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Role of Nitrogen and Carbon Transport, Regulation, and Metabolism Genes for *Saccharomyces cerevisiae* Survival In Vivo†

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Saccharomyces cerevisiae **is both an emerging opportunistic pathogen and a close relative of pathogenic *Candida* species. To better understand the ecology of fungal infection, we investigated the importance of pathways involved in uptake, metabolism, and biosynthesis of nitrogen and carbon compounds for survival of a clinical *S. cerevisiae* strain in a murine host. Potential nitrogen sources in vivo include ammonium, urea, and amino acids, while potential carbon sources include glucose, lactate, pyruvate, and fatty acids. Using mutants unable to either transport or utilize these compounds, we demonstrated that no individual nitrogen source was essential, while glucose was the most significant primary carbon source for yeast survival in vivo. Hydrolysis of the storage carbohydrate glycogen made a slight contribution for in vivo survival compared with a substantial requirement for trehalose hydrolysis. The ability to sense and respond to low glucose concentrations was also important for survival. In contrast, there was little or no requirement in vivo in this assay for any of the nitrogen-sensing pathways, nitrogen catabolite repression, the ammonium- or amino acid-sensing pathways, or general control. By using auxotrophic mutants, we found that some nitrogenous compounds (polyamines, methionine, and lysine) can be acquired from the host, while others (threonine, aromatic amino acids, isoleucine, and valine) must be synthesized by the pathogen. Our studies provide insights into the yeast-host environment interaction and identify potential antifungal drug targets.**

Saccharomyces cerevisiae is an emerging opportunistic pathogen that can cause clinically relevant infections in a variety of patient types and at different body sites (32, 65, 75). This feature, together with the genetic tractability, well annotated genome sequence, close phylogenetic relationship to the more clinically prevalent *Candida* species (5, 58), and ability to infect mice (10, 14), has recently led researchers to develop clinical *S. cerevisiae* isolates as a model to identify fungal and host features that are important for fungal virulence and survival in vivo. Specifically, this model has been used to determine that some genes or processes important for survival of other fungal pathogens in vivo are also important for *S. cerevisiae* survival in vivo (survival at high temperature [64], *ADE2*, and *URA3* [28]) and has identified novel survival or virulence determinants (*LEU2*, respiration, the mitochondrial genome [28], and *SSD1* [100]). In addition, complement has been identified as an important host factor for resistance to *S. cerevisiae* infection (10), as it is for other fungi (33, 34, 80).

In addition to virulence factors, which are required for evasion of host defenses, invasion of host tissue, and causing symptoms of disease, factors that permit the survival and proliferation of a fungal pathogen once infection is established are also essential for fungal pathogenicity. To better understand the ecology of fungal infection, we are investigating yeast genes and pathways required for survival in vivo. The ability of a microbe to survive in vivo is determined by a complex interplay between the host environment and the microbe. For example, maintaining a constant supply and metabolism of carbon and nitrogen compounds by yeast in vivo first depends on the concentration and quality of nitrogen and carbon sources available to yeast in that environment. Second, nitrogen and carbon compound uptake and assimilation by yeast is influenced by environmental factors such as pH and temperature. Third, the capacity of the yeast to assimilate and metabolize nitrogen and carbon sources present in vivo is determined by yeast-specific genes and pathways.

As sources of nitrogen and carbon are essential for survival, various yeast gene products and pathways required for maintaining a constant supply of nitrogen and carbon will be necessary for yeast survival in vivo. Establishing which nitrogen and carbon metabolism genes are required for survival in the mouse environment should provide insights into conditions present in the in vivo niche inhabited by yeast, which nitrogen and carbon sources are important for yeast survival in that environment, which genes and pathways may be important for the survival of other clinically relevant fungi, and identifying potential antifungal drug targets.

Because serum is the best analyzed in vivo compartment with respect to composition, the mode of infection is intravenous, and survival in serum is necessary for dissemination, serum concentrations of various nitrogen and carbon compounds were used as a guide to identify compounds and conditions that may be relevant for yeast survival in vivo. Based on serum composition, this study investigated the relevance of utilization of primary nitrogen sources (ammonium, urea, and amino acids), primary carbon sources (glucose, lactate, pyruvate, and fatty acids), and storage carbohydrates (trehalose and glycogen) for yeast survival in vivo. The role of the nitrogen- and carbon-sensing and regulatory pathways, including nitrogen catabolite repression, the amino acid-sensing pathway, am-

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monium- and glucose-sensing pathways, and general control, was also examined. Finally, the importance of various genes involved in nitrogen compound metabolism, particularly amino acid and polyamine biosynthesis, was investigated.

MATERIALS AND METHODS

Strains, media, and growth conditions. All *S. cerevisiae* strains used in this study are listed in Table S1 in the supplemental material and are isogenic with YJM145 (63). Yeast culture media, including yeast extract peptone dextrose (YPD), synthetic dextrose (SD), pantothenate-free medium, and minimal L-citrulline medium, were prepared as described previously (49, 79, 85). When growth on a particular compound as a sole carbon or nitrogen source was tested, media consisted of SD with dextrose or ammonium sulfate replaced by the carbon or nitrogen source of interest, respectively. Where specified, media were supplemented with amino acids (85), nourseothricin (100 μg ml^{-1}; Hans Knöll Institute für Naturstoff-Forschung, Jena, Germany), hygromycin B (300 μg ml^{-1}; Calbiochem), Geneticin (200 μg ml^{-1}; Life Technologies), or flucytosine (1 mM; Sigma). Other supplements were also used as indicated in the text. Unless specified otherwise, *S. cerevisiae* cultures were incubated at 30°C.

Gene deletion and strain construction. *S. cerevisiae* genes were replaced with either the natMX4, kanMX4, or hphMX4 cassette by PCR-mediated gene deletion (29). When single-gene deletions were made, strains were typically constructed from the homothallic strain YAG129. As YAG129 is diploid, transformation resulted in heterozygous gene deletions, thus, transformants were sporulated at 30°C, tetrads were dissected, and drug-resistant segregants were selected. When two or more gene deletions were made in one strain, deletions were typically made singly in *lys2* (YJM155 or YJM631) or *lys5* (YJM385 or YJK365) mutant strains, and homozygous mutants were obtained as described for YAG129. Sporulated *lys2* and *lys5* strains containing different gene deletions were then crossed, Lys$^+$ diploids were selected, strains were sporulated, and tetrads were dissected to obtain strains with multiple homozygous deletions. Alternatively, strains with multiple gene deletions were obtained by crossing strains with genes replaced by different drug markers. Strains YJM610 (*lyp1*) and YJM360 (*can1*) were spontaneous mutants, selected for resistance to 100 μg ml^{-1} S-2-aminoethyl-L-cysteine and 40 μg ml^{-1} canavanine, respectively.

To create the S15A mutation within Hxk2p, a plasmid (pAG92) containing the *HXK2* open reading frame and 600-bp flanking sequence cloned into the SalI site of the *HO*-poly-KanMX4-*HO* plasmid (97) was mutated using the QuickChange XL-Site-Directed Mutagenesis kit (Stratagene) and mutagenic primers (GCCAGAAA GGGTGCCATGGCCGATGTG and CACATCGGCCATGGCACCCTTTCTG GC), according to manufacturer's instructions. The mutation was confirmed by sequencing (Duke Comprehensive Cancer Center Sequencing Facility). The *HO-hxk2*^{S15A}-kanMX4-*HO* region of the resulting plasmid was liberated by NotI digestion and was introduced into YAG438b (*hxk2*Δ/*hxk2*Δ), resulting in strain YAG621 (*HO*/*ho*Δ::*hxk2*^{S15A}-kanMX4 *hxk2*Δ::natMX4/*hxk2*Δ::natMX4).

To reconstitute mutant strains with wild-type alleles, strains were transformed with a PCR product containing the wild-type gene and flanking sequence. Transformants were selected by acquisition of the wild-type phenotype and screened for replacement of one copy of the deleted region by the wild-type allele. When no selection for the wild-type allele was available, strains were first constructed in which a dominant drug marker was inserted approximately 200 bp downstream of the wild-type allele of interest. The wild-type allele, together with the drug marker, were PCR-amplified using primers homologous to sequence that flanked sequence deleted in the mutant. The PCR product was introduced into the mutant strain, and transformants were selected that had acquired resistance conferred by the newly introduced drug marker. To reconstitute *hxk2*Δ mutants, the *HXK2* gene together with 600 bp flanking sequence and the kanMX4 marker were liberated from pAG92 by NotI digestion and introduced into YAG438b to replace an *HO* allele.

