INFECTION AND IMMUNITY, Nov. 1998, p. 5301–5306 Vol. 66, No. 11
0019-9567/98/$04.00+0

Altered Expression of Selectable Marker *URA3* in Gene-Disrupted *Candida albicans* Strains Complicates Interpretation of Virulence Studies

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Received 17 April 1998/Returned for modification 3 June 1998/Accepted 1 September 1998

The ura-blaster technique for the disruption of *Candida albicans* genes has been employed in a number of studies to identify possible genes encoding virulence factors of this fungal pathogen. In this study, the *URA3*-encoded orotidine 5′-monophosphate (OMP) decarboxylase enzyme activities of *C. albicans* strains with ura-blaster-mediated genetic disruptions were measured. All strains harboring genetic lesions via the ura-blaster construct showed reduced OMP decarboxylase activities compared to that of the wild type when assayed. The activity levels in different gene disruptions varied, suggesting a positional effect on the level of gene expression. Because the *URA3* gene of *C. albicans* has previously been identified as a virulence factor for this microorganism, our results suggest that decreased virulence observed in strains constructed with the ura-blaster cassette cannot accurately be attributed, in all cases, to the targeted genetic disruption. Although revised methods for validating a *URA3*-disrupted gene as a target for antifungal drug development could be devised, it is clearly desirable to replace *URA3* with a different selectable marker that does not influence virulence.

Fungal species have become an increasingly common cause of human infection, with *Candida albicans* emerging as the predominant fungal pathogen in both superficial and systemic infections (7). This polymorphic fungus is carried as a commensal by many individuals; however, in patients rendered immunocompromised by circumstances such as AIDS, chemotherapy, and organ transplantation, *C. albicans* readily becomes pathogenic.

The development of effective strategies for the treatment of *C. albicans* infections continues to be a major challenge. Effective antifungal agents may prove to be those which target virulence mechanisms important in the pathogenesis of this microorganism. This approach to drug development utilizes molecular biological strategies to identify fungal virulence genes for the determination of targets (23). Unfortunately, induction and analysis of specific genetic lesions required to identify these virulence factors are difficult tasks. Many conventional genetic techniques utilized in the study of bacterial pathogens cannot be applied to *C. albicans* due to its asexual life cycle and diploid genome (26). Fortunately, proven techniques used with the haploid yeast *Saccharomyces cerevisiae* are applicable, in certain cases, to the study of *C. albicans*.

A construct termed the ura-blaster was developed for the disruption of *S. cerevisiae* genes by Alani et al. (1). It consists of the *URA3* gene of *S. cerevisiae* flanked by direct repeats of a *Salmonella hisG* sequence and portions of the target gene which can then be used for the disruption of a targeted gene of interest by integrative transformation with *URA3* as a selectable marker. Once introduced into the genome, the *hisG* direct repeats may undergo mitotic recombination to eliminate the *URA3* gene, leaving behind a single copy of the *hisG* repeat sequence at the site of the original integration in the target gene. This disruption technique became attractive in the study of *C. albicans* because strains could be constructed with targeted disruptions by using the *C. albicans URA3* gene as the single selectable marker in a uracil auxotrophic background (*ura3*/*ura3*) (8). The final result of the ura-blaster genetic disruption method is an intact copy of the *C. albicans URA3* gene located within the ura-blaster cassette at the position in the genome corresponding to one allele of the target gene.

The *URA3* gene of *C. albicans* encodes orotidine 5′-monophosphate (OMP) decarboxylase, the enzyme that catalyzes the conversion of OMP to uridine 5′-monophosphate (UMP), the last step in the de novo pyrimidine biosynthetic pathway. Alterations within this pathway, such as disrupting *URA3*, result in significant decreases in the in vivo growth rate and pathogenicity of *C. albicans* (15). Therefore, CAI4, the strain used for the creation of ura-blaster genetic disruptions in *C. albicans*, is not virulent due to the absence of a functional *URA3* gene. Any strain created to test the effect of a gene on virulence must have an intact, functional *URA3* gene.

