Ectopic Expression of URA3 Can Influence the Virulence Phenotypes and Proteome of Candida albicans but Can Be Overcome by Targeted Reintegration of URA3 at the RPS10 Locus

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Uridine auxotrophy, based on disruption of both URA3 alleles in diploid Candida albicans strain SC5314, has been widely used to select gene deletion mutants created in this fungus by “Ura-blasting” and PCR-mediated disruption. We compared wild-type URA3 expression with levels in mutant strains where URA3 was positioned either within deleted genes or at the highly expressed RPS10 locus. URA3 expression levels differed significantly and correlated with the specific activity of Ura3p, orotidine 5’-monophosphate decarboxylase. Reduced URA3 expression following integration at the GCN4 locus was associated with an attenuation of virulence. Furthermore, a comparison of the SC5314 (URA3) and CAI-4 (ura3) proteomes revealed that inactivation of URA3 caused significant changes in the levels of 14 other proteins. The protein levels of all except one were partially or fully restored by the reintegration of a single copy of URA3 at the RPS10 locus. Transcript levels of genes expressed ectopically at this locus in reconstituted heterozygous mutants also matched the levels found when the genes were expressed at their native loci. Therefore, phenotypic changes in C. albicans can be associated with the selectable marker rather than the target gene. Reintegration of URA3 at an appropriate expression locus such as RPS10 can offset most problems related to the phenotypic changes associated with gene knockout methodologies.

Genes encoding enzymes in the uridine biosynthetic pathway are used as positive selectable markers in gene disruption strategies for a wide range of eukaryotic organisms. For the major human pathogenic fungus Candida albicans, this approach, based on the URA3 gene, was the only means available for gene disruption for more than 10 years. C. albicans is diploid and has no known natural sexual cycle that leads to a haploid form. Therefore, molecular genetic evidence associating phenotypic changes with individual gene functions requires the successive disruption of two copies of the gene of interest. “Ura-blasting,” used for gene disruption in C. albicans (28), is based on a similar approach in Saccharomyces cerevisiae (1) and makes use of a C. albicans ura3 auxotroph. Transformants are selected on the basis of uridine prototrophy. The marker is then recycled by use of 5-fluoroorotic acid to select for Ura- segregants that have lost URA3 through a reciprocal crossover between identical flanking DNA repeats. The “Ura-blaster” can then be used to disrupt the second allele in these ura3 segregants. PCR-based gene disruption protocols also exploit the URA3 marker but often do not recycle it (90).

One area of investigation that has made extensive use of specific gene disruption based on the URA3 selection strategy is the study of virulence in C. albicans. At least 50 genes have been implicated as putative virulence factors in this fungus based on results of mouse intravenous challenge experiments in which the homozygous C. albicans null mutants of interest were shown to be less lethal than wild-type controls (61). In most of these studies, the strain tested in mice had been the Ura+ transformant obtained after the second round of Ura-blasting (e.g., yfgΔ::hisG/yfg1Δ::hisG-URA3-hisG).

“Molecular Koch’s postulates” suggest that confirmation of a microbial virulence factor requires that (i) inactivation of the gene should attenuate virulence and (ii) reintegration of the gene into the null mutant should restore wild-type virulence. In C. albicans, this process depends predominantly on the use of URA3 as a selectable marker. However, the use of Ura selection creates a technical problem. These procedures result in the transfer of URA3 from its normal location on chromosome 3 to a nonnative chromosomal location. Hence, mutant and control strains used in virulence experiments often differ in two (not one) respects: disruption of the target locus and the genomic location of URA3.

A number of publications have documented problems arising from the use of URA3 as a selectable marker following gene disruption. Lay et al. (48) demonstrated that specific activities of orotidine 5’-monophosphate (OMP) decarboxylase (Ura3p) differed between strains of C. albicans harboring one or two copies of URA3 in a way that was not simply related to the gene dosage. They showed an inverse correlation between OMP decarboxylase activity and the survival times of mice infected with these mutants. Sundstrom et al. (82) showed that the location of URA3 at the disruption site affected the virulence phenotype of hisp1/hisp1 mutants. Bain et al. (7) demonstrated that the URA3 status of cell wall mutants affected their ability to adhere to buccal epithelial cells. Cheng et al. showed that changing the location of URA3 in the C. albicans genome affected OMP decarboxylase activity, hyphal morphogenesis, buccal cell adherence, and lethality for mice (19). In addition, the phenotypic effects that have been correlated with the disruption of four C. albicans genes were shown...
to be a consequence of the genomic relocation of \( URA3 \) in three of these cases (19). Staab and Sundstrom reviewed the importance of \( URA3 \) positional effects in influencing phenotypes of \( C. albicans \) mutants (81). They propose that a "wild-type" strain of \( C. albicans \) comprising CAI-4 with \( URA3 \) reintegrated at a highly expressed locus, ENO1, is a more appropriate control for Ura-blasted mutants than either of the CAI-4 parental strains, SC5314 and CAF2-1.