Gene deletions and reconstitutions were confirmed by colony PCR and by acquisition or loss of a phenotype, where possible. Specifically, *aro7*Δ, *lys9*Δ, *ilv2*Δ, *met3*Δ, *hom3*Δ, and *spe3*Δ mutants were confirmed by acquisition of aromatic amino acid (2), lysine (90), isoleucine and valine (22), methionine (12), methionine and threonine (76), and spermidine and pantothenate auxotrophy (101), respectively. *dur3*Δ mutants were able to use urea as a nitrogen source when provided at high (2.5 g liter^{-1}) but not low (380 mg liter^{-1}) concentrations (91), while *dur1,2*Δ mutants could not use urea as a nitrogen source at either concentration (92). *mep2*Δ mutants did not form pseudohyphae (57), and *mep1*Δ *mep2*Δ *mep3*Δ mutants were unable to grow on low-ammonium media (14 mg liter^{-1}) (61). *gap1*Δ mutants were unable to use citrulline as a sole nitrogen source (31); *gcn4*Δ mutants were sensitive to 3-amino triazole and sulfometuron methyl in a disk diffusion assay (38); *ath1*Δ mutants were unable to use trehalose (20 g liter^{-1}) as a sole carbon source (69); *nth1*Δ mutants recovered less well than the wild type following incubation at 50°C (68); and *snf3*Δ, *hxt2*Δ, *hxt5*Δ, and *hxt7*Δ mutants grew less well than the wild type in media containing less than 0.5% (wt/vol) dextrose; specifically, 0.125 and 0.0625% (wt/vol).

Experimental infections. The in vivo survival of isogenic clinical diploid *S. cerevisiae* wild-type and mutant strains were compared following infection by lateral tail vein injection of 4- to 6-week-old male CD-1 mice (outbred, immune competent; Charles River Laboratories), performed as described previously (14, 28). The inocula consisted of an equal mix of differentially marked, isogenic strains (typically, one wild-type [YAG40 or YAG214b] control and two mutants) that had been grown separately to mid-log phase in YPD medium, washed in sterile phosphate-buffered saline (PBS), and resuspended in PBS. The inocula were diluted and plated to selective media to determine the exact proportion of each strain present. Typically, for each experiment, 15 mice were each infected with approximately 2×10^7 CFU of an inoculum. At 4 h, 1 week, and 2 weeks following infection, five mice per time point were euthanized by CO_2 inhalation, and since the brain is the predominant organ inhabited by yeast in CD-1 mice (14), the brains were recovered. Brains were homogenized in 5 ml PBS supplemented with 100 μg ml^{-1} ampicillin and streptomycin, pelleted by centrifugation ($700 \times g$ for 10 min), resuspended in 1 to 2 ml PBS, and plated to selective media to determine the relative numbers of each strain present. Results for each mutant and at each time point ($t = x$) were expressed in Table 1 as competitive index (CI) values (9, 13), a measure of $[(\text{mutant/wild type})_{t=x}]/[(\text{mutant/wild type})_{t=0}]$. Mutants with CI values between 0.75 and 1.38, between 0.75 and 0.2, or <0.2 were considered phenotypically equivalent, slightly attenuated, or severely attenuated, respectively, compared with the wild type (28).

Mice were fed ad libitum for the course of the experiment. The design of animal experiments met with institutional guidelines and was approved by the institutional animal care and use committee.

RESULTS

Urea, ammonium, and amino acid utilization by yeast in vivo. Since urea is abundant in serum at concentrations of about 350 to 380 mg liter^{-1} (16), it may be an important nitrogen source for pathogenic fungal survival in vivo. To investigate whether urea is a required nitrogen source for yeast survival in vivo, we tested the in vivo survival of mutants unable to grow on medium containing urea as the sole nitrogen source at the concentration found in serum. Mutants were constructed that contained deletions of either *DUR3*, encoding the urea permease (active at urea concentrations up to 0.25 mM [20]), or the urea amidolyase gene, *DUR1,2*, required for metabolizing urea to carbon dioxide and ammonium (27, 92, 102). Two weeks after infection of CD-1 mice with *dur1,2*Δ (YJK251) and *dur3*Δ (YJK255 and YJK258) mutants, the CI values were 1.20 and 0.89, respectively (Table 1). Therefore, by the criteria described in Materials and Methods, *dur1,2*Δ and *dur3*Δ mutants survived as well as the wild type in vivo, indicating that urea was not important as a sole nitrogen source for *S. cerevisiae* survival in vivo.

To investigate the importance of ammonium as a nitrogen source and the ability to sense external ammonium for yeast survival in vivo, the in vivo survival of ammonium permease mutants were tested. Ammonium is present at low concentrations in serum (100 to 800 μg liter^{-1}) (16). Yeast transports ammonium by three permeases, Mep1p (high capacity, moderate affinity; K_m, 5 to 10 μM), Mep2p (low capacity and high affinity; K_m, 1 to 2 μM), and Mep3p (high capacity and low affinity; K_m, 1.4 to 2.1 mM) (61). In addition, Mep2p functions as an ammonium sensor required for pseudohyphal growth in response to nitrogen starvation (57). When provided with ammonium at the concentration found in serum as the sole nitrogen source in vitro, *mep1*Δ (YJK240 and YJK242) and