Strains constructed by the ura-blaster disruption technique have the *URA3* gene of *C. albicans* inserted into the targeted gene of interest. These genetically engineered strains are then used to test for altered virulence in a murine model of infection to ascertain whether the targeted gene is a virulence factor. The attenuated virulence frequently observed when investigating these strains is generally attributed to the targeted genetic disruption via the ura-blaster. However, an alteration in *URA3* activity within the disruption cassette may also contribute to a decrease in virulence. We undertook the analysis of *URA3* gene expression within a variety of strains genetically disrupted by the ura-blaster technique to determine if the ura-blaster mode of disruption alters the activity of the *URA3* gene product.

MATERIALS AND METHODS

Strains and media. The *C. albicans* strains utilized in this study and their relevant genotypes are listed in Table 1. Some of these strains were acquired from other investigators including William A. Fonzi, Gerald R. Fink, and Judith Berman. For the maintenance of *C. albicans* strains, YPD medium (1% yeast

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TABLE 1. *C. albicans* strains used in this study

Strain	Parent strain	Genotype	Reference or source
SC5314[a]	Clinical isolate	*URA3/URA3*	9a
CAF2-1[a]	SC5314	Δ*ura3*::imm434/URA3	8
CAI4[a]	CAF2-1	Δ*ura3*::imm434/Δura3::imm434	8
CAI4-2312L	CAI4	Δ*ura3*::imm434/Δura3::imm434 + URA3[b]	3
CAI4-01	CAI4	Δ*Camdr1::hisG-URA3-hisG/CaMDR1*	3
CAI4-116	CAI4-01	Δ*Camdr1::hisG-URA3-hisG*/Δ*Camdr1::hisG*	3
CJC01[c]	CAI4	Δ*ben38::hisG-URA3-hisG-URA3-hisG/BEN38*	Unpublished data
CJC024[c]	CJC01	Δ*ben38::hisG-URA3-hisG*/Δ*ben38::hisG*	Unpublished data
CAI4-10	CAI4	Δ*rsr1::hisG-URA3-hisG/RSR1*	29
CAI4-5	CAI4-10	Δ*rsr1::hisG-URA3-hisG*/Δ*rsr1::hisG*	29
CWJ429	CAI4	Δ*topoI::hisG-URA3-hisG/TOPOI*	13
CWJ430	CAI4	Δ*topoI::hisG-URA3-hisG/TOPOI*	13
JKC19[d]	JKC17	Δ*cphI::hisG-URA3-hisG*/Δ*cphI::hisG*	18
CACB8B-5	CACB8B-6	Δ*chs3-3::hisG-URA3-hisG-URA3-hisG*/Δ*chs3-2::hisG*	5
CACB8B-6	CAI4	Δ*chs3-3::hisG-URA3-hisG-URA3-hisG/CHS3*	5
CACB10B-8	CAI4	Δ*chs3-1::hisG-URA3-hisG/CHS3*	5
CACB10B-10	CACB10B-8	Δ*chs3-1::hisG-URA3-hisG*/Δ*chs3-2::hisG*	5
CAG1[e]	CAI4	Δ*int1::hisG-URA3-hisG/INT1*	9
CAG3[e]	CAG1	Δ*int1::hisG-URA3-hisG*/Δ*int1::hisG*	9
CAG4[e]	CAG3	Δ*int1::hisG*/Δ*int1::hisG*	9
CAG5[e]	CAG4	Δ*int1::hisG*/Δ*int1::hisG + INT1-URA3*[f]	9
RSM3[c]	CAI4	Δ*cdr1::hisG-URA3-hisG-URA3-hisG/CDR1*	Unpublished data
RSM4[c]	CAI4	Δ*cdr1::hisG-URA3-hisG/CDR1*	Unpublished data
RSM7[c]	RSM3	Δ*cdr1::hisG-URA3-hisG*/Δ*cdr1::hisG*	Unpublished data
RSM11[c]	RSM4	Δ*cdr1::hisG-URA3-hisG*/Δ*cdr1::hisG*	Unpublished data
RSM17[c]	CAI4-116	Δ*Camdr1::hisG*/Δ*Camdr1::hisG*/Δ*cdr1::hisG-URA3-hisG*/Δ*cdr1::hisG*	Unpublished data

[a] Received from William A. Fonzi.
[b] + *URA3* indicates that *URA3* was reintegrated into the chromosome at the *URA3* locus.
[c] Received from Millennium Pharmaceuticals, Inc., Cambridge, Mass.
[d] Received from Gerald R. Fink.
[e] Received from Judith Berman.
[f] +*INTI-URA3* indicates that *INT1-URA3* was reintegrated into the chromosome at the *INT1* locus.

extract, 2% Bacto Peptone, 2% dextrose) was used as a complex growth medium. For minimal medium, SD medium (0.67% Difco yeast nitrogen base without amino acids, 2% dextrose) lacking uridine supplementation was used. Two percent agar was added for the solidification of both YPD and SD-ura medium.