We have further assessed the significance of \( URA3 \) expression as an influence on virulence and other phenotypic characteristics in \( C. albicans \). Our experiments make use of the Clp10 vector, which reintegrates \( URA3 \) at the high-expression \( RPS10 \) locus (59), producing mutant and control strains with isogenic backgrounds. We show that expression levels of \( URA3 \) correlate with OMP decarboxylase activities in three mutants created using the Ura-blasted protocol. We have tested the association between OMP decarboxylase activities and mouse virulence in several \( C. albicans \) mutants and clinical isolates and studied the global effects of \( URA3 \) deletion on the \( C. albicans \) proteome in a pairwise comparison of SC5314 and CAI-4. We show, for a wide range of mutant strains, that ectopic expression of \( URA3 \) is likely to have an effect on the virulence phenotype in as many as 30% of published cases but that the phenotype can be corrected by expression of \( URA3 \) at the \( RPS10 \) locus.

### MATERIALS AND METHODS

**C. albicans**, strains, culture media, and growth conditions. The \( C. albicans \) strains used in this study are listed in Table 1. \( C. albicans \) strains were routinely grown and maintained on Sabouraud agar. For reverse transcription-PCR (RT-PCR) experiments, strains were grown with shaking at 37°C in SD (6.7 g of yeast nitrogen base per liter, 20 g of glucose per liter) to an optical density at 600 nm (OD<sub>600</sub>) of 0.95. For analysis by two-dimensional (2D) gel electrophoresis, strains were grown to an OD<sub>600</sub> of 0.65 at 37°C in YEPD (10 g of yeast extract [Oxoid] per liter, 20 g of mycological peptone [Oxoid] per liter, 20 g of glucose

### TABLE 1. \( C. albicans \) strains used in this study

| Strain | Description or name | Genotype | Source or reference |
|--------|---------------------|----------|---------------------|
| ATCC 44858 | Isolate from parrot | originally from Janssen Pharmaceutica |
| NCPF 3153 | Clinical isolate | reference antigen strain, Public Health Mycology Reference Laboratory |
| RV4688 | Clinical isolate | originally from Janssen Pharmaceutica |

### Strain Description or Name

- AM2003-020: Isolate from healthy carrier
- 73/034: Isolate from healthy carrier
- SC5314: Clinical isolate
- CAI-4: Clinical isolate
- SC5314 Clinical isolate 28
- AM2003-020 Isolate from healthy carrier
- RV4688 Clinical isolate Originally from Janssen Pharmaceutica

### Genotype

- Saul at the high-expression

### Source or Reference

- Originally from Janssen Pharmaceutica
- Reference antigen strain, Public Health Mycology Reference Laboratory
- Originally from Janssen Pharmaceutica

### Mutations

- \( URA3 \) reintegrants
- Multicopy reintegrants

### Additional Information

- \( URA3 \) correlation with OMP decarboxylase activities in three mutants
- Testing the correlation with OMP decarboxylase activities in three mutants

### References

- Staab and Sundstrom reviewed the importance of \( URA3 \) positional effects in influencing phenotypes of \( C. albicans \) mutants (81).
- They propose that a "wild-type" strain of \( C. albicans \) comprising CAI-4 with \( URA3 \) reintegrated at a highly expressed locus, ENO1, is a more appropriate control for Ura-blasted mutants than either of the CAI-4 parental strains, SC5314 and CAF2-1.

### Notes

- \( URA3 \) at the high-expression
- \( RPS10 \) locus
- Multicopy reintegrants
- Deletion spans MN1, MN2, and intervening ORF IPF6318 (putative \( \beta \)-glucosidase).
per liter) supplemented with 25 μg of uridine per ml. For inoculum preparation in animal experiments, strains were grown for 24 h in 5-ml lots of NGY (1 g of neopeptone [Difco] per liter, 4 g of glucose per liter, 1 g of yeast extract [Difco] per liter) rotated at 30°C at 20 rpm in a test tube wheel set at an angle of 5° from the horizontal. For OMP decarboxylase assays, cells were either grown in SD, as for RT-PCR experiments, or shaken in flask cultures of NGY at 37°C until growth reached an OD600 of 0.6. Pilot experiments showed that there was no significant difference in enzyme activity from cells grown under the two conditions.

**RNA isolation and cDNA synthesis.** Total RNA was extracted by the method of Schaller et al. (72). mRNA was isolated from total RNA using a Dynabeads mRNA purification kit (Dynal A.S., Oslo, Norway). cDNA was synthesized using 400 U of Superscript II RNase H– reverse transcriptase kit (Invitrogen) as specified by the manufacturer. cDNA was quantified by measurement of OD_{260} and diluted to give a stock concentration of 500 ng/μl.

**Multiplex RT-PCR and estimation of URA3 mRNA abundance.** Fragments of URA3, GCN4, MNN4, and MNT5 cDNAs were each coamplified with the cDNA of elongation factor B1 (54, 72). Primers for PCR are listed in Table 2. PCR conditions were 94°C for 10 s followed by 27 cycles of 94°C for 45 s, annealing temperature for 60 s (Table 2), and 72°C for 45 s. Approximately 500 ng of cDNA was used as template. The PCR products from sequential PCR cycles were visualized on 2% agarose gels, and the difference in cycle number between the appearance of the first band for EFB1 and that of the gene of interest was recorded for three independent samples for each strain. Within each RT-PCR experiment, the relative abundance of URA3 mRNA was estimated from the mean cycle number difference.