TABLE 1. Competitive indices (CI) of *S. cerevisiae* mutant strains

Disrupted function	Genotype	Strain	CI values at[a]:		
			4 h	7 days	14 days
Nitrogen compound transport and utilization					
Urea metabolism	*dur1,2Δ/dur1,2Δ*	YJK251	1.19	1.13	1.20
Urea transport	*dur3Δ/dur3Δ*	YJK255, YJK258	1.17	1.03	0.89
Ammonium transport	*mep1Δ/mep1Δ*	YJK240, YJK242	1.72	1.39	1.10
Ammonium transport and sensing	*mep2Δ/mep2Δ*	YJK243	1.22	1.58	1.38
Ammonium transport and sensing	*mep1Δ/mep1Δ mep2Δ/mep2Δ mep3Δ/mep3Δ*	YJK282	1.03	1.04	0.84
Amino acid transport	*agp1Δ/agp1Δ*	YJK231, YJK233	0.99	1.04	0.95
Amino acid transport	*gap1Δ/gap1Δ*	YJK228, YJK229	0.93	*0.73*	*0.73*
Lysine transport	*lyp1/lyp1*	YJM610	*0.73*	*0.46*	*0.51*
Arginine transport	*can1/can1*	YJM360	*0.64*	*0.53*	*0.50*
Nitrogen regulation					
Amino acid sensing	*ssy1Δ/ssy1Δ*	YJK236, YJK238	0.94	*0.71*	*0.72*
Amino acid transport and sensing	*gap1Δ/gap1Δ agp1Δ/agp1Δ ssy1Δ/ssy1Δ*	YJK296	1.24	1.10	1.11
General control transcriptional activation	*gcn4Δ/gcn4Δ*	YJK318, YJK319	1.18	1.09	0.85
Nitrogen catabolite repression transcriptional activation	*gln3Δ/gln3Δ*	YJK321a, YJK295c	*0.33*	*0.41*	*0.38*
Nitrogen compound biosynthesis					
Lysine biosynthesis	*lys9Δ/lys9Δ*	YJK788, YJK794	0.92	0.99	1.08
Spermidine biosynthesis	*spe3Δ/spe3Δ*	YJK801, YJK804	*0.54*	0.91	*0.62*
Methionine biosynthesis	*met3Δ/met3Δ*	YJK384a, YJK463b	*0.69*	*0.72*	*0.57*
Methionine and threonine biosynthesis	*hom3Δ/hom3Δ*	YJK487a, YJK495a	0.83	***0.001***	***<0.008***
	HOM3/hom3Δ	YJK543, YJK560	1.04	0.85	*0.71*
Isoleucine and valine biosynthesis	*ilv2Δ/ilv2Δ*	YJK365a, YJK464a	1.26	***0.005***	***<0.008***
	ILV2/ilv2Δ	YJK539	1.09	0.83	*0.65*
Tyrosine and phenylalanine biosynthesis	*aro7Δ/aro7Δ*	YJK398a, YJK470	*0.35*	***<0.0005***	***<0.001***
	ARO7/aro7Δ	YJK508	*0.63*	***0.15***	***0.12***
Glucose utilization					
High affinity hexose transport	*hxt2Δ/hxt2Δ hxt5Δ/hxt5Δ hxt7Δ/hxt7Δ*	YAG406b	0.94	*0.49*	*0.39*
High glucose sensing	*rgt2Δ/rgt2Δ*	YAG310c, YAG312c	1.03	ND[b]	*0.55*
Low glucose sensing	*snf3Δ/snf3Δ*	YAG315b, YAG317c	0.93	***0.14***	***0.14***
	SNF3/snf3Δ	YAG444	1.57	1.00	0.90
Glycolysis (hexokinase 1, glucokinase)	*hxk1Δ/hxk1Δ glk1Δ/glk1Δ*	YAG451, YAG452	1.05	1.14	0.91
Glycolysis, glucose signaling (hexokinase 2)	*hxk2Δ/hxk2Δ*	YAG436b, YAG438b	*0.45*	***0.17***	***0.18***
	HXK2/hxk2Δ	YAG487	0.79	*0.50*	*0.62*
Glucose signaling	*hxk2*^{S15A} *hxk2Δ/hxk2Δ*	YAG621	*0.42*	*0.23*	*0.47*
Fatty acid, lactic acid, and pyruvate utilization					
Lactate and pyruvate transport	*jen1Δ/jen1Δ*	YAG592d, YAG594d	1.00	0.78	0.77
2,3 Carbon utilization	*icl1Δ/icl1Δ icl2Δ/icl2Δ*	YAG610	0.88	ND	0.84
Storage carbohydrate utilization					
Glycogen utilization	*gph1Δ/gph1Δ*	YJK388a, YJK468b	0.88	*0.59*	*0.47*
Trehalose utilization (acid trehalase)	*ath1Δ/ath1Δ*	YJK338a	1.52	ND	*0.53*
Trehalose utilization (neutral trehalase 1)	*nth1Δ/nth1Δ*	YJK410	0.82	ND	*0.22*
Trehalose utilization (neutral trehalase 2)	*nth2Δ/nth2Δ*	YJK337a	0.84	ND	0.90
Trehalose utilization	*ath1Δ/ath1Δ nth1Δ/nth1Δ*	YJK421	0.92	ND	***0.17***
Trehalose utilization	*ath1Δ/ath1Δ nth2Δ/nth2Δ*	YJK424	0.80	ND	***0.13***
Trehalose utilization	*nth1Δ/nth1Δ nth2Δ/nth2Δ*	YJK418	1.23	ND	*0.42*
Trehalose utilization	*ath1Δ/ath1Δ nth1Δ/nth1Δ nth2Δ/nth2Δ*	YJK639, YJK644	1.22	*0.38*	***0.06***
Trehalose utilization	*ATH1/ath1Δ nth1Δ/nth1Δ nth2Δ/nth2Δ*	YJK769	0.93	ND	*0.35*
Trehalose utilization	*ath1Δ/ath1Δ NTH1/nth1Δ nth2Δ/nth2Δ*	YJK759	1.05	ND	***0.10***
Trehalose utilization	*ath1Δ/ath1Δ nth1Δ/nth1Δ NTH2/nth2Δ*	YJK784	1.24	ND	***0.14***

[a] CI = [(mutant/wild type)$_{t=x}$]/[(mutant/wild type)$_{t=0}$]. CI values between 0.75 and 1.38, between 0.75 and 0.2, or <0.2 were considered phenotypically equivalent, slightly attenuated (italics), or severely attenuated (italics and boldface), respectively, compared to the wild type. Results were averaged when two mutants with the same gene deleted were tested.

[b] ND, not determined.

*mep2*Δ (YJK243) mutants grew, while *mep1*Δ *mep2*Δ *mep3*Δ (YJK282) mutants did not. Unlike *mep1*Δ mutants, *mep2*Δ mutants did not form pseudohyphae (data not shown). However, when these mutants were tested in mice, each mutant survived as well as the wild type 2 weeks postinoculation (Table 1). Thus, utilization of ammonium as a sole nitrogen source and the ability to sense external ammonium were not critical for yeast survival in vivo.

Since amino acids are relatively abundant in serum at levels comparable to those used in synthetic media (10 to 60 mg liter^{-1}) (16), the importance of amino acids as nitrogen sources for in vivo survival was also examined. Yeast possesses at least 20 amino acid permeases, which differ with respect to transport capacity, substrate specificity, affinity, uptake velocity, and regulation (30, 40, 59, 78). Since it was impractical to disrupt all permeases to completely eliminate amino acid transport, four were selected for disruption and testing their significance for survival in vivo. Specifically, the general amino acid permeases Gap1p and Agp1p were selected, since these have the broadest substrate specificity, are significant transporters of the preferentially used nitrogen sources asparagine and glutamine, and are controlled by the nitrogen source availability, making them well adapted for transporting amino acids for a catabolic role (78, 84). The importance of high-affinity, narrow substrate permeases, better suited for providing amino acids for an anabolic role, was also tested, using *LYP1*-mutated (encoding the high-affinity lysine permease [93]) and *CAN1*-mutated (encoding the high-affinity arginine permease [1, 39]) strains. The *gap1*Δ (YJK228 and YJK229) and *agp1*Δ (YJK231 and YJK233) mutants were found to survive nearly as well, or as well as, the wild type in vivo, while *lyp1* (YJM610) and *can1* (YJM360) mutant survival was slightly attenuated, at about 50% of wild-type levels (Table 1). Therefore, there was little or no requirement for the Lyp1p, Can1p, Gap1p, and Agp1p permeases for in vivo survival.

The role of regulators of nitrogen metabolism in vivo. The lack of a significant effect on survival in vivo by preventing yeast utilization or uptake of urea, ammonium, or amino acids suggests that yeast can utilize a variety of nitrogen sources in vivo. Thus, disruption of regulators that control multiple nitrogen uptake or metabolism processes might have a more significant effect on yeast survival in vivo.

Ssy1p is a membrane-embedded protein that responds to external amino acid concentrations by transmitting intracellular signals and is necessary for maximal transcription of genes of a number of peptide and amino acid permeases, including *AGP1*, when amino acids are present (18, 45, 51). However, the amino acid-sensing pathway was not important for yeast survival in vivo, as *ssy1*Δ mutants (YJK236 and YJK238) survived nearly as well as the wild type in vivo on average (Table 1). Since minimal transcription of *AGP1* is still present and *GAP1* transcription is increased in an *ssy1*Δ mutant (51), we also tested the survival in vivo of a triple *ssy1*Δ *agp1*Δ *gap1*Δ mutant (YJK296). This mutant also survived as well as the wild type. Thus, reducing or eliminating amino acid and peptide transport from a number of permeases had no effect on yeast survival in vivo.

Nitrogen catabolite repression is the physiological response, whereby genes encoding permeases and enzymes for catabolism of poorly used nitrogen sources are repressed in the presence of readily utilizable nitrogen sources and activated in their absence. The transcriptional activator, Gln3p, is required for the high-level expression of a wide variety of nitrogen catabolite repressible genes, including *AGP1*, *GAP1*, *CAN1*, *MEP2*, *DUR1*,*2*, and *DUR3*, that were not required for survival of yeast in vivo individually but may have an effect when repressed together in a *gln3*Δ mutant. Consistent with this hypothesis, *gln3*Δ mutants (YJK321a and YJK295c) were slightly attenuated in vivo, with recoverable CFU at approximately one-third of wild-type levels after 2 weeks in mice (Table 1).

Starvation for any of 11 amino acids or purines elicits a response called the general control or the starvation response, which results in increased transcription of at least 539 genes by 2- to 10-fold, including at least 35 amino acid biosynthetic genes (37, 46, 66). To assess the importance of the starvation response for survival of yeast in vivo, the survival of strains deleted for the general control transcriptional activator gene, *GCN4*, were assessed in vivo. The *gcn4*Δ mutants (YJK318 and YJK319) on average survived as well as the wild type in vivo (Table 1), thus, the starvation response was not necessary for yeast survival in vivo.