Materials. OMP, potassium phosphate (monobasic), and dithiothreitol (DTT) were obtained from Sigma Chemical Co., St. Louis, Mo. β-Mercaptoethanol and potassium phosphate (dibasic) were acquired from Mallinckrodt Speciality Chemicals Co., Paris, Ky. The protein assay reagent used in determining protein concentrations was acquired from Bio-Rad Laboratories, Hercules, Calif.

Assay of OMP decarboxylase enzyme activity. Enzyme activity was monitored by using a modified version of assays described previously (22, 30). Cells from a 50-ml culture were collected at mid-log phase by centrifugation, washed once with 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM DTT, and resuspended to a final volume of 4 ml in the same buffer. The entire suspension was added to 3 g of glass beads (0.50-mm diameter; B. Braun Biotech International) and vortexed for 2 min at room temperature. The resulting extract was transferred to a clean tube, placed on ice, and cleared of particulate matter by centrifugation at 14,000 × *g* for 20 min at 4°C. The volume of the final cell lysate, which was stored at −80°C in 30% glycerol for later use, was approximately 2 ml. Protein concentrations within the lysates were determined by the method described by Bradford (4) with bovine serum albumin (BSA) as the standard. The conversion of OMP to UMP was measured spectrophotometrically at 23°C by observing the decrease in absorbance of the OMP substrate at 285 nm for a period of 25 min. The assay mixture in a total volume of 0.5 ml consisted of the cell lysate (150 μl), 0.1 M potassium phosphate buffer (pH 6.0), 1.0 μmol of β-mercaptoethanol, and 0.15 μmol of the OMP substrate. The concentration of OMP in the assay mixture was calculated by Beer's law with a molar extinction coefficient of 1.65×10^3 cm^{-1} M^{-1} (22). A unit of enzyme activity was defined as the quantity of enzyme which catalyzed the conversion of 1 μmol of OMP to UMP per min. Specific activity was defined as units of enzyme activity per milligram of protein. Each assay with a particular cell extract was repeated at least three times, with specific activities reported as means ± standard deviations of the means.

Growth rate determination. The doubling times of *C. albicans* strains were calculated in 2× YPD medium and SD-ura medium at 37°C. An overnight culture grown at 37°C was diluted 1 to 500 into 50 ml of either fresh 2× YPD or SD-ura medium. The culture was incubated with shaking at 37°C. The optical density at 600 nm (OD_{600}) of each culture was determined every hour. The doubling times shown in Tables 2 and 3 are the averages of three independent experiments.

RESULTS

OMP decarboxylase activities of *C. albicans* strains. OMP decarboxylase enzyme activity was first assayed for control strains SC5314 (Ura^+) and CAI4 (Ura^-). SC5314, a clinical isolate, is the parent strain of CAF2-1 and the grandparent strain of CAI4. SC5314 was used as the relevant wild-type control in this work, since CAI4 is the beginning strain used in the construction of the disrupted strains assayed in this study. OMP decarboxylase assays of SC5314 and CAI4 were performed with three different cell extracts, each yielding results highly comparable to each other (Table 2). The enzyme activity of the *C. albicans* wild-type strain SC5314 was not significantly affected by the growth of the culture in SD-ura minimal medium. The specific activity of OMP decarboxylase was significantly decreased in the CAI4 strain which contained deletions of both genomic *URA3* copies. There was no detectable conversion of OMP to UMP when the substrate was incubated in the presence of buffer alone or with boiled cell extract. The small amount of conversion of OMP to UMP observed in the CAI4 assays was probably due to enzymes of nucleotide anabolism other than OMP decarboxylase.

