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, iso-leucine, 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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† Supplemental material for this article may be found at http://ec.asm.org/.
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), norspermidine (100 µg ml−1; Hans Knoll Institute für Naturstoff-Forschung, Jena, Germany), hygromycin B (300 µg ml−1; Calbiochem), Genetecin (200 µg ml−1; Life Technologies), or fluocytosine (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.** Genes to be deleted 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 homohygro mutants. Alternatively, strains with multiple gene deletions were obtained by crossing strains with genes replaced by different drug markers. Strains YJM630 (sp1) and YJM634 (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 HXX2 open reading frame and 600-bp flanking sequence cloned into the Sall site of the HO-poly-KanMX4-HO plasmid (97) was mutated using the QuickChange XL-Site-Directed Mutagenesis kit (Stratagene) and mutagenic primers (CCCAAGAAA GGTTGCCATGGCCGATG and CACATCCGCAATGACCCCTTTCG GC), according to manufacturer’s instructions. The mutation was confirmed by sequencing (Duke Comprehensive Cancer Center Sequencing Facility). The HO-hxk2515A-kanMX4-HO region of the resulting plasmid was liberated by NotI digestion and was introduced into YAG438b (hsk2Δ/hxk2Δ), resulting in strain YAG621 (HO/hoxk2515A-kanMX4 hsk2Δ/ntmx4 hsk2Δ/ntmx4).

To reconstitute mutant strains with wild-type alleles, strains were transformed with a HXX2-containing plasmid 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 hsk2Δ mutants, the HXX2 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 recombinations were confirmed by colony PCR and by acquisition or loss of a phenotype, where possible. Specifically, *aro7A*, *lys9A*, *ihv2Δ*, *met3Δ*, *hom3A*, and *spe1Δ* 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* 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 *dur2*Δ mutants could not use urea as a nitrogen source at either concentration (92). *mep2*Δ mutants did not form pseudohyphae (57), and *mep1A* and *mep2A* mutants were unable to grow on low-ammonium media (14 mg liter−1) (61). *gap1Δ* mutants were unable to use citrulline as a sole nitrogen source (31); *gen4A* mutants were sensitive to 3-amino triazole and sulfometuron methyl in a disk diffusion assay (38); *nhl1*Δ mutants were unable to use trehalose (20 g liter−1) as a sole carbon source (69); *nhl1*Δ mutants recovered less well than the wild type following incubation at 50°C (68); and *smp1Δ* and *hxt2Δ* mutants showed less growth 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 × 10⁶ CFU of an inoculum. At 4 h, 1 week, and 2 weeks following infection, five mice per time point were euthanized by CO₂ 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 × 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 ([mutant/wild type]−1)[(mutant/wild type)]−1. 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 (26).

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*, required for metabolizing urea to carbon dioxide and ammonium (27, 92, 102). Two weeks after infection of CD-1 mice with *dur1Δ* (YJK251) and *dur3Δ* (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, *dur1Δ* and *dur3Δ* 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 ml−1) (16). Yeast transports ammonium by three permeases, Mep1p (high capacity, moderate affinity; *Kₘ*, 5 to 10 µM), Mep2p (low capacity and high affinity; *Kₘ*, 1 to 2 µM), and Mep3p (high capacity and low affinity; *Kₘ*, 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, *mep1Δ* (YJK240 and YJK242) and...
TABLE 1. Competitive indices (CI) of *S. cerevisiae* mutant strains