The role of nitrogen biosynthetic genes in vivo. An alternative way to examine the levels of essential nitrogenous compounds available to yeast in vivo is by assessing whether nitrogen compound biosynthetic mutants can survive in vivo. Survival of a biosynthetic mutant in vivo would indicate that the compound that the strain is auxotrophic for is present and sufficiently transported from the in vivo environment. We determined the importance of the methionine, methionine and threonine, isoleucine and valine, tyrosine and phenylalanine, lysine, and spermidine biosynthetic pathways for survival of yeast in vivo.

The in vivo survival of methionine-auxotrophic ATP sulfurylase (*met3*Δ), methionine- and threonine-auxotrophic aspartate kinase (*hom3*Δ), isoleucine- and valine-auxotrophic acetolactate synthase (*ilv2*Δ), spermidine- and pantothenate-auxotrophic spermidine synthase (*spe3*Δ), tyrosine- and phenylalanine-auxotrophic chorismate mutase (*aro7*Δ), and lysine-auxotrophic saccharopine dehydrogenase (*lys9*Δ) mutants were compared to the wild type. The *lys9*Δ (YJK788 and YJK794) mutants survived as well as the wild type in vivo, while *met3*Δ (YJK384a and YJK463b) and *spe3*Δ (YJK801 and YJK804) mutants were slightly attenuated after 2 weeks (Table 1).

Unlike the *met3*Δ, *lys9*Δ, and *spe3*Δ mutants, the *ilv2*Δ (YJK365a and YJK464a) and *hom3*Δ (YJK487a and YJK495a) mutants were severely attenuated following 1 week in vivo and were eliminated 2 weeks postinfection (Table 1). Moreover, the *aro7*Δ mutants (YJK398a and YJK470) were depleted to one-third of wild-type levels after only 4 h and were eliminated 1 week postinfection. To prove that the inability to survive in vivo was due to the loss of *ILV2*, *ARO7*, or *HOM3*, these alleles were added back to replace a mutant *ilv2*Δ, *aro7*Δ, or *hom3*Δ allele, respectively. Reconstitution of *ilv2*Δ mutants with *ILV2* (YJK539) and *hom3*Δ mutants with *HOM3* (YJK543 and YJK560) restored the ability of strains to survive nearly as well as the wild type in vivo. However, while the restoration of *ARO7* to *aro7*Δ mutants recovered the strain's ability to survive in vivo, survival was still significantly attenuated compared to the wild-type strain after 2 weeks, indicating possible haploinsufficiency.

Glucose utilization in vivo. The preferred carbon source utilized by yeast, glucose, is also the predominant sugar present in murine serum, at a concentration of approximately 0.7 g liter^{-1} (16). Thus, we predicted that glucose would be the most significant carbon source for yeast survival in vivo. While complete elimination of glucose transport at these concentrations would require disruption of multiple hexose transporters (103), reduction of glucose transport by deletion of several high-affinity hexose transporter genes (*HXT2*, *HXT5*, and *HXT7*) attenuated yeast survival (YAG406b) to approximately a third of wild-type levels after 2 weeks in vivo (Table 1), supporting the importance of glucose utilization in vivo.

Utilization of glucose at concentrations present in serum requires the low-glucose sensor Snf3p, which is important for the expression of the high-affinity hexose transporter genes, while the high-glucose sensor Rgt2p is involved in repression of these transporters and induction of low-affinity hexose transporters when glucose is abundant (70, 71). Consistent with a critical requirement for glucose utilization in vivo, *snf3*Δ mutant (YAG315b and YAG317c) survival was severely attenuated in vivo, while *RGT2* disruption (YAG310c and YAG312c) had a slight effect (Table 1). Interestingly, while *snf3*Δ mutant survival was reduced to approximately 1/10th of wild-type levels after 1 week, no further decline in CI values was observed after 2 weeks, suggesting a requirement for *SNF3* in only the early stages of infection. *SNF3* reconstitution of a *snf3*Δ mutant restored the ability of the strain (YAG444) to survive to wild-type levels.

The first step in glucose utilization (glycolysis) involves the phosphorylation of glucose at C-6, which can be catalyzed by the hexokinases Hxk1p and Hxk2p as well as the glucokinase Glk1p (3, 15, 98). *glk1*Δ *hxk1*Δ mutants (YAG451 and YAG452) were found to survive as well as the wild type in vivo (Table 1). In contrast, survival of *hxk2*Δ mutants (YAG436b and YAG438b) was severely attenuated and could be restored to almost wild-type levels by reintroduction of the wild-type *HXK2* allele (strain YAG487). In addition to its hexokinase catalytic role, Hxk2p also has glucose-signaling properties (62, 74). To test which property was important for survival in vivo, a phosphorylation-negative mutant, reportedly deficient in glucose signaling, in which serine 15 was replaced with alanine (77), was constructed, and its in vivo survival phenotype was assessed. The survival of the *hxk2*^{S15A} (YAG621) mutant was also attenuated in vivo, although to a lesser degree than that of the *hxk2*Δ mutant, suggesting that both functions of Hxk2p may be relevant for survival in the mouse model of systemic infection.

Pyruvate, lactate, and fatty acid utilization in vivo. Since glucose is present in mouse serum in concentrations low enough to induce derepression in yeast cells in vitro, and since a subset of *snf3*Δ and *hxk2*Δ mutants still survived in vivo after 2 weeks, other nonglucose carbon sources present in the in vivo environment may also play a significant role in *S. cerevisiae* growth and survival in vivo. Lactate is present in mouse serum at concentrations of 0.14 to 0.17 g liter^{-1} (16); the uptake of both lactate and pyruvate is mediated by the pyruvate/lactate transporter Jen1p. However, the *jen1*Δ mutants YAG592d and YAG594d survived at levels similar to those of the wild type in vivo, thus, lactate and pyruvate were not critical carbon sources for yeast survival in vivo (Table 1).

Fatty acids comprise another carbon source available to yeast in vivo. While two-carbon compounds metabolized by the glyoxylate cycle were not a significant carbon source for yeast in vivo due to the lack of requirement for isocitrate lyase (Icl1p) (28), it remained possible that yeast use odd-chained fatty acids, requiring 2-methylisocitrate lyase (Icl2p) for metabolism. However, deletion of both *ICL1* and *ICL2* together (strain YAG610) did not affect the ability of yeast to survive in vivo (Table 1). Thus, fatty acids were not an important carbon source for yeast survival in vivo.

The importance of storage carbohydrate utilization for in vivo survival. *S. cerevisiae* accumulates the storage carbohydrates glycogen and trehalose during various nutrient deprivation (48, 54, 72) and stress (21, 42, 73) conditions. In addition to acting as a storage carbohydrate, trehalose protects cell membranes and proteins from aggregation and denaturation during stress, and trehalose hydrolysis is required for recovery from stress (17, 41, 43, 73, 87, 99, 104). We therefore investigated whether trehalose and glycogen hydrolysis influenced yeast survival in vivo.

Glycogen hydrolysis to glucose and glucose-6-phosphate requires glycogen phosphorylase (Gph1p) (44). Survival of *gph1*Δ mutants (YJK388a and YJK468b) was slightly attenuated in vivo, at approximately 50% of wild-type levels 2 weeks postinfection. Thus, there was a slight requirement for glycogen hydrolysis by *S. cerevisiae* survival in vivo (Table 1).

Hydrolysis of trehalose into glucose is performed by three trehalases: the cytosolic or neutral trehalase Nth1p, and possibly its homolog Nth2p, are responsible for mobilization and/or recycling of stored trehalose, while the periplasmic Ath1p is important for growth on trehalose as an external carbon source (47, 67, 69). Strains were constructed containing each combination of single, double, or triple deletions of the trehalase genes, and their in vivo survival was tested. In general, each consecutive trehalase gene deletion had a deleterious effect on survival in vivo, ranging from no effect of *NTH2* deletion alone (YJK337a) to severe attenuation after 2 weeks for strains lacking all three trehalases (YJK639 and YJK644) (Table 1). Consistent with this, increased survival was observed following introduction of the wild-type trehalase genes individually to the triple mutant. Therefore, trehalose hydrolysis plays a substantial role in yeast survival in vivo.