Also assayed were *C. albicans* CAF2-1 and CAI4-2312L (Table 2). CAF2-1, a strain heterozygous for the *URA3* gene, and CAI4-2312L, a Δ*ura3*/Δ*ura3* strain with one copy of *URA3* reintegrated into the genome, both showed specific activities comparable to the SC5314-positive control strain.

Assay results of *C. albicans* strains constructed with ura-blaster-mediated genetic disruptions. Twenty-three constructed *C. albicans* strains containing one or more genetic disruptions via the ura-blaster construct were assayed for OMP decarboxylase enzyme activity (Table 3). Compared to the

TABLE 2. OMP decarboxylase activities of various *C. albicans* strains

Strain[a]	Phenotype[b]	Sp act[c]	No. of copies of *URA3* per genome	Doubling time 2× YPD (hr)[d]	Doubling time SD-ura (hr)[d]
SC5314	Ura$^+$	16.00 ± 0.47 17.23 ± 0.31 17.53 ± 0.73	2	1.06 ± 0.11	
SC5314 (SD-ura medium)	Ura$^+$	15.09 ± 0.42	2		1.38 ± 0.08
CAI4	Ura$^-$	0.60 ± 0.39 0.40 ± 0.32 0.71 ± 0.14	0	0.89 ± 0.08	
CAF2-1	Ura$^+$	19.61 ± 1.26	1	0.85 ± 0.09	1.41 ± 0.04
CAI4-2312L	Ura$^+$	17.61 ± 0.35	1	0.96 ± 0.12	

[a] All strains were grown in YPD medium unless noted.
[b] For genotype, see Table 1.
[c] Reported as micromoles of OMP converted to UMP per minute per milligram of protein. Values are means ± standard deviations.
[d] Reported as the averages of three independent cultures ± standard deviations.

wild-type SC5314 strain, the test strains assayed showed 2- to 18-fold less OMP decarboxylase enzyme activity. Repeated assays of various test strains with cell extracts prepared from several independent cultures yielded highly comparable values for specific enzyme activity (data not shown). To examine the possible effect of growth medium on these strains, the enzyme activity of RSM11 grown in SD-ura medium was also determined. YPD or SD-ura growth medium had no apparent effect on the specific activity obtained for test strain RSM11 (Table 3).

Growth rate data and germ tube formation of *C. albicans* strains. In order to determine whether growth rate or germ tube formation was affected by the decrease in OMP decarboxylase enzyme activity within the *C. albicans* strains constructed with the ura-blaster, the doubling times in 2× YPD medium and the ability of each strain to form germ tubes in serum were examined. The doubling times of the control strains SC5314 (Ura$^+$) and CAI4 (Ura$^-$) did not vary significantly in 2× YPD medium at the $P > 0.10$ level (Table 2). In addition, with the exception of strain JKC19, the doubling times of the constructed strains were not significantly different from one another ($P > 0.03$) in 2× YPD and appeared to be somewhat lower in their growth rates than that of the CAI4 strain from which they were derived. The doubling times of several strains in SD-ura medium were also determined (Tables 2 and 3). The doubling times of strains RSM3 and CACB8B-6 were not statistically different from those of the Ura$^+$ control strains SC5314 and CAF2-1 at the $P > 0.10$ level. However, the doubling times of strains RSM7, RSM17, and CAI4-5, which all display low levels of OMP decarboxylase enzyme activity, were significantly different from that of the wild-type SC5314 strain.

By a method described previously (12), the strains in the study were examined for their ability to form germ tubes. The