**OMP decarboxylase assays.** Protein extraction from cells that had been disrupted by vortexing with 0.45-mm-diameter glass beads (Sigma-Aldrich Ltd., Poole, United Kingdom) and OMP decarboxylase assays were based on the methods described by Lay et al. (48) and Yashimoto et al. (95). The assay mixture, in a total volume of 850 μl, contained approximately 100 μg of cell lysate protein, 0.1 M phosphate buffer (pH 6.0), 1.0 μM of d-beta-methylcothanolamin and 0.15 μM of OMP substrate (Sigma-Aldrich). Conversion of OMP to UMP was measured spectrophotometrically at room temperature as the decrease in absorbance of the substrate at 285 nm over a specified period. The concentration of OMP in the assay was calculated from a molar extinction coefficient of 0.14 cm⁻¹ M⁻¹. One unit of enzyme was defined as the amount of enzyme required to convert 1 μmol of OMP to UMP per min. Specific activity was defined as units of enzyme activity per milligram of protein. The protein concentration was determined with a Coomassie protein assay reagent kit (Pierce, Rockford, Ill.). Enzyme activity was assayed for three independent samples per strain. C. albicans SC5314 was included as a reference control in each experiment. To compare data from different experiments, specific activities of OMP decarboxylase were normalized to the activity measured for SC5314, which was set at 100%. The OMP decarboxylase specific activities reported previously for CAF2-1 (48) differed by approximately 1,000-fold from those we have measured.

**Protein extraction and 2D gel electrophoresis.** The COGEME Proteome Research Facility 1 (http://www.abdn.ac.uk/cogeme) undertook all protein extraction and 2D gel electrophoresis experiments. Samples were resolved in the first dimension on 24-cm Immobiline DryStrip gels (pH 4 to 7) (Amersham Biosciences, Chalfont St. Giles, United Kingdom) in an Ettan IPGphor isoelectric focusing unit (Amersham Biosciences) and in the second dimension on precast sodium dodecyl sulfate–12.5% polyacrylamide gels with an Ettan Dalt system (Amersham Biosciences).

**Image analysis and protein identification.** Images from three independent samples per strain were analyzed with Phoretix 2D software (Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, United Kingdom) to identify features that were reproducibly altered between strains. Spots that displayed more than a twofold change in relative volume with a statistical significance of P ≤ 0.05 (Student’s t test) were analyzed further. Peptide mass fingerprints were generated from spots by MALDI-TOF mass spectrometry and used to interrogate the CandidaDB database (http://genolist.pasteur.fr/CandidaDB). Data are available at the COGEME PRF website (http://www.cogeme.abdn.ac.uk).

**Experimental infections in mice.** Experimentation was done under the terms of United Kingdom Home Office licenses for research on animals. Female DBA/2 and BALB/c mice (Harlan) with a weight range from 17 to 23 g were maintained in individually ventilated cages under conditions specified by the Health and Safety Executive for level 2 biohazard containment. The animals were supplied with food and water ad libitum. Challenge inocula were grown overnight at 30°C in NGY, centrifuged, washed twice with sterile distilled water, and re suspended in sterile saline. The inocula were standardized by spectrophotometry, and their concentrations were adjusted according to the experimental design. The true concentrations of yeast inocula were routinely determined by viable counts made from the suspensions used for (intra)venous challenge.

Mice were challenged by i.v. inoculation of C. albicans suspensions via a lateral tail vein. For BALB/c mice the challenge dose was in the range of 4.2 to 5.0 log_{10} CFU/g of body weight (median, 10.4.5 CFU/g), and for DBA/2 mice the dose range was 2.4 to 3.3 log_{10} CFU/g of body weight (median, 10.2.8 CFU/g). Differences in mouse survival and organ burdens between C. albicans strains were not attributable to the variations in challenge dose. Correlation coefficients between challenge dose and mean survival times or mean organ burdens ranged from -0.1 to 0.3 for both mouse strains.

For all animals, the postchallenge day of death or of euthanasia for animals exhibiting signs of serious illness was recorded and the C. albicans tissue burden in both kidneys and brain was determined at the time of demise by viable counting of 100-μl samples homogenized in 0.5-mM volumes of sterile saline. Detection limits for tissue burdens under these conditions were 150 CFU (kid-
nees) and 80 CFU (brain). Culture-negative samples were scored as 0.5 log_{10} unit lower than these limits for statistical purposes. All experiments ended 28 days after challenge; animals surviving at this time were humanely killed, and tissue burdens were determined by plating on Sabouraud agar.