DISCUSSION

As nitrogen and carbon compounds are critical for survival, genes involved in maintaining a constant supply of nitrogen and carbon compounds would be expected to be important for the survival of a fungal pathogen in vivo. It is unknown, however, which nitrogen and carbon compounds fungi utilize in vivo. Therefore, to better understand fungal pathogenesis and to identify potential antifungal drug targets, we investigated the importance of fungal genes required for nitrogen and carbon uptake, metabolism, and regulation for the survival of a pathogenic fungus in vivo. These processes are best understood in the easily manipulated yeast *S. cerevisiae*, which is a close relative of *Candida* species, and is itself an emerging pathogen; thus, our study utilized a host-pathogen model involving the survival of a clinical *S. cerevisiae* strain in a murine host (28).

Carbon sources present in serum include glucose, two-car-

bon compounds, fatty acids, lactate, and pyruvate. Consistent with glucose being the preferred carbon source used by *S. cerevisiae* in vitro, our results indicate that it is also likely the most important carbon source for survival in vivo. The lack of requirement in vivo for utilization of odd-chained fatty acids (*ICL2*) together with even-chained fatty acids and two-carbon compounds (*ICL1*) further validates earlier findings that the glyoxylate cycle is not required for *S. cerevisiae* survival in vivo (28). While apparently required for full virulence of *C. albicans* (56), the glyoxylate cycle is also not required for *Cryptococcus neoformans* virulence (82). These species-specific effects may reflect differences in niche-specific requirements. While two-carbon compounds, fatty acids, lactate, and pyruvate were not necessary as sole carbon sources for *S. cerevisiae* in vivo, it remains possible that these compounds may be supplementary carbon sources in vivo, where low glucose concentrations (for example, 0.7 g liter^{-1} in serum) likely result in derepression of genes for the utilization of alternative carbon sources such as *ICL1* (36), *ICL2* (35), and *JEN1* (11).

Another aspect of carbohydrate metabolism is the accumulation and hydrolysis of storage carbohydrates. We found a small benefit for glycogen hydrolysis, while trehalose hydrolysis was highly significant for yeast survival in vivo. *S. cerevisiae* accumulates both carbohydrates in conditions such as those likely to be experienced by yeast in vivo, including low carbohydrate levels, nitrogen starvation, and heat or osmotic stress (21, 42, 48, 54, 72, 73). Since hydrolysis of either trehalose or glycogen can enhance survival during carbon starvation (86), the highly significant role of trehalose hydrolysis only in vivo suggests that the important function of trehalases may be due to their additional role in the correct renaturation of proteins during stress recovery (17, 73, 87). While trehalose synthesis has been implicated in *C. albicans* pathogenesis (95, 106), no role was found for trehalose hydrolysis, although the lack of effect may be due to residual trehalase activity present in the trehalase mutant studied (19).

Expression of a large number of genes superfluous for survival in a particular environment is energetically expensive and thus detrimental to survival. Hence, yeast rapidly responds to changing environments by reprogramming gene expression. The shift from in vitro to in vivo environments likely represents a significant change for yeast with respect to the carbon and nitrogen environment, thus, it is not surprising that we found a requirement in vivo during the initial stages of infection for Snf3p, important for sensing and responding to low glucose concentrations, while there was only a slight requirement for the high-glucose sensor Rgt2p. In contrast, besides a small effect for *GLN3* deletion, processes important for sensing and responding to quality and concentration of nitrogen compounds were not required for yeast survival in vivo. While glucose was important as a sole carbon source, no single nitrogen source present in serum was important individually for yeast survival in vivo (although amino acid transport could only be reduced and not eliminated), indicating that yeast can use a variety of nitrogen sources in vivo. Therefore, differences in effects of nitrogen versus carbon regulation genes may be attributable to the inability of any one nitrogen regulator to completely control utilization of all nitrogen sources compared with the importance of Snf3p in utilization of the most important carbon source, glucose.

In addition to influencing nitrogen source utilization by yeast, nitrogen regulation genes involved in this study also control dimorphic transition in *S. cerevisiae* and/or other fungi (*MEP2* [57, 88], *SSY1* [7], *GAP1* [4], and *GCN4* [6, 94]), a response critical for the pathogenicity of many fungi (83). Our results, combined with earlier findings (28), indicate that pseudohyphal formation is not important for *S. cerevisiae* survival in vivo. Indeed, this species may not form pseudohyphae in vivo (14), perhaps contributing to the virulence differences between *S. cerevisiae* and *C. albicans* (28). Dimorphism in *C. albicans* is triggered by multiple factors (8). In contrast, *S. cerevisiae* dimorphism requires low-nitrogen conditions and is further enhanced by poorly utilized carbon sources (26). Since our results indicate that nitrogen sources are not limiting for *S. cerevisiae* in vivo and the most important carbon source comprises easily utilized glucose, the inability of *S. cerevisiae* (and *C. glabrata* [23]) to form pseudohyphae in vivo could be attributable to these environmental conditions. Therefore, it is possible that some nitrogen regulation genes will be important in vivo for fungi in which dimorphic transition is important for pathogenicity.

The most critical genes for yeast survival in vivo revealed by this study were required for amino acid biosynthesis. Aromatic amino acid (*ARO7*), threonine and methionine (*HOM3*), and branched-chain amino acid biosynthetic genes (*ILV2*) were essential for the survival of *S. cerevisiae* in vivo, although there was little or no requirement for genes involved in biosynthesis of methionine alone (*MET3*), lysine (*LYS9*), or the polyamine spermidine (*SPE3*). Methionine, lysine, spermidine, and spermine are therefore present and transported by *S. cerevisiae* at sufficient concentrations from the in vivo environment, as has been reported for histidine and tryptophan (28). Similarly, methionine auxotrophy does not reduce the virulence of *C. albicans* (60). However, the in vivo survival phenotypes of *S. cerevisiae* *met3*Δ, *lys9*Δ, and *spe3*Δ mutants contrast with those observed for the respective mutants in *Cryptococcus neoformans*, which were all significantly attenuated in virulence and survival in vivo (49, 50, 105). Differences in in vivo survival could be due to species differences in the primary in vivo niche inhabited, nitrogen compound transport, or requirement levels. Also, unlike *S. cerevisiae*, *C. neoformans lys9*, *spe3*, and *met3* mutants had defects in other cryptococcal virulence and in vivo survival factors: growth at 37°C, capsule formation, and/or melanin formation (49, 50, 105).

The necessity of *ARO7*, *HOM3*, and *ILV2* for *S. cerevisiae* survival in vivo indicates that threonine, aromatic amino acids, isoleucine, and/or valine are either present or transported at limiting concentrations for yeast survival in vivo, or, alternatively, *aro7*Δ, *hom3*Δ, or *ilv2*Δ mutants have other deleterious phenotypes that could influence their survival in vivo. For example, there is evidence that inhibition of bacterial acetolactate synthase results in toxic accumulation of the intermediate 2-ketobutyate (52, 53, 96), which may also explain in vitro defects observed for *S. cerevisiae* and *C. neoformans ilv2* mutants (50). *C. neoformans ILV2* was also essential for virulence and survival in mice (50). The extreme in vivo survival phenotypes of *ilv2*Δ, *hom3*Δ, and *aro7*Δ mutants, together with the absence of these pathways from humans, the conservation of these pathways throughout fungi, and the availability of various amino acid biosynthetic inhibitors used successfully as herbi-

cides (89), validate the isoleucine and valine, threonine, and aromatic amino acid biosynthetic pathways as ideal potential antifungal drug targets.

The in vivo survival profile of nitrogen and carbon transport, utilization, and regulation gene mutants revealed by this study was interesting compared with gene expression profiles of *S. cerevisiae* and *C. albicans* genes following exposure to serum, neutrophils, and macrophages (24, 25, 55, 56, 81). Thirteen of the genes tested in this study were induced greater than twofold following transfer of *S. cerevisiae* from YPD medium to RPMI medium with serum (56 and Table S1), none of which were highly significant for in vivo survival. Expression profiles following exposure to whole blood rather than serum may be a better indication of genes expressed in vivo, since *C. albicans* expression profiles following intravenous infection of mice closely resembled profiles following incubation in whole blood (25), which in turn strongly correlated with neutrophil phagocytosis profiles (24). When phagocytosed by neutrophils, *S. cerevisiae* and *C. albicans* elicit an amino acid deprivation response, resulting in the Gcn4p-dependent induction of arginine biosynthetic pathway genes. In addition, transcription of other genes unimportant for yeast survival in vivo (*MET3*, *DUR1,2*, *DUR3*, *MEP2*, and *GAP1*) was induced in the neutrophil. Similarly, there was little correlation between *S. cerevisiae* or *C. albicans* genes induced upon exposure to macrophages (55, 56) and *S. cerevisiae* genes shown to be important for in vivo survival. If these genes and processes are important for yeast survival in neutrophils or macrophages, our results may indicate that the neutrophil or macrophage is not a major environment inhabited by *S. cerevisiae* in vivo. Alternatively, if intracellular survival is an important part of *S. cerevisiae* survival in the murine host, the lack of importance of these genes for yeast survival in vivo indicates that gene induction is not always a reliable indicator of gene product necessity in a particular environment.