TABLE 3. *C. albicans* strains constructed with the ura-blaster

Strain[a]	Sp act[b]	Fold reduction in enzyme activity compared to wild type[c]	Gene copy no.		Doubling time in 2× YPD medium (h)[d]	Doubling time in SD-ura medium (h)[d]
			URA3	Target gene		
RSM3	12.87 ± 0.87	1.3	2	1	1.41 ± 0.10	1.46 ± 0.01
RSM4	8.98 ± 0.44	1.9	1	1	1.39 ± 0.04	
RSM11 (SD-ura)	8.75 ± 1.96	1.9	1	0		
CAI4-01	8.28 ± 1.33	2.1	1	1	1.27 ± 0.05	
RSM11	8.11 ± 0.37	2.1	1	0	1.38 ± 0.08	
CWJ430	7.85 ± 0.19	2.2	1	1	1.23 ± 0.11	
JKC19	7.53 ± 0.94	2.3	1	0	0.90 ± 0.06	
CAI4-10	7.49 ± 0.98	2.3	1	1	1.34 ± 0.14	
CACB8B-6	7.49 ± 0.73	2.3	2	1	1.35 ± 0.15	1.42 ± 0.05
CACB8B-5	7.19 ± 0.13	2.4	2	1	1.22 ± 0.05	
CACB10B-8	6.46 ± 0.96	2.6	1	1	1.25 ± 0.03	
CAI4-116	6.22 ± 0.37	2.7	1	0	1.33 ± 0.11	
CJC01	6.05 ± 0.97	2.8	2	1	1.21 ± 0.10	
RSM7	5.53 ± 1.01	3.1	1	0	1.36 ± 0.11	1.70 ± 0.06
CAG1	4.55 ± 0.41	3.7	1	1	1.23 ± 0.13	
CJC024	3.75 ± 0.20	4.5	1	0	1.24 ± 0.08	
CAG5	3.31 ± 0.38	5.1	1	1	1.24 ± 0.10	
CAI4-5	3.24 ± 0.60	5.2	1	0	1.39 ± 0.07	2.11 ± 0.03
CACB10B-10	3.16 ± 0.41	5.4	1	0	1.30 ± 0.12	
CAG3	2.13 ± 0.10	8.0	1	0	1.30 ± 0.08	
CWJ429	1.62 ± 0.76	10	1	1	1.15 ± 0.09	
RSM17	1.23 ± 1.06	18	1	0	1.40 ± 0.06	1.87 ± 0.05
CAG4	0.56 ± 0.11	30	0	0	1.27 ± 0.05	

[a] For genotype, see Table 1. Strains were grown in YPD medium unless otherwise noted.
[b] Reported as micromoles of OMP converted to UMP per minute per milligram of protein. Values are means ± standard deviations.
[c] For purposes of comparison, wild-type specific enzyme activity was 17.
[d] Reported as the averages of three independent cultures ± standard deviations.

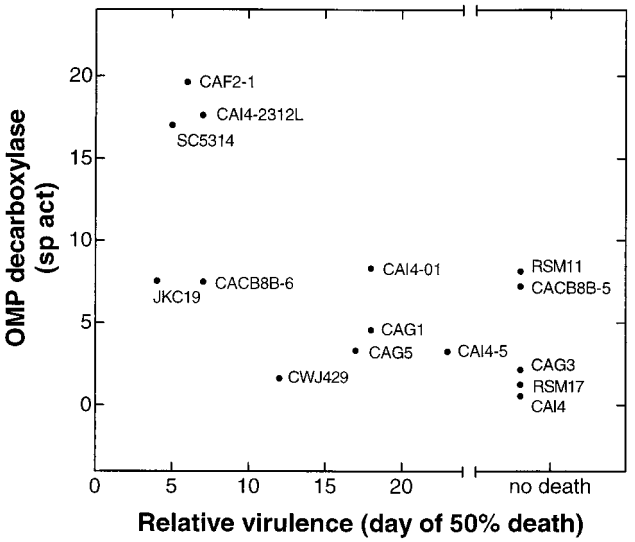

FIG. 1. Relationship between OMP decarboxylase specific activities in *C. albicans* strains and the known experimental virulence of the strains previously reported. Relative virulence is represented as the day of death for 50% of a group of immunocompetent mice injected with 10^6 *C. albicans* cells via the lateral tail vein. Mice injected with avirulent strains remain healthy and do not achieve 50% death after 24 to 30 days of observation. OMP decarboxylase specific activities are reported as units of enzyme activity per milligram of protein within the assayed cell lysate of each strain.

control strains SC5314 (Ura^+) and CAI4 (Ura^-) formed germ tubes at rates indistinguishable from one another, indicating that the level of *URA3* gene activity does not directly affect the ability of cells to form germ tubes (data not shown). With the exception of strains CAG1, CAG3, CAG4, CAG5, and CAI4-5, the strains constructed with the ura-blaster were able to form germ tubes at wild-type levels (data not shown). Strains CAG1, CAG3, CAG4, CAG5, and CAI4-5 contain disruptions in one or both copies of the *INT1* or *RSR1* gene, both of which are involved in germ tube formation (9, 29).