**RESULTS**

**OMP decarboxylase activity correlates with URA3 mRNA abundance.** Ura3p specific activities were measured for cell extracts from a range of strains created with the Ura-blaster methodology and containing one or two copies of URA3. OMP decarboxylase activity reflected the URA3 gene copy number in the control strains SC5314 and CAF2-1 and was not affected by growing the cells in increasing concentrations of exogenous uridine (Table 3). No activity was detected in strain CAI-4, consistent with the lack of a functional URA3 allele in this strain. In mutant strains derived from the SC5314–CAF2-1–CAI-4 lineage, OMP decarboxylase activity varied depending on the genomic location of the URA3 gene and was lower than that of CAF2-1 in all mutants. However, OMP decarboxylase activity was restored to the level of CAF2-1 when URA3 was reintegrated at the RPS10 locus by using CIP10. There was one exception (NGY145), which had significantly higher activity
than CAF2-1 (Student’s t test; \( P = 0.022 \)), although this was only 73% of the activity of SC5314. A selection of five wild-type clinical isolates of *C. albicans* had OMP decarboxylase specific activities of the same order as that of SC5314 (Table 3). Therefore, the chromosomal location of *URA3* significantly influenced Ura3p specific activity.

**URA3 expression levels depend on chromosomal location but are restored to the CAF2-1 equivalent by reintegration at the RPS10 locus.** To establish whether the expression of ectopically placed *URA3* was influenced by the normal level of transcriptional activity at target loci, *MNT3, MNN4,* and *GCN4* mRNA abundance was measured relative to *URA3* mRNA abundance for strain CAF2-1 (*URA3/ura3*) by semiquantitative RT-PCR. The results (Table 4) showed that *GCN4* mRNA levels were considerably higher than those for *URA3*. The *MNN4* mRNA levels were similar to those of *URA3*, and expression of *MNT5* was 25-fold lower than that of *URA3*.

Notwithstanding these differences in the expression of the native genes, *URA3* mRNA abundance was relatively low at all of these loci compared with *URA3* expression at its native locus (in CAF2-1 [Fig. 1; Table 4]). This indicated that expression levels of *URA3* at ectopic loci do not reflect the expression level of the gene native to each locus. When Clp10 was used to transform the *mnt5, mnn4,* and *gcn4* mutants, producing strains with *URA3* at the RPS10 locus, *URA3* mRNA abundance was restored essentially to the same levels as in CAF2-1 (Fig. 1). Hence, the reinsertion of *URA3* at the RPS10 locus restored *URA3* mRNA to normal (heterozygote) levels, regardless of which chromosomal locus had been disrupted by Ura-blasting. There was a strong positive correlation between *URA3* mRNA level and OMP decarboxylase activity in the same strains (Tables 3 and 4; \( r = 0.75 \)).

**Influence of URA3 expression on virulence of*C. albicans* mutants.** To determine the importance of *URA3* expression levels for the virulence of *C. albicans*, a series of strains derived from CAI-4 were tested by i.v. challenge in DBA/2 mice for differences in survival times and tissue burdens of *C. albicans*. The strains tested carried a single copy of *URA3*: CAF2-1 (*URA3/ura3*), NGY152 (CAI-4/Cbp10), GTC41 (*GCN4/gcn4*), GTC43 (*gcn4*/Δ), GTC45 (*gcn4*/Δ/Clp10), and GTC49 (*gcn4*/Δ/Cbp10-*GCN4*). The results are shown in Fig. 2 and Table 5. A marked reduction in virulence for both the heterozygous *GCN4/gcn4* mutant (GTC41) and the homozygous *gcn4*Δ mutant (GTC43) was found relative to the parent strain (CAF2-1). The effect was equally evident from both the long survival (Fig. 2) and the

| C. albicans strain | OMP decarboxylase activity (nmol/mg/min; a) | Activity relative to SC5314 (%) | Strain transformed with Clp10 | OMP decarboxylase activity (nmol/mg/min; a) | Activity relative to SC5314 (%) |
|-------------------|---------------------------------------------|-------------------------------|--------------------------------|---------------------------------------------|-------------------------------|
| SC5314            | 29.7 ± 3.7                                  | 100.0                         |                                |                                             |                               |
| CAF2-1            | 15.4 ± 1.4                                  | 51.7                          |                                |                                             |                               |
| CAF2-1 + uridine (25 μg/ml) | 14.7 ± 0.8                                 | 49.5                          |                                |                                             |                               |
| CAF2-1 + uridine (250 μg/ml) | 15.0 ± 2.0                                 | 50.5                          |                                |                                             |                               |
| CAI-4             | <1.0f                                       | <1.0f                         | NGY152                         | 16.7 ± 1.3                                  | 56.2                          |
| CAI-4 + uridine (25 μg/ml) | <1.0f                                       | <1.0f                         | NGY152                         | 13.0 ± 1.2                                  | 43.8                          |
| CAI-4 + uridine (250 μg/ml) | <1.0f                                       | <1.0f                         | NGY152                         | 13.0 ± 2.8                                  | 43.8                          |
| NGY23 (mnt1Δ)     | 8.8 ± 0.5                                   | 29.6                          | NGY158                         | 16.8 ± 0.7                                  | 56.6                          |
| NGY105 (mnt2Δ)    | 13.6 ± 1.5                                  | 45.8                          | NGY145                         | 21.6 ± 2.9                                  | 72.7                          |
| NGY63 (mnt3Δ)     | 2.6 ± 0.8                                   | 14.1                          | NGY146                         | 12.9 ± 1.9                                  | 43.4                          |
| NGY87 (mnt4Δ)     | 2.7 ± 1.1                                   | 9.1                           | NGY313                         | 14.4 ± 1.4                                  | 48.5                          |
| NGY71 (mnt5Δ)     | 5.9 ± 0.3                                   | 19.9                          | NGY147                         | 17.7 ± 1.2                                  | 59.6                          |
| DHS5 (mnt6Δ)      | 7.0 ± 0.9                                   | 23.6                          | DH15                           | 12.3 ± 1.1                                  | 41.4                          |
| GTC43 (gcn4Δ)     | 8.8 ± 0.9                                   | 29.6                          | GTC45                          | 15.5 ± 0.8                                  | 52.1                          |
| BH1-1-1 (sup3Δ)   | 14.1 ± 0.9                                  | 47.5                          | VC3.9                          | 15.4 ± 0.0                                  | 51.9                          |
| 73004             | 38.7 ± 0.2                                  | 130.3                         |                                |                                             |                               |
| AM2003-020        | 29.4 ± 1.0                                  | 99.0                          |                                |                                             |                               |
| 3153              | 25.0 ± 3.2                                  | 84.2                          |                                |                                             |                               |
| B2360             | 24.9 ± 2.9                                  | 83.8                          |                                |                                             |                               |