The discrepancy between expression profile results and genes shown to be required in vivo highlights the reality that there is no substitute to testing mutants in vivo to determine which genes and processes are important for virulence and survival in vivo. In particular, this study has further demonstrated the utility of clinical *S. cerevisiae* strains as model pathogens to identify gene products required for fungal survival in the mammalian host environment, and hence potential antifungal drug targets. The in vivo importance of some genes may be fungal species specific, reflecting unique regulation of metabolic pathways or the habitation of different in vivo niches. However, the conserved nature of most fungal pathways ensures that many of the genes required for *S. cerevisiae* survival in vivo will also be important for the survival of other clinically important but less genetically tractable fungi, such as *Candida* or cryptococcal species.

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REFERENCES

1. **Ahmad, M., and H. Bussey.** 1986. Yeast arginine permease: nucleotide sequence of the *CAN1* gene. Curr. Genet. **10:**587–592.
2. **Ball, S. G., R. B. Wickner, G. Cottarel, M. Schaus, and C. Tirtiaux.** 1986. Molecular cloning and characterization of *ARO7-OSM2*, a single yeast gene necessary for chorismate mutase activity and growth in hypertonic medium. Mol. Gen. Genet. **205:**326–330.
3. **Bianconi, M. L.** 2003. Calorimetric determination of thermodynamic parameters of reaction reveals different enthalpic compensations of the yeast hexokinase isozymes. J. Biol. Chem. **278:**18709–18713.
4. **Biswas, S., M. Roy, and A. Datta.** 2003. N-Acetylglucosamine-inducible *CaGAP1* encodes a general amino acid permease which co-ordinates external nitrogen source response and morphogenesis in *Candida albicans*. Microbiology **149:**2597–2608.
5. **Bowman, B. H., J. W. Taylor, and T. J. White.** 1992. Molecular evolution of the fungi: human pathogens. Mol. Biol. Evol. **9:**893–904.
6. **Braus, G. H., O. Grundmann, S. Bruckner, and H. U. Mosch.** 2003. Amino acid starvation and Gcn4p regulate adhesive growth and *FLO11* gene expression in *Saccharomyces cerevisiae*. Mol. Biol. Cell **14:**4272–4284.
7. **Brega, E., R. Zufferey, and C. B. Mamoun.** 2004. *Candida albicans* Csy1p is a nutrient sensor important for activation of amino acid uptake and hyphal morphogenesis. Eukaryot. Cell **3:**135–143.
8. **Brown, A. J., and N. A. Gow.** 1999. Regulatory networks controlling *Candida albicans* morphogenesis. Trends Microbiol. **7:**333–338.
9. **Brown, J. S., A. Aufauvre-Brown, J. Brown, J. M. Jennings, H. Arst, Jr., and D. W. Holden.** 2000. Signature-tagged and directed mutagenesis identify PABA synthetase as essential for *Aspergillus fumigatus* pathogenicity. Mol. Microbiol. **36:**1371–1380.
10. **Byron, J. K., K. V. Clemons, J. H. McCusker, R. W. Davis, and D. A. Stevens.** 1995. Pathogenicity of *Saccharomyces cerevisiae* in complement factor five-deficient mice. Infect. Immun. **63:**478–485.
11. **Chambers, P., A. Issaka, and S. P. Palecek.** 2004. *Saccharomyces cerevisiae JEN1* promoter activity is inversely related to concentration of repressing sugar. Appl. Environ. Microbiol. **70:**8–17.
12. **Cherest, H., P. Kerjan, and Y. Surdin-Kerjan.** 1987. The *Saccharomyces cerevisiae MET3* gene: nucleotide sequence and relationship of the 5′ noncoding region to that of *MET25*. Mol. Gen. Genet. **210:**307–313.
13. **Chiang, S. L., and J. J. Mekalanos.** 1998. Use of signature-tagged transposon mutagenesis to identify *Vibrio cholerae* genes critical for colonization. Mol. Microbiol. **27:**797–805.
14. **Clemons, K. V., J. H. McCusker, R. W. Davis, and D. A. Stevens.** 1994. Comparative pathogenesis of clinical and nonclinical isolates of *Saccharomyces cerevisiae*. J. Infect. Dis. **169:**859–867.
15. **Clifton, D., R. B. Walsh, and D. G. Fraenkel.** 1993. Functional studies of yeast glucokinase. J. Bacteriol. **175:**3289–3294.
16. **Crispens, C. G.** 1975. Section IV. Blood, p. 93–123. Handbook on the laboratory mouse. Charles C. Thomas, Springfield, Ill.
17. **De Virgilio, C., T. Hottiger, J. Dominguez, T. Boller, and A. Wiemken.** 1994. The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. Eur. J. Biochem. **219:**179–186.
18. **Didion, T., B. Regenberg, M. U. Jorgensen, M. C. Kiellandbrandt, and H. A. Andersen.** 1998. The permease homologue Ssy1p controls the expression of amino acid and peptide transporter genes in *Saccharomyces cerevisiae*. Mol. Microbiol. **27:**643–650.
19. **Eck, R., C. Bergmann, K. Ziegelbauer, W. Schonfeld, and W. Kunkel.** 1997. A neutral trehalase gene from *Candida albicans*: molecular cloning, characterization and disruption. Microbiology **143:**3747–3756.
20. **ElBerry, H. M., M. L. Majumdar, T. S. Cunningham, R. A. Sumrada, and T. G. Cooper.** 1993. Regulation of the urea active transporter gene (*DUR3*) in *Saccharomyces cerevisiae*. J. Bacteriol. **175:**4688–4698.
21. **Eleutherio, E. C., P. S. Araujo, and A. D. Panek.** 1993. Protective role of trehalose during heat stress in *Saccharomyces cerevisiae*. Cryobiology **30:**591–596.
22. **Falco, S. C., K. S. Dumas, and K. J. Livak.** 1985. Nucleotide sequence of the yeast *ILV2* gene which encodes acetolactate synthase. Nucleic Acids Res. **13:**4011–4027.
23. **Fidel, P. L., Jr., J. A. Vazquez, and J. D. Sobel.** 1999. *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. Clin. Microbiol. Rev. **12:**80–96.
24. **Fradin, C., P. De Groot, D. MacCallum, M. Schaller, F. Klis, F. C. Odds, and B. Hube.** 2005. Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. Mol. Microbiol. **56:**397–415.
25. **Fradin, C., M. Kretschmar, T. Nichterlein, C. Gaillardin, C. d'Enfert, and B. Hube.** 2003. Stage-specific gene expression of *Candida albicans* in human blood. Mol. Microbiol. **47:**1523–1543.
26. **Gagiano, M., F. F. Bauer, and I. S. Pretorius.** 2002. The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*. FEMS Yeast Res. **2:**433–470.
27. **Genbauffe, F. S., and T. G. Cooper.** 1991. The urea amidolyase (*DUR1,2*) gene of *Saccharomyces cerevisiae*. DNA Seq. **2:**19–32.
28. **Goldstein, A. L., and J. H. McCusker.** 2001. Development of *Saccharomyces cerevisiae* as a model pathogen. A system for the genetic identification of