Relationship between OMP decarboxylase activity and virulence of *C. albicans* strains. Virulence data for 14 of the strains assayed in this study are available (3, 5, 9, 18, 29). To compare results from these previous studies, the day of death of 50% of the mice within a group of immunocompetent mice injected with 10^6 *C. albicans* cells via the lateral vein was related to the OMP decarboxylase enzyme activity within the strain (Fig. 1).

Strains SC5314, CAF2-1, and CAI4-2312L, which contain at least one functional copy of the *URA3* gene in its native locus, are fully virulent, whereas CAI4, a strain lacking *URA3*, is avirulent and has only trace OMP decarboxylase activity. However, an examination of the *C. albicans* strains with ura-blaster-mediated genetic disruptions does not reveal a definitive relationship between enzyme activity and virulence. Most notably, two avirulent strains (CAG3 and RSM17) have markedly decreased OMP decarboxylase enzyme activities. Other avirulent strains (RSM11 and CACB8B-5), as well as two fully virulent strains (JKC19 and CACB8B-6), have intermediate levels of enzyme activity. Strains with intermediate virulence (CAI4-01, CAG1, CAG5, and CWJ429) vary from intermediate to low levels of enzyme activity.

Relationship between *URA3* gene copy number and OMP decarboxylase specific activity. Four *C. albicans* strains assayed in this study—RSM3, CACB8B-6, CACB8B-5, and CJC01—contain an alternative ura-blaster-mediated disruption in which one allele contains an additional copy of *hisG* and *URA3* (see Table 1 for exact genotypes). The existence of tandem duplications has been noted previously in the process of disrupting *Candida* genes via the ura-blaster cassette (5, 10), possibly due to the formation of a circularized intermediate form of the linear ura-blaster cassette during transformation.

OMP decarboxylase specific activities and the numbers of *URA3* copies were compared to determine whether these extra copies of *URA3* within the disruption cassette at the targeted gene in strains RSM3, CACB8B-6, CACB8B-5, and CJC01 affected enzyme activity (Table 3). There is no definitive correlation between OMP decarboxylase activity and *URA3* gene copy number within the genome of each strain. Strains CAF2-1 and CAI4-2312L show that wild-type enzyme activity is achieved with one functional copy of *URA3* at its native locus. *C. albicans* strains containing one copy of *URA3* in the targeted gene locus within the *hisG-URA3-hisG* disruption cassette have OMP decarboxylase activities ranging from 1.23 to 8.98. Compared to disrupted strains harboring one copy of the *URA3* gene, an extra copy of *URA3* present within the disrupted alleles of RSM3, CACB8B-6, CACB8B-5, and CJC01 did not notably increase OMP decarboxylase activity in all of the strains. RSM3, a Δ*cdr1*/*CDR1* strain with a *hisG-URA3-hisG-URA3-hisG* sequence present within the disrupted gene, did show the highest enzyme activity of any of the disrupted strains assayed. However, since the three other strains containing the *hisG-URA3-hisG-URA3-hisG* sequence did not have high activities as well, the observed OMP decarboxylase activity of RSM3 probably cannot be attributed to an extra copy of *URA3* within the disrupted allele.

Relationship between OMP decarboxylase activity and total number of genetic disruptions. *C. albicans* strains with one, two, or four ura-blaster-mediated genetic disruptions were assayed in this study (see Table 1 for exact genotypes). The OMP decarboxylase specific activities of the strains were compared to the numbers of genes disrupted with the ura-blaster cassette in order to determine if enzyme activity was affected by the number of times the ura-blaster cassette was used for disruption. No positive correlation between OMP decarboxylase specific activities and the numbers of genes disrupted was observed. Homozygous knockout strains do not, in general, show less activity than their heterozygous counterparts. For example, CACB8B-5, a Δ*chs3*/Δ*chs3* strain, has an activity similar to that of CACB8B-6, a Δ*chs3*/*CHS3* strain. RSM17, a *C. albicans* strain in which the ura-blaster cassette has been used to disrupt two copies of the *CDR1* gene and two copies of the *CaMDR1* gene, has a very low OMP decarboxylase specific activity. However, the low enzyme activity of RSM17 cannot definitely be attributed to the fact that it contains four ura-blaster-mediated genetic disruptions.