| Disrupted function | Genotype | Strain       | 4 h  | 7 days | 14 days |
|--------------------|----------|--------------|------|--------|---------|
| Nitrogen compound transport and utilization | dur2Δ/dur1Δ | YJK251 | 1.19 | 1.13  | 1.20    |
| Urea metabolism    | dur3Δ/dur3Δ | YJK225, YJK258 | 1.17 | 1.03  | 0.89    |
| Urea transport     | mep2Δ/mep2Δ | YJK240, YJK242 | 1.72 | 1.39  | 1.10    |
| Ammonium transport | mep23Δ/mep22Δ | YJK243 | 1.22 | 1.58  | 1.38    |
| Ammonium transport and sensing | mep1Δ/mep1Δ mep2Δ/mep2Δ | YJK282 | 1.03 | 1.04  | 0.84    |
| Ammonium transport and sensing | mep1Δ/mep1Δ mep2Δ/mep2Δ | YJK231, YJK233 | 0.99 | 1.04  | 0.95    |
| Amino acid transport | gap1Δ/gap1Δ | YJK228, YJK229 | 0.93 | 0.73  | 0.73    |
| Lysine transport   | bpl1/bpl1 | YJM610 | 0.73 | 0.46  | 0.51    |
| Lysine utilization | can1/can1 | YJM360 | 0.64 | 0.53  | 0.50    |
| Nitrogen regulation | ssy1Δ/ssy1Δ | YJK236, YJK238 | 0.94 | 0.71  | 0.72    |
| Amino acid transport and sensing | gap1Δ/gap1Δ | YJK296 | 1.24 | 1.10  | 1.11    |
| General control transcriptional activation | gcn4Δ/gcn4Δ | YJK318, YJK319 | 1.18 | 1.09  | 0.85    |
| Nitrogen catabolite repression | gln3Δ/gln3Δ | YJK321a, YJK295c | 0.33 | 0.41  | 0.38    |
| Glycogen utilization | gph1 | YJK356a, YJK464a | 1.26 | <0.0005 | <0.0005 |
| Glucose utilization | hxt2Δ/hxt2Δ | YJK539 | 1.09 | 0.83  | 0.65    |
| Glycolysis (hexokinase 1, glucokinase) | hxt5Δ/hxt5Δ | YAG406b | 0.94 | 0.49  | 0.39    |
| High affinity hexose transport | hxt7Δ/hxt7Δ | YAG406b | 0.94 | 0.49  | 0.39    |
| High glucose sensing | rgt2Δ/rgt2Δ | YAG310c, YAG312c | 1.03 | ND⁵ | 0.55    |
| Low glucose sensing | snf3Δ/snf3Δ | YAG315b, YAG317c | 0.93 | 0.14  | 0.14    |
| Methionine biosynthesis | met3Δ/met3Δ | YJK801, YJK004 | 0.54 | 0.91  | 0.62    |
| Methionine and threonine biosynthesis | hom3Δ/hom3Δ | YJK384a, YJK463b | 0.69 | 0.72  | 0.57    |
| Glycolysis (hexokinase 1, glucokinase) | hxt5Δ/hxt5Δ | YAG444 | 1.57 | 1.00  | 0.90    |
| Glycolysis, glucose signaling (hexokinase 2) | hxt12Δ/hxt12Δ | YAG451, YAG452 | 1.05 | 1.14  | 0.91    |
| Glycogen signaling | hxt2Δ/hxt2Δ | YAG436b, YAG438b | 0.45 | 0.17  | 0.18    |
| Fatty acid, lactic acid, and pyruvate utilization | hxt7Δ/hxt7Δ | YAG487 | 0.79 | 0.50  | 0.62    |
| Lactate and pyruvate transport | jen1Δ/jen1Δ | YAG406b | 0.94 | 0.49  | 0.39    |
| 2,3 Carbon utilization | icl1Δ/icl1Δ icl2Δ/icl2Δ | YAG406b | 0.94 | 0.49  | 0.39    |
| Storage carbohydrate utilization | icl1Δ/icl1Δ | YAG406b | 0.94 | 0.49  | 0.39    |
| Glycogen utilization | gph1Δ/gph1Δ | YAG592d, YAG594d | 1.00 | 0.78  | 0.77    |
| Trehalose utilization (acid trehalase) | ath1Δ/ath1Δ | YJK388a, YJK468b | 0.88 | 0.59  | 0.47    |
| Trehalose utilization (neutral trehalase 1) | ath1Δ/ath1Δ | YJK388a, YJK468b | 0.88 | 0.59  | 0.47    |
| Trehalose utilization (neutral trehalase 2) | nfh1Δ/nfh1Δ | YJK410 | 0.82 | ND  | 0.22    |
| Trehalose utilization | nfh2Δ/nfh2Δ | YJK377a | 0.84 | ND  | 0.90    |
| Trehalose utilization | ath1Δ/ath1Δ nfh1Δ/nfh1Δ | YJK421 | 0.92 | ND  | 0.17    |
| Trehalose utilization | ath1Δ/ath1Δ nfh2Δ/nfh2Δ | YJK424 | 0.80 | ND  | 0.13    |
| Trehalose utilization | nfh1Δ/nfh1Δ nfh2Δ/nfh2Δ | YJK418 | 1.23 | ND  | 0.42    |
| Trehalose utilization | ath1Δ/ath1Δ nfh1Δ/nfh1Δ | YJK639, YJK464 | 1.22 | 0.38  | 0.06    |
| Trehalose utilization | ath1Δ/ath1Δ nfh1Δ/nfh1Δ | YJK639, YJK644 | 1.22 | 0.38  | 0.06    |
| Trehalose utilization | nfh2Δ/nfh2Δ | YJK639, YJK644 | 1.22 | 0.38  | 0.06    |
| Trehalose utilization | ath1Δ/ath1Δ nfh2Δ/nfh2Δ | YJK759 | 1.05 | ND  | 0.10    |

⁴ CI = [(mutant/wild type) - 1]/[(mutant/wild type) + 1]. 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.