\( ^{a} \) Results are expressed as mean ± standard error of the mean.

\( ^{b} \) Limit of detection.
TABLE 4. Expression of C. albicans genes and expression of UR43 in ectopic gene loci measured on the basis of relative RNA abundance by RT-PCR

| Gene   | Expression of target genes in CAF2-1 | Strain   | Strain   | Strain   |
|--------|-------------------------------------|----------|----------|----------|
| URA3   | Increased 6.4-fold                  | NGY152   | Decreased 6.8-fold | Increased 1.34-fold |
| MNT5   | Decreased 26-fold                   | GTC49    | Increased 435-fold | Decreased 2.0-fold |
| GCN4   | Increased 435-fold                  | GTC43    | Decreased 2.0-fold | Decreased 2.0-fold |
| URA3   | Decreased 0.6-fold                  | NGY147   | Decreased 2.0-fold | Decreased 1.33-fold |
| MNN4   | Decreased 8.0-fold                  | DH15     | Decreased 1.33-fold | Decreased 2.0-fold |
| GTC49  | Decreased 1.33-fold                 | GTC45    | Decreased 2.0-fold | Decreased 2.0-fold |

* Measured as mRNA abundance relative to URA3 mRNA.

relatively low tissue burdens (Table 5) of mice challenged with these strains. However, integration of Clp10 containing only the URA3 gene into the gcn4Δ null mutant (GTC45) restored wild-type virulence, with survival curves and tissue burdens matching those of CAF2-1 and NGY152. Reintegration of one copy of GCN4 at the RPS10 locus using Clp10 also produced a mutant with wild-type virulence restored.

Figure 3 shows scatter plots of OMP decarboxylase specific activities for a range of C. albicans clinical isolates and mutants versus their mean survival times in the mouse and versus mean fungal burdens in the kidneys and brains of infected animals. For mutants derived from the SC5314–CAF2-1–CAI-4 lineage by Ura-blasting, a weak negative association was apparent between relative OMP decarboxylase specific activity and mean survival time (r = −0.38) and a weak positive association was apparent between enzyme activity and kidney burdens (r = 0.40) and brain burdens (r = 0.38). Data for strains RV4688 and 73/034 show clearly that strains of relatively low virulence the deletion of URO1 as well as UR43 (31), we also examined the proteome of strain NGY152, in which one copy of UR43 is reintegrated at the RPS10 locus. As predicted, Ura3p was present on SC5314 gels at a position consistent with its pl and molecular mass (pl = 5.24, molecular mass = 24 kDa) but was not detected on CAI-4 gels. Exogenous uridine was supplied for all strains to facilitate growth of CAI-4. The presence of Ura3p in SC5314 gels under these growth conditions was consistent with the finding in this study that exogenous uridine supplied to growing cells did not affect OMP decarboxylase activity. Fourteen other proteins showed altered expression in CAI-4 (Table 6). Levels of all proteins except the transcription factor, Toa2p, were partially or fully restored by the reintroduction of a single copy of UR43 in strain NGY152, indicating that the deletion of UR43, and not the truncation of IRO1, accounts for the majority of proteomic changes observed for CAI-4. Of the 13 proteins displaying UR43-dependent changes, 3 (Hpt1p, Ade2p, and Ura5p) are involved in purine and pyrimidine metabolism. These pathways may be perturbed by an altered flux of metabolic intermediates in the absence of OMP decarboxylase, the last of six enzymes in the uridine de novo biosynthetic pathway. Other changes in expression suggest that heme biosynthesis (Ald5p and Hem13p) and aromatic amino acid turnover (Aro8p and Aro10p) may also be perturbed by the loss of Ura3p. Another group of proteins with related function that showed altered expression in CAI-4 comprised Rps12p, Etf2p, and IPF6037p, which are involved in translation or transcription. Therefore, inactivation of UR43 appears to influence C. albicans metabolism in unexpected ways.