DISCUSSION

We have shown that the OMP decarboxylase enzyme activities of *C. albicans* strains assayed in this study are significantly reduced and quite variable compared to the wild-type enzyme activity observed in Ura^+ control strains. In each of these strains, the ura-blaster cassette, which contains either one or two tandem copies of the *C. albicans URA3* gene, disrupts the targeted gene of interest in a Δ*ura3*/Δ*ura3* background. It is of critical importance that the OMP decarboxylase enzyme, as encoded by the *URA3* gene within the ura-blaster cassette, retains sufficient activity, since both uracil auxotrophy and mutations in the pyrimidine biosynthetic pathway have previously been shown to diminish virulence in *C. albicans* (15).

The ura-blaster disruption technique uses *URA3*, a gene known to affect the virulence of *C. albicans*, within a *hisG-*

URA3-hisG disruption cassette, in order to identify other genes involved in pathogenesis. Our results indicate that it is necessary to closely examine the OMP decarboxylase activity of a ura-blaster-constructed strain before determining its virulence in order to determine if the gene of interest is actually related to *C. albicans* pathogenesis. Our results also indicate that the level of *URA3* gene activity does not by itself influence the process of germ tube formation. However, reduced enzyme activity contributed to the lower growth rate of some of the tested strains in SD-ura medium. This lower rate of growth in some of the strains due to inadequate OMP decarboxylase activity may cause a decrease in virulence in vivo because a more slowly growing strain can be eliminated by the host's defenses more effectively than a strain which is rapidly reproducing. However, when considering the growth rate data, it is important to note that two strains examined, RSM3 and CACB8B-6, have reduced levels of OMP decarboxylase enzyme activity but are able to grow in SD-ura medium at rates similar to those of the wild-type strain. These results suggest that a wild-type level of enzyme activity is not necessary to maintain a wild-type rate of growth in SD-ura medium.

One strain in this study, JKC19 (Δ*cphI*/Δ*cphI*), showed decreased OMP decarboxylase activity but retained wild-type virulence. Since the engineered disruption with the ura-blaster within this strain does not affect its virulence, it can be concluded that the targeted gene within JKC19, *CPHI*, is not a virulence factor. The reduced OMP decarboxylase activity of JKC19 does not affect its virulence, implying that *C. albicans* strains may not require wild-type enzyme activity to maintain a wild-type level of virulence. Therefore, some strains with intermediate levels of enzyme activity are able to maintain wild-type virulence (JKC19) or wild-type rates of growth in minimal medium (RSM3 and CACB8B-6). The OMP decarboxylase enzyme levels within these strains may represent a threshold or adequate level of enzyme activity necessary for these strains to behave like their wild-type parental strains. However, our results do not accurately define the adequate or sufficient amount of OMP decarboxylase enzyme activity required by *C. albicans* strains.

Strains RSM11 and CACB8B-5, which are both avirulent, have OMP decarboxylase specific activities similar to that of JKC19. Therefore, RSM11 and CACB8B-5 may have sufficient OMP decarboxylase activity, and the avirulence observed is due to the targeted genetic disruptions. RSM17 and CAG3 are avirulent strains which showed very low OMP decarboxylase activities compared to that of the wild type. In this situation, the reason for the apparent lack of virulence is difficult to assign. Avirulence could be the result of decrease in OMP decarboxylase activity, the targeted genetic disruption, or a combination of both factors. The same problem arises with several other strains in the study, such as CAI4-01 and CAG1, which display intermediate virulence in murine models and also have decreased OMP decarboxylase activities. Again, a distinction between the factors which could result in this decreased virulence cannot be determined.

The *hisG-URA3-hisG* disruption cassettes present within each strain are all derived from the same plasmid (8) and are presumably identical. Therefore, the reduction of OMP decarboxylase activity in the disrupted *C. albicans* strains is most likely due to altered *URA3* expression within the ura-blaster construct. Several phenomena could result in this altered *URA3* expression. After insertion into the targeted gene for disruption, the *URA3* gene no longer resides at its native locus in the genome. Epigenetic gene regulation or different states of gene expression caused by differential effects of chromosome or chromatin packaging have been observed for many eukaryotes, including fungi (14, 28). An example of this effect is commonly referred to as position effect variegation (11). The *S. cerevisiae URA3* gene, which shares 66.8% sequence identity with that of *C. albicans* (20), is known to be regulated at the level of transcription (2) and is susceptible to positional and orientation effects when manipulated (24). It is possible that the *URA3* gene within the ura-blaster construct is not in close proximity to its proper regulatory elements, causing the observed decrease in OMP decarboxylase activity. Positional effects on *URA3* expression and regulation may also explain the variability in enzyme activities observed within the constructed *C. albicans* strains.