gene products required for survival in the mammalian host environment. Genetics **159:**499–513.
29. **Goldstein, A. L., and J. H. McCusker.** 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast **15:**1541–1553.
30. **Grenson, M.** 1992. Amino acid transporters in yeast: structure, function and regulation, p. 219–245. *In* J. J. H. H. M. D. Pont (ed.), Molecular aspects of transport proteins. Elsevier Science Publishers, Amsterdam, The Netherlands.
31. **Grenson, M., C. Hou, and M. Crabeel.** 1970. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. IV. Evidence for a general amino acid permease. J. Bacteriol. **103:**770–777.
32. **Hazen, K. C.** 1995. New and emerging yeast pathogens. Clin. Microbiol. Rev. **8:**462–478.
33. **Hector, R. F., J. E. Domer, and E. W. Carrow.** 1982. Immune responses to *Candida albicans* in genetically distinct mice. Infect. Immun. **38:**1020–1028.
34. **Hector, R. F., E. Yee, and M. S. Collins.** 1990. Use of DBA/2N mice in models of systemic candidiasis and pulmonary and systemic aspergillosis. Infect. Immun. **58:**1476–1478.
35. **Heinisch, J. J., E. Valdes, J. Alvarez, and R. Rodicio.** 1996. Molecular genetics of *ICL2*, encoding a non-functional isocitrate lyase in *Saccharomyces cerevisiae*. Yeast **12:**1285–1295.
36. **Herrero, P., R. Fernandez, and F. Moreno.** 1985. Differential sensitivities to glucose and galactose repression of gluconeogenic and respiratory enzymes from *Saccharomyces cerevisiae*. Arch. Microbiol. **143:**216–219.
37. **Hinnebusch, A. G.** 1986. The general control of amino acid biosynthetic genes in the yeast *Saccharomyces cerevisiae*. CRC Crit. Rev. Biochem. **21:**277–317.
38. **Hinnebusch, A. G.** 2005. Translational regulation of *GCN4* and the general amino acid control of yeast. Annu. Rev. Microbiol. **59:**407–450.
39. **Hoffmann, W.** 1985. Molecular characterization of the *CAN1* locus in *Saccharomyces cerevisiae*. A transmembrane protein without N-terminal hydrophobic signal sequence. J. Biol. Chem. **260:**11831–11837.
40. **Horak, J.** 1997. Yeast nutrient transporters. Biochim. Biophys. Acta **1331:** 41–79.
41. **Hottiger, T., C. De Virgilio, M. N. Hall, T. Boller, and A. Wiemken.** 1994. The role of trehalose synthesis for the acquisition of thermotolerance in yeast. II. Physiological concentrations of trehalose increase the thermal stability of proteins *in vitro*. Eur. J. Biochem. **219:**187–193.
42. **Hottiger, T., P. Schmutz, and A. Wiemken.** 1987. Heat-induced accumulation and futile cycling of trehalose in *Saccharomyces cerevisiae*. J. Bacteriol. **169:**5518–5522.
43. **Hounsa, C. G., E. V. Brandt, J. Thevelein, S. Hohmann, and B. A. Prior.** 1998. Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. Microbiology **144:**671–680.
44. **Hwang, P. K., S. Tugendreich, and R. J. Fletterick.** 1989. Molecular analysis of *GPH1*, the gene encoding glycogen phosphorylase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**1659–1666.
45. **Iraqui, I., S. Vissers, F. Bernard, J. O. De Craene, E. Boles, A. Urrestarazu, and B. Andre.** 1999. Amino acid signaling in *Saccharomyces cerevisiae*: a permease-like sensor of external amino acids and F-box protein Grr1p are required for transcriptional induction of the *AGP1* gene, which encodes a broad-specificity amino acid permease. Mol. Cell. Biol. **19:**989–1001.
46. **Jones, E. W., and G. R. Fink.** 1982. Regulation of amino acid and nucleotide biosynthesis in yeast. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Jules, M., V. Guillou, J. Francois, and J. L. Parrou.** 2004. Two distinct pathways for trehalose assimilation in the yeast *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **70:**2771–2778.
48. **Kane, S. M., and R. Roth.** 1974. Carbohydrate metabolism during ascospore development in yeast. J. Bacteriol. **118:**8–14.
49. **Kingsbury, J. M., Z. Yang, T. M. Ganous, G. M. Cox, and J. H. McCusker.** 2004. A novel chimeric spermidine synthase-saccharopine dehydrogenase (*SPE3-LYS9*) gene in the human pathogen *Cryptococcus neoformans*. Eukaryot. Cell **3:**752–763.
50. **Kingsbury, J. M., Z. Yang, T. M. Ganous, G. M. Cox, and J. H. McCusker.** 2004. *Cryptococcus neoformans* Ilv2p confers resistance to sulfometuron methyl and is required for survival at 37°C and *in vivo*. Microbiology **150:** 1547–1558.
51. **Klasson, H., G. R. Fink, and P. O. Ljungdahl.** 1999. Ssy1p and Ptr3p are plasma membrane components of a yeast system that senses extracellular amino acids. Mol. Cell. Biol. **19:**5405–5416.
52. **LaRossa, R. A., and T. K. Van Dyk.** 1987. Metabolic mayhem caused by 2-ketoacid imbalances. Bioessays **7:**125–130.
53. **LaRossa, R. A., T. K. Van Dyk, and D. R. Smulski.** 1987. Toxic accumulation of α-ketobutyrate caused by inhibition of the branched-chain amino acid biosynthetic enzyme acetolactate synthase in *Salmonella typhimurium*. J. Bacteriol. **169:**1372–1378.
54. **Lillie, S. H., and J. R. Pringle.** 1980. Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. J. Bacteriol. **143:** 1384–1394.
55. **Lorenz, M. C., J. A. Bender, and G. R. Fink.** 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. Eukaryot. Cell **3:**1076–1087.
56. **Lorenz, M. C., and G. R. Fink.** 2001. The glyoxylate cycle is required for fungal virulence. Nature **412:**83–86.
57. **Lorenz, M. C., and J. Heitman.** 1998. The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. EMBO J. **17:**1236–1247.
58. **Lott, T. J., R. J. Kuykendall, and E. Reiss.** 1993. Nucleotide sequence analysis of the 5.8S rDNA and adjacent *ITS2* region of *Candida albicans* and related species. Yeast **9:**1199–1206.
59. **Magasanik, B., and C. A. Kaiser.** 2002. Nitrogen regulation in *Saccharomyces cerevisiae*. Gene **290:**1–18.
60. **Manning, M., C. B. Snoddy, and R. A. Fromtling.** 1984. Comparative pathogenicity of auxotrophic mutants of *Candida albicans*. Can. J. Microbiol. **30:**31–35.
61. **Marini, A. M., S. Soussi-Boudekou, S. Vissers, and B. Andre.** 1997. A family of ammonium transporters in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **17:**4282–4293.
62. **Mayordomo, I., and P. Sanz.** 2001. Hexokinase PII: structural analysis and glucose signalling in the yeast *Saccharomyces cerevisiae*. Yeast **18:**923–930.
63. **McCusker, J. H., K. V. Clemons, D. A. Stevens, and R. W. Davis.** 1994. Genetic characterization of pathogenic *Saccharomyces cerevisiae* isolates. Genetics **136:**1261–1269.
64. **McCusker, J. H., K. V. Clemons, D. A. Stevens, and R. W. Davis.** 1994. *Saccharomyces cerevisiae* virulence phenotype as determined with CD-1 mice is associated with the ability to grow at 42°C and form pseudohyphae. Infect. Immun. **62:**5447–5455.
65. **Murphy, A., and K. Kavanagh.** 1999. Emergence of *Saccharomyces cerevisiae* as a human pathogen: implications for biotechnology. Enzyme Microb. Technol. **25:**551–557.
66. **Natarajan, K., M. R. Meyer, B. M. Jackson, D. Slade, C. Roberts, A. G. Hinnebusch, and M. J. Marton.** 2001. Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol. Cell. Biol. **21:**4347–4368.
67. **Nwaka, S., and H. Holzer.** 1998. Molecular biology of trehalose and the trehalases in the yeast *Saccharomyces cerevisiae*. Prog. Nucleic Acid Res. Mol. Biol. **58:**197–237.
68. **Nwaka, S., B. Mechler, M. Destruelle, and H. Holzer.** 1995. Phenotypic features of trehalase mutants in *Saccharomyces cerevisiae*. FEBS Lett. **360:** 286–290.
69. **Nwaka, S., B. Mechler, and H. Holzer.** 1996. Deletion of the *ATH1* gene in *Saccharomyces cerevisiae* prevents growth on trehalose. FEBS Lett. **386:** 235–238.
70. **Ozcan, S., J. Dover, and M. Johnston.** 1998. Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. EMBO J. **17:**2566–2573.
71. **Ozcan, S., J. Dover, A. G. Rosenwald, S. Wolfl, and M. Johnston.** 1996. Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proc. Natl. Acad. Sci. USA **93:**12428–12432.
72. **Parrou, J. L., B. Enjalbert, L. Plourde, A. Bauche, B. Gonzalez, and J. Francois.** 1999. Dynamic responses of reserve carbohydrate metabolism under carbon and nitrogen limitations in *Saccharomyces cerevisiae*. Yeast **15:**191–203.
73. **Parrou, J. L., M. A. Teste, and J. François.** 1997. Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. Microbiology **143:**1891–1900.
74. **Petit, T., J. A. Diderich, A. L. Kruckeberg, C. Gancedo, and K. Van Dam.** 2000. Hexokinase regulates kinetics of glucose transport and expression of genes encoding hexose transporters in *Saccharomyces cerevisiae*. J. Bacteriol. **182:**6815–6818.
75. **Pontón, J., R. Rüchel, K. V. Clemons, D. C. Coleman, R. Grillot, J. Guarro, D. Aldebert, P. Ambroise-Thomas, J. Cano, A. J. Carrillo-Muñoz, J. Gené, C. Pinel, D. A. Stevens, and D. J. Sullivan.** 2000. Emerging pathogens. Med. Mycol. **38**(Suppl. 1)**:**225–236.
76. **Rafalski, J. A., and S. C. Falco.** 1988. Structure of the yeast *HOM3* gene which encodes aspartokinase. J. Biol. Chem. **263:**2146–2151.
77. **Randez-Gil, F., P. Sanz, K. D. Entian, and J. A. Prieto.** 1998. Carbon source-dependent phosphorylation of hexokinase PII and its role in the glucose-signaling response in yeast. Mol. Cell. Biol. **18:**2940–2948.
78. **Regenberg, B., L. During-Olsen, M. C. Kielland-Brandt, and S. Holmberg.** 1999. Substrate specificity and gene expression of the amino-acid permeases in *Saccharomyces cerevisiae*. Curr. Genet. **36:**317–328.
79. **Regenberg, B., and J. Hansen.** 2000. *GAP1*, a novel selection and counter-selection marker for multiple gene disruptions in *Saccharomyces cerevisiae*. Yeast **16:**1111–1119.
80. **Rhodes, J. C., L. S. Wicker, and W. J. Urba.** 1980. Genetic control of susceptibility to *Cryptococcus neoformans* in mice. Infect. Immun. **29:**494–499.
81. **Rubin-Bejerano, I., I. Fraser, P. Grisafi, and G. R. Fink.** 2003. Phagocytosis