TABLE 5. Tissue burdens of C. albicans in left kidneys and brains from mice challenged i.v. with mutants derived from CAF2-1.

| Strain           | C. albicans burden (log10 CFU/g) | Left kidney | Brain |
|------------------|---------------------------------|-------------|-------|
| CAF2-1           | 6.6 ± 0.6                       | 4.4 ± 0.5   |       |
| NGY152 (CAI-4/Clp10) | 6.4 ± 0.6                  | 4.3 ± 0.4   |       |
| GTC41 (GCN4/#gn4 Δ) | 3.2 ± 1.2                     | 2.9 ± 1.1   |       |
| GTC43 (gn4 Δ)    | 3.2 ± 1.2                       | 1.7 ± 1.0   |       |
| GTC45 (gn4 Δ/Clp10) | 6.5 ± 0.2                   | 4.5 ± 0.1   |       |
| GTC49 (gn4 Δ/Clp10/GCN4) | 6.7 ± 0.1           | 4.3 ± 0.2   |       |

* There were 6 mice per group except for CAF2-1, which had 10 mice per group.

b Results are expressed as mean ± standard deviation.
DISCUSSION

We show that the chromosomal location of _URA3_ has a significant impact on its expression, the resulting specific activity of the gene product, the cellular proteome, and consequently the virulence of _C. albicans_. The Ura-blast approach to disruption of diploid genes is highly effective as a molecular tool, but phenotypic alterations recorded in mutants created by Ura-blasting cannot be reliably attributed to the target gene unless care is taken to ensure that the mutant expresses adequate levels of Ura3p. Ectopic expression of _URA3_ from within a disrupted gene cannot be depended on to provide adequate Ura3p levels, presumably because of positional effects of the target locus on _URA3_ gene expression. Only by specific insertion of the marker gene at a common genomic locus in different strains can a scientifically comparable set of parents and mutants be created. Sundstrom and her colleagues have recommended (81) and implemented (4, 5) specific insertion of the _URA3_ gene at the _ENO1_ locus as a means of circumventing positional effects on _URA3_ expression. Our own preference is for placement of _URA3_ at the _RPS10_ locus, since the plasmid _Clp10_ makes the necessary transformation technically very simple and we have confirmed that this locus enables near-normal levels of gene expression to occur.

One area of study in which the difficulties associated with ectopic expression of _URA3_ may have greatly affected the outcome of experiments is in the delineation of molecular virulence factors in _C. albicans_. At least 70 _C. albicans_ genes (Table 7) have been implicated in virulence on the basis of evidence of attenuation in the mouse i.v. challenge model for Ura-blasted homozygous null mutants. However, in the majority of these animal studies, _URA3_ was expressed from within the locus of the disrupted gene (Table 7). We show a dramatic example of this, where attenuated virulence in _C. albicans_ was attributable to decreased _URA3_ expression rather than disruption of the _GCN4_ locus (Fig. 2, compare NGY152 [wild type], GTC45 [gen42] and GTC45 [gen4Δ/Clp10]). Our evidence supporting a role for Gcn4p in virulence satisfies the molecular Koch’s postulates. However, the virulence of the mutant containing only _URA3_ reintegrated at the _RPS10_ locus proves that the attenuated phenotype in the homozygous null mutant was the result of ectopic expression of _URA3_ and not of deletion of _GCN4_ (Fig. 2, compare NGY152 [wild type], GTC45 [gen4Δ/Clp10], and GTC49 [gen4Δ/Clp10-GCN4]). We have similar data for two other _C. albicans_ loci (unpublished data). It seems

![FIG. 3. Scatter plots of OMP decarboxylase specific activity in wild-type and mutant _C. albicans_ strains versus three parameters of virulence in a mouse i.v. challenge model: mean survival time (a), mean kidney burden (b), and mean brain burden (c). Each point represents the result with a single strain tested for BALB/c mice (open circles) or DBA/2 mice (solid circles) in groups of 6 animals, except for ATCC 44858. Data for brain burdens were not available for ATCC 44858.](image)

**TABLE 6. Effect of _URA3_ deletion on the cytosolic proteome**

| Protein (Reference) | Change in expression in CAI-4 | Expression in NGY152 (URA3 reintegrated at _RPS10_ locus) |
|---------------------|-----------------------------|----------------------------------------------------------|
| Ura3p (33)          | Not detected                | Partially restored                                        |
| Hpt1p (34)          | Not detected                | Fully restored                                            |
| Ald5p (2 spots) (46, 68) | Only 1 spot present        | Fully restored                                            |
| Sgt2p (22, 45)      | Only 1 spot present         | Fully restored                                            |
| Pmn1p (78)          | 4.5-fold increase           | Fully restored                                            |
| IPF6037             | 4.5-fold increase           | Fully restored                                            |
| Aro6p (40)          | 3.8-fold increase           | Fully restored                                            |
| Ade2p (73)          | 3.8-fold increase           | Partially restored                                        |
| Ura5p (25)          | 3.3-fold increase           | Fully restored                                            |
| Efi2p (55)          | 2.9-fold increase           | Partially restored                                        |
| Aro10p (87)         | 2.9-fold increase           | Partially restored                                        |
| Hemi3p (98)         | 2.1-fold increase           | Partially restored                                        |
| Rps12p (63)         | 4.2-fold decrease           | Fully restored                                            |
| Toa2p (43)          | 3.2-fold decrease           | Not restored (same as CAI-4)                              |
| IPF4328             | 2.6-fold decrease           | Fully restored                                            |