The presence of flanking *hisG* direct repeats within the disruption cassette may also alter the *URA3* gene in these constructed *C. albicans* strains. A proportion of the cells may have lost *URA3* due to mitotic recombination between the *hisG* repeats, resulting in an overall decrease in OMP decarboxylase activity. Repeat-induced mutation (RIP) should also be noted when examining the ura-blaster construct. RIP is a phenomenon observed in some fungal species in which the presence of heterologous flanking repeated sequences increases the frequency of mutation of the gene being flanked (27). It is possible that the *URA3* gene within the ura-blaster construct undergoes frequent mutation due to the presence of flanking *hisG* repeats from *Salmonella*, resulting in decreased OMP decarboxylase activity. Finally, it has not escaped our attention that the altered expression of *URA3*, as measured by us in *C. albicans* cell extracts, may not reflect expression during host infection when many environmental factors may modulate enzyme activity.

Since its application to the study of *C. albicans*, the ura-blaster technique for sequential gene disruption has been used extensively in searching for genes responsible for the virulence of this fungal pathogen (3, 5, 6, 13, 16, 18, 19, 21, 25, 31). The importance of *C. albicans* as a significant infectious microorganism in both healthy and immunocompromised individuals warrants the proper identification of genes necessary for its pathogenesis. Our results imply that the decreased in virulence of *C. albicans* strains constructed with the ura-blaster cassette cannot be definitively attributed, in all cases, to the targeted genetic disruption. The decrease in OMP decarboxylase activities within these strains may contribute to an attenuation in virulence. We conclude that the interpretation of virulence data obtained from this method of genetic disruption must take into account the decreased activity of the *URA3* gene product.

There is currently a trend when examining *C. albicans* strains to reintroduce the wild-type gene back into a strain with both copies of the targeted gene disrupted. One strain employed in this study, CAG5, is representative of this technique. It contains both copies of the *INT1* gene disrupted by a *hisG* sequence with one copy of *INT1* and one copy of *URA3* reintegrated into the genome. Strain CAG5 displays intermediate virulence and reduced OMP decarboxylase enzyme activity, whereas CAG3, the homozygous disruptant *INT1* strain, is avirulent and has reduced enzyme activity. Restoration of one copy of the *INT1* gene in CAG5 resulted in a partial recovery of virulence, indicating that *INT1* could be a virulence factor. However, the problem of reduced *URA3* gene activity still persists. It is possible that full, wild-type virulence was not restored in CAG5 due to inadequate OMP decarboxylase activity.

Alternative methods should most likely be employed in constructing a *C. albicans* strain harboring genetic disruptions to identify possible virulence factors. One revised ura-blaster disruption method would use a stable plasmid to reintroduce the

URA3 gene encoding wild-type levels of OMP decarboxylase. Such a method has been reported elsewhere (17), but plasmid stability may not be optimized until an authentic *C. albicans* centromere is identified and incorporated into a plasmid. Another revised method would reintroduce the *URA3* gene into its native locus, where it should be properly regulated and expressed. Preliminary data have shown that returning *URA3* to its native location in the genome of a Ura^- strain restores both wild-type OMP decarboxylase enzyme activity and wild-type virulence (data not shown). However, these additional genetic manipulations have the disadvantage of introducing mutations complicating the analysis of virulence studies. Perhaps the most desirable alternative to the construction of strains for virulence studies would be to use a selectable marker that encodes an activity not required for virulence or host survival.

ACKNOWLEDGMENTS

We thank the personnel of the Becker laboratory at the University of Tennessee for their support. We are especially grateful to Melinda Hauser for her input in numerous fruitful discussions regarding this project. We also thank Gerald Fink, William Fonzi, and Judith Berman for the strains used in this study.

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Editor: T. R. Kozel