purine biosynthesis; FAS1 and FAS2 (fatty acid synthase complex, Zhao et al., 1997; Xu et al., 2009); OLE1 (fatty acid desaturase, Xu et al., 2009). In the case of OLE1, increases in dietary monounsaturated fatty acids failed to reverse, even partially, the avirulence of its genetic inactivation in hematogenously disseminated candidiasis (Xu et al., 2009). It is most likely that the nutritional conditions in a mammalian host are such that only the levels of (certain) amino acids are compatible with the requirements of auxotrophic fungal cells. The in vivo essentiality of other conditional essential genes clearly demonstrates the nutritional constraints that the commensal C. albicans has to overcome in order to establish and maintain a systemic infection. The glyoxylate cycle serves as another example that illustrates the host nutritional constraints imposed on the pathogen (Lorenz & Fink, 2001). These metabolic enzymes confer alternative antifungal targets based on their essentiality in vivo.
heterozygotes tested were hypersensitive to methotrexate (data not shown), we suspect that both alleles are functionally equivalent. Furthermore, adenine rescued ade17/ade17 mutants on YNBD medium fully (unpublished data), whereas the homozygous disruption of ADE8 conferred slow growth under the same conditions (Fig. 2b), a defect rescued by exogenous folic acid (Fig. 3c). These results suggest a more prominent role for Ade8p (as opposed to Ade17p) in the C1-folate pool, as the conversion of 10-formyl-THF to THF is crucial in the flow of the C1 pool. A perturbation of the de novo source of THF by methotrexate could preferentially impair the growth of a strain in which one allele of ADE8 (but not ADE17) is deleted. The observed haploinsufficiency (phenotypic defects associated with a heterozygous deletion) reflects the susceptibility of a 50% loss of gene dosage to specific chemical perturbation (e.g. Rodriguez-Suarez et al., 2007). We speculate that the attenuated virulence of the ade8/ADE8 mutant (Fig. 6a; i.e. haploinsufficiency in vivo) could be related to the metabolic constraints encountered in the animal host, which reduces its fitness.

The drug 6AU reduces the intracellular GTP and UTP levels by inhibiting the biosynthetic enzymes IMPDH (Imh3p) and orotidylate decarboxylase (Ura3p), respectively (Exinger & Lacroute, 1992). The gua1/GUA1 strain was hypersensitive to 6AU, but not to MPA, another inhibitor of IMPDH (Fig. 5). However, 6AU is suppressed by uridine, but not by guanine. MPA, on contrast, is suppressed by guanine (unpublished data). This raises the possibility that the hypersensitivity of the ADE8/GUA1 mutant is due to one copy of URA3 (similar to haploinsufficiency), or a different level of URA3 expression at the GUA1 locus, or the synergistic effect of heterozygosity of both genes. However, the Ade8p/GART mutant displayed a susceptibility similar to that of SC5314 (Fig. 5b). The precise mechanistic cause of the hypersensitivity of the GUA1 heterozygote to 6AU remains to be determined. Caution must be exercised when interpreting these results. These ambiguities argue in favor of the disruption methods by Noble & Johnson (2005), and interpreting these results. These ambiguities argue in favor of the disruption methods by Noble & Johnson (2005), illustrating the intrinsic problems associated with disrupting/deleting genes for metabolic enzymes with other nutritional selective markers and the biological consequences (e.g. synthetic effect of homozygous deletion and low levels of URA3 expression).

Ade8p/GART as an antifungal target

In humans, GART is part of a large trifunctional enzyme that also catalyzes the second and fifth steps of the de novo purine biosynthesis. These three steps are catalyzed by three enzymes in bacteria and plants, but two in fungi (Supporting Information, Fig. S1a). Because the GART uses 10-formyl-THF as a cofactor, this part of the human trifunctional enzyme has been an anticancer target of THF analogues (Kaye, 1998). The fungal GARTs are phylogenetically diverged from the human and other enzymes (Fig. S1b and c). They are relatively small in size (~220 amino acids). As demonstrated, ADE8 is most likely essential for systemic candidiasis in mice (Fig. 6a), and the corresponding enzyme could be an antifungal drug target. Furthermore, the enhanced susceptibility of the ade8/ADE8 mutant to methotrexate (Fig. 3) suggests that inhibitors of Ade8p should also elicit specific hypersensitivity in the heterozygote. If so, it provides a convenient chemical genetic means for screening such inhibitors. Suppression by exogenous adenine or folic acid can then be used to distinguish whether candidate compounds affect the activities of Ade8p or the metabolism of folate. The question remains as to whether it is possible to identify inhibitors of the GART that are specific to the fungal enzyme, given that these enzymes are likely functionally conserved.

Acknowledgements

We wish to thank other members of the group for their valuable comments and technical assistance during the course of this study. We also thank L. Kasprzak for critically reading the manuscript. This work was supported by the National Natural Science Foundation of China grants (no. 30870107 and 30571047).

References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Comparison of GARTs.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.