* Results are shown for proteins with a greater than twofold change in expression between _SC5314_ and CAI-4. Cells were grown in YPD with 25 μg of uridine per ml at 30°C to _OD_600 = 0.6. Total solubilized protein was resolved by 2D gel electrophoresis and subjected to proteomic analysis. Proteins were identified by MALDI-TOF mass spectrometry and interrogation of the CandidaDB database.
| Gene   | Function                              | Locus containing URA3<sup>a</sup> | Reference(s) |
|--------|---------------------------------------|-----------------------------------|--------------|
| AAF1   | Unknown function                      | AAF1                              | 66           |
| ADE2   | Phosphoribosylaminomimidazole carboxylase | ADE2                             | 27           |
| ALO1   | α-Arabinono-1,4-lactone oxidase       | ALO1                              | 36           |
| ALS1   | Surface adhesin                       | ALS1                              | 29           |
| ASH1   | Transcription factor                  | ASH1                              | 39           |
| BGL2   | (1-3) β-Glucosyltransferase          | BGL2                              | 71           |
| CAP1   | Adenyl cyclase regulator              | eno::URA3                         | 4, 5         |
| CAT1   | Catalase                              | CAT1                              | 91           |
| CDC10  | Septin                               | ARG4                              | 88           |
| CDC11  | Septin                               | ARG4                              | 88           |
| CDC24  | GDP,GTP exchange factor               | RPS10                             | 8            |
| CDC35  | Adenyl cyclase regulator              | part of plasmid pVEC              | 67           |
| CDC42  | G protein (GTPase)                    | RPS10                             | 8            |
| CHS1   | Chitin synthase                       | CHS1 under mal promoter           | 58           |
| CHS3   | Chitin synthase                       | CHS3                              | 14, 58       |
| CLA4   | Ser/Thr protein kinase                | CLA4                              | 50           |
| CNA    | Calci neurin A                        | CNA                               | 70           |
| CNB1   | Calci neurin B                        | CNB1                              | 11           |
| COS1   | Histidine kinase                      | COS1                              | 75           |
| CPP1   | Protein phosphatase                   | CPP1                              | 51, 29       |
| CRK1   | Cdc2 kinase                           | CRK1                              | 18           |
| CSP37  | 37-kDa surface protein                | CSP37                             | 76           |
| CST20  | MEKK kinase                           | CST20                             | 49           |
| EFG1   | Transcription factor                  | EFG1                              | 52           |
| FAS2   | Fatty acid synthase                   | FAS2                              | 98           |
| FTR1   | High-affinity iron permease           | FTR1                              | 64           |
| GNA1   | Glucosamine-6-phosphate acetyltransferase | GNA1                           | 56           |
| GP17   | Transcription factor                  | GP17                              | 65           |
| HEM3   | Uroporphyrin I synthase               | URA3                              | 44           |
| HKI1   | Histidine kinase                      | HKI1                              | 17, 93       |
| HOG1   | Mitogen-activated protein kinase      | MKC1                              | 2            |
| HWP1   | Transglutaminase                      | HWP1, ENO1                        | 21, 80, 82, 85 |
| ICL1   | Isocitrate lyase                      | ICL1                              | 53           |
| INT1   | Surface protein                       | INT1                              | 10, 30       |
| KEX2   | Kexin (subtilase)                     | KEX2                              | 62           |
| LIG4   | DNA ligase                            | LIG4                              | 3            |
| MAD2   | Spindle assembly factor               | MAD2                              | 6            |
| MDR1   | Membrane efflux pump                  | MDR1                              | 9            |
| MKC1   | Mitogen-activated protein kinase      | MKC1                              | 26           |
| MNT1   | α-1,2-Mannosyl transferase            | MNT1                              | 15           |
| NAG1   | N-Acetylglucosamine-6-phosphate deaminase | NAG1                           | 77, 94       |
| NAG2   | N-Acetylglucosamine phosphate deacetylase | NAG2                           | 94           |
| NAG6   | N-Acetylglucosamine kinase            | NAG6                              | 94           |
| NIK1   | Histidine kinase                      | NIK1                              | 93           |
| NMT1   | N-Myristoyl transferase               | NMT1                              | 89           |
| NRG1   | Transcriptional repressor             | NRG1                              | 60           |
| PHR1   | pH-regulated expression               | PHR1                              | 24, 32       |
| PLB1   | Phospholipase B                       | PLB1                              | 51, 57       |
| PMP1   | Protein O mannosylation               | PMP1                              | 83           |
| RAS1   | Regulates gene expression             | Part of plasmid pVEC              | 49           |
| RGB1   | Hypha-specific wall protein           | RBP1                              | 12           |
| RGT4   | Unknown function                      | RGT4                              | 12           |
| RFG1   | Transcription factor                  | RFG1                              | 42           |
| RIM101 | Transcription factor                  | NotI-digested pRS-ARG-URA-BN      | 23           |
| RM8    | Transcription factor                  | NotI-digested pRS-ARG-URA-BN      | 23           |
| RSR1   | GTase                                 | RSR1                              | 92           |
| SAP1   | Aspartyl proteinase                   | SAP1                              | 35           |
| SAP2   | Aspartyl proteinase                   | SAP2                              | 35           |
| SAP3   | Aspartyl proteinase                   | SAP3                              | 35           |
| SAP4   | Aspartyl proteinases                  | SAP5                              | 69           |
| SAP4–6 | Aspartyl proteinases                  | SAP5                              | 35           |
| SLN1   | Histidine kinase                      | SLN1                              | 93           |
| SOD1   | Cu/Zn superoxide dismutase            | SOD1                              | 38           |
| SPT3   | Transcriptional activator             | SPT3                              | 47           |
| SSK1   | Two-component response regulator      | SSK1                              | 16           |
| SSN6   | Transcriptional corepressor           | SSN6                              | 37           |
| TEC1   | Hyphal transcription factor           | Part of plasmid pVEC              | 74           |
| TOP1   | Topoisomerase                         | Upstream of TOP1, part of inducible promoter construct | 41 |
| TPK2   | Protein kinase A subunit              | TPK2                              | 79           |
| TPS1   | Trehalose phosphate synthase          | TPS1                              | 97           |
| TPS2   | Trehalose phosphate phosphatase       | TPS2                              | 86           |
| TUP1   | Transcription regulator               | TUP1                              | 60           |
| URA3   | Nucleotide synthesis                  | URA3                              | 44           |
| VPS34  | Phosphoinositol-3-kinase              | VPS34                             | 13           |