⁵ ND, not determined.
mutants (YJK243) grew, while mep1Δ mep2Δ mep3Δ (YJK282) mutants did not. Unlike mep1Δ mutants, mep2Δ 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 gap1Δ (YJK228 and YJK229) and agp1Δ (YJK231 and YJK233) mutants were found to survive nearly as well, or as well as, the wild type in vivo, while hpl1 (YJM610) and can1 (YJM630) 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 ssy1Δ 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 ssy1Δ mutant (51), we also tested the survival in vivo of a triple sss1Δ agp1Δ gap1Δ 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 their 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 gln3Δ mutant. Consistent with this hypothesis, gln3Δ 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 gcn4Δ 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 (met3Δ), methionine- and threonine-auxotrophic aspartate kinase (hom3Δ), isoleucine- and valine-auxotrophic acetalactate synthase (ilv2Δ), spermidine- and pantothenate-auxotrophic spermidine synthase (spe3Δ), tyrosine- and phenylalanine-auxotrophic chorismate mutase (aro7Δ), and lysine-auxotrophic saccharopine dehydrogenase (lys9Δ) mutants were compared to the wild type. The hsp9Δ (YJK788 and YJK794) mutants survived as well as the wild type in vivo, while met3Δ (YJK384a and YJK463b) and spe3Δ (YJK801 and YJK894) mutants were slightly attenuated after 2 weeks (Table 1).

Unlike the met3Δ, hsp9Δ, and spe3Δ mutants, the ilv2Δ (YJK365a and YJK464a) and hom3Δ (YJK487a and YJK495a) mutants were severely attenuated following 1 week in vivo and were eliminated 2 weeks postinfection (Table 1). Moreover, the aro7Δ 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 ilv2Δ, aro7Δ, or hom3Δ allele, respectively. Reconstitution of ilv2Δ mutants with ILV2 (YJK539) and hom3Δ 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 aro7Δ 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 (HXK2, 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, the snf3Δ mutant (YAG315b and YAG317c) survival was severely attenuated in vivo, while RGT2 disruption (YAG310c and YAG312c) had a slight effect (Table 1). Interestingly, while the snf3Δ 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 snf3Δ 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 Hk1p and Hxk2p as well as the glucokinase Glk1p (3, 15, 98). glk1Δ hsk1Δ mutants (YAG451 and YAG452) were found to survive as well as the wild type in vivo (Table 1). In contrast, survival of hsk2Δ mutants (YAG436b and YAG438b) was severely attenuated and could be restored to almost wild-type levels by reintroduction of the wild-type HXX2 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 hxx2<sup>STαΔ</sup> (YAG621) mutant was also attenuated in vivo, although to a lesser degree than that of the hsk2Δ 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 snf3Δ and hsk2Δ 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 jen1Δ 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 glycerogen 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 glycerogen hydrolysis influenced yeast survival in vivo.

Glycerogen hydrolysis to glucose and glucose-6-phosphate requires glycerogen phosphorylase (Gph1p) (44). Survival of gph1Δ 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 glycerogen 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 effects 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* *met3Δ*, *lys9Δ*, and *spe3Δ* 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, *aro7Δ*, *hom3Δ*, or *ilv2Δ* 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-ketobutyrate (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 *ilv2Δ*, *hom3Δ*, and *aro7Δ* 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 genes 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 two-fold following transfer of S. cerevisiae from YPD medium to RPMI medium with serum (56 and Table S1), none of which were highly significant in vivo. 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, DUR3, MEP2, and GAPI) 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 habituation 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.

ACKNOWLEDGMENTS

We thank T. Mitchell and J. Heitman for comments on the manuscript and A. Kruckenberg for helpful discussion.

This work was funded by NIH grants R01-GM58476, R01-GM707541, and P01-AI44975.

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