by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. Proc. Natl. Acad. Sci. USA **100:**11007–11012.
82. **Rude, T. H., D. L. Toffaletti, G. M. Cox, and J. R. Perfect.** 2002. Relationship of the glyoxylate pathway to the pathogenesis of *Cryptococcus neoformans*. Infect. Immun. **70:**5684–5694.
83. **San-Blas, G., L. R. Travassos, B. C. Fries, D. L. Goldman, A. Casadevall, A. K. Carmona, T. F. Barros, R. Puccia, M. K. Hostetter, S. G. Shanks, V. M. Copping, Y. Knox, and N. A. Gow.** 2000. Fungal morphogenesis and virulence. Med. Mycol. **38**(Suppl. 1)**:**79–86.
84. **Schreve, J. L., J. K. Sin, and J. M. Garrett.** 1998. The *Saccharomyces cerevisiae YCC5* (YCL025C) gene encodes an amino acid permease, Agp1, which transports asparagine and glutamine. J. Bacteriol. **180:**2556–2559.
85. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1974. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
86. **Sillje, H. H., J. W. Paalman, E. G. ter Schure, S. Q. Olsthoorn, A. J. Verkleij, J. Boonstra, and C. T. Verrips.** 1999. Function of trehalose and glycogen in cell cycle progression and cell viability in *Saccharomyces cerevisiae*. J. Bacteriol. **181:**396–400.
87. **Singer, M. A., and S. Lindquist.** 1998. Multiple effects of trehalose on protein folding in vitro and in vivo. Mol. Cell **1:**639–648.
88. **Smith, D. G., M. D. Garcia-Pedrajas, S. E. Gold, and M. H. Perlin.** 2003. Isolation and characterization from pathogenic fungi of genes encoding ammonium permeases and their roles in dimorphism. Mol. Microbiol. **50:** 259–275.
89. **Stetter, J.** 1994. Herbicides inhibiting branched chain amino acid biosynthesis - recent developments, vol. 10. Springer-Verlag, Berlin, Germany.
90. **Storts, D. R., and J. K. Bhattacharjee.** 1987. Purification and properties of saccharopine dehydrogenase (glutamate forming) in the *Saccharomyces cerevisiae* lysine biosynthetic pathway. J. Bacteriol. **169:**416–418.
91. **Sumrada, R., M. Gorski, and T. Cooper.** 1976. Urea transport-defective strains of *Saccharomyces cerevisiae*. J. Bacteriol. **125:**1048–1056.
92. **Sumrada, R. A., and T. G. Cooper.** 1982. Urea carboxylase and allophanate hydrolase are components of a multifunctional protein in yeast. J. Biol. Chem. **257:**9119–9127.
93. **Sychrova, H., and M. R. Chevallier.** 1993. Cloning and sequencing of the *Saccharomyces cerevisiae* gene *LYP1* coding for a lysine-specific permease. Yeast **9:**771–782.
94. **Tripathi, G., C. Wiltshire, S. Macaskill, H. Tournu, S. Budge, and A. J. Brown.** 2002. Gcn4 co-ordinates morphogenetic and metabolic responses to amino acid starvation in *Candida albicans*. EMBO J. **21:**5448–5456.
95. **Van Dijck, P., L. De Rop, K. Szlufcik, E. Van Ael, and J. M. Thevelein.** 2002. Disruption of the *Candida albicans TPS2* gene encoding trehalose-6-phosphate phosphatase decreases infectivity without affecting hypha formation. Infect. Immun. **70:**1772–1782.
96. **Van Dyk, T. K., D. R. Smulski, and Y. Y. Chang.** 1987. Pleiotropic effects of *poxA* regulatory mutations of *Escherichia coli* and *Salmonella typhimurium*, mutations conferring sulfometuron methyl and alpha-ketobutyrate hypersensitivity. J. Bacteriol. **169:**4540–4546.
97. **Voth, W. P., J. D. Richards, J. M. Shaw, and D. J. Stillman.** 2001. Yeast vectors for integration at the *HO* locus. Nucleic Acids Res. **29:**E59–E69.
98. **Walsh, R. B., G. Kawasaki, and D. G. Fraenkel.** 1983. Cloning of genes that complement yeast hexokinase and glucokinase mutants. J. Bacteriol. **154:** 1002–1004.
99. **Wera, S., E. De Schrijver, I. Geyskens, S. Nwaka, and J. M. Thevelein.** 1999. Opposite roles of trehalase activity in heat-shock recovery and heat-shock survival in *Saccharomyces cerevisiae*. Biochem. J. **343:**621–626.
100. **Wheeler, R. T., M. Kupiec, P. Magnelli, C. Abeijon, and G. R. Fink.** 2003. A *Saccharomyces cerevisiae* mutant with increased virulence. Proc. Natl. Acad. Sci. USA **100:**2766–2770.
101. **White, W. H., P. L. Gunyuzlu, and J. H. Toyn.** 2001. *Saccharomyces cerevisiae* is capable of *de novo* pantothenic acid biosynthesis involving a novel pathway of β-alanine production from spermine. J. Biol. Chem. **276:**10794–10800.
102. **Whitney, P. A., and T. G. Cooper.** 1972. Urea carboxylase and allophanate hydrolase: two components of a multienzyme complex in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **49:**45–51.
103. **Wieczorke, R., S. Krampe, T. Weierstall, K. Freidel, C. P. Hollenberg, and E. Boles.** 1999. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Lett. **464:**123–128.
104. **Wiemken, A.** 1990. Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie Leeuwenhoek **58:**209–217.
105. **Yang, Z., R. C. Pascon, A. Alspaugh, G. M. Cox, and J. H. McCusker.** 2002. Molecular and genetic analysis of the *Cryptococcus neoformans MET3* gene and a *met3* mutant. Microbiology **148:**2617–2625.
106. **Zaragoza, O., M. A. Blazquez, and C. Gancedo.** 1998. Disruption of the *Candida albicans TPS1* gene encoding trehalose-6-phosphate synthase impairs formation of hyphae and decreases infectivity. J. Bacteriol. **180:**3809–3815.