<sup>a</sup>The URA3 position in the mutants is indicated according to the information published.
likely that many of the attenuated virulence phenotypes attributed to particular genes in the studies listed in Table 7 may also have resulted from (inadequate) ectopic expression of URA3, and hence these virulence phenotypes require reexamination. In some of the instances tabulated, the evidence for a virulence role of the gene concerned has been confirmed in experiments not dependent on the ectopic expression of URA3, including reintegration of a disrupted gene without altering the ectopic locus of URA3 (11), but there remain many examples where this is not the case. Based on the analysis of the number of strains in which Ura3p specific activities were below 40% of that found when the URA3 was at the native locus and which were significantly attenuated as measured by mean survival time or kidney burdens (Fig. 3), we estimate that the virulence of at least 30% of all mutants generated to date may have to be formally reassessed after correction for ectopic URA3 expression. Of the 73 genes for which attenuated virulence has been associated with specific disruptions (Table 7), at least 60 were tested in experiments where URA3 was expressed ectopically. Our data suggest that more than 20 of these genes may have been incorrectly designated virulence factors in C. albicans.

Ura3p enzyme activities correlated well with URA3 mRNA levels in C. albicans mutants. Our results relating relative OMP deacetylase activity with mean survival time (Fig. 3) are consistent with those of Lay et al. (48). However, any relationship between the enzyme activity and virulence can be interpolated only for mutants in the C. albicans SC5314 lineage. The existence of naturally attenuated wild-type strains with normal levels of OMP deacetylase shows that a virulence phenotype (as measured by mouse mean survival times) is polygenic. Our experiments with an isogenic set of mutants have allowed us to determine specifically the influence of URA3 expression on virulence. We interpret the data in Fig. 3 as indicating that a minimum necessary level of OMP deacetylase activity (URA3 expression) of around 40% of wild-type (SC5314) levels is required for an SC5314-derived mutant used in a mouse virulence assay.

Iron uptake is thought to be important for the growth and survival of a pathogen such as C. albicans (64). Hence, the fact that the disruption of URA3 in CAI-4 also removed a portion of the IRO1 locus (31) could be argued as another cause of the phenotypic changes that we and others have attributed to URA3 underexpression. However, we could reestablish wild-type virulence in our homozygous gcn4 and mnn4 mutants by reintegrating URA3 alone at the RPS10 locus. Proteomic analysis showed that changes in the expression of only one protein appeared to be URA3 independent, suggesting that its lowered expression in CAI-4 and NGY152 was related to the truncation of IRO1. Taken together, these data strongly suggest that URA3 and not IRO1 is the more important regulator of phenotype, at least for the mouse lethality phenotype.

Our proteomic data (Table 6) show that the expression of 13 proteins is altered in CAI-4 in a URA3-dependent manner, because reintegration of a single copy of URA3 at the RPS10 locus either partially or fully restored expression levels to those of SC5314. While some of the proteins that are collaterally affected by URA3 deletion are obviously related to purine and pyrimidine metabolism (Hpt1p, Ade2p, and Ura5p), others are not. These findings further reinforce the main conclusion of this study and its predecessors, where phenotypic effects resulting from URA3 disruption were demonstrated (7, 19, 48, 81, 82). Namely, the use of auxotrophic selectable markers can result in misleading phenotypes that arise from unpredicted collateral effects on cell metabolism and physiology.

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