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Single-Copy *IMH3* Allele Is Sufficient To Confer Resistance to Mycophenolic Acid in *Candida albicans* and To Mediate Transformation of Clinical *Candida* Species

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Parasexual genetic analysis of *Candida albicans* utilized the dominant selectable marker that conferred resistance to mycophenolic acid. We cloned and sequenced the *IMH3*^{r} gene from *C. albicans* strain 1006, which was previously identified as resistant to mycophenolic acid (MPA) (A. K. Goshorn and S. Scherer, Genetics 123:213–218, 1989). MPA is an inhibitor of IMP dehydrogenase, an enzyme necessary for the de novo biosynthesis of GMP. G. A. Kohler et al. (J. Bacteriol. 179:2331–2338, 1997) have shown that the wild-type *IMH3* gene, when expressed in high copy number, will confer resistance to this antibiotic. We demonstrate that the *IMH3*^{r} gene from strain 1006 has three amino acid changes, two of which are nonconservative, and demonstrate that at least two of the three mutations are required to confer resistance to MPA. We used this gene as a dominant selectable marker in clinical isolates of *C. albicans* and *Candida tropicalis*. We also identified the presence of autonously replicating sequence elements that permit autonomous replication in the promoter region of this gene. Finally, we found the excision of a ϕ-type long terminal repeat element outside the *IMH3* open reading frame of the gene in some strains. We used the *IMH3*^{r} allele to disrupt one allele of *ARG4* in two clinical isolates, WO-1 and FC18, thus demonstrating that a single ectopic integration of this dominant selectable marker is sufficient to confer resistance to MPA.

Candida albicans, an asexual, diploid yeast, has emerged as the primary fungal pathogen of medical importance (18). This polymorphic yeast normally exists as a harmless commensal. However, in patients immunocompromised due to AIDS, organ transplantation, or chemotherapy, *C. albicans* can cause significant morbidity and mortality. Despite the increasing clinical importance of *C. albicans* and other *Candida* spp., we lack a clear understanding of *Candida* pathogenesis and the etiology of candidiasis (4). Much of this lack of understanding of the basic biology of *Candida* is due to the facts that it is diploid and has no known sexual cycle, although recent evidence suggests that a genetic system may exist (11, 17). Many of the classical genetic approaches that have been successful in elucidating the pathogenicity of bacteria and of some species of phytopathogenic fungi cannot be successfully applied to *Candida*. In particular, it has been difficult to demonstrate unequivocally the role of particular genes in the pathogenic process (16).

Presently, the use of molecular techniques such as transformation has circumvented some of the problems of genetic analysis (24). The Ura-blaster cassette, which was originally developed by Alani et al. (1) for the sequential disruption of genes in *Saccharomyces cerevisiae*, was adopted by Irwin and Fonzi (5) for use in *C. albicans*. This procedure utilizes a selectable marker (in this case, the *URA3* gene, encoding orotidine 5′-monophosphate [OMP] decarboxylase) flanked by direct repeats of heterologous sequence (the *Salmonella hisG* gene). This cassette is used to disrupt or replace the gene of interest. The construct is then used for disruption of the intended gene by homologous integration into a *ura3* strain, and cells are selected on the basis of *URA3* prototrophy. Mitotic recombination between the *hisG* repeat sequences results in a return to uridine auxotrophy, with the presence of a *hisG* "footprint" disrupting one allele of the gene of interest. This process is then repeated to disrupt the second allele. To date, most molecular studies of *C. albicans* biology have utilized the Ura-blaster approach in a *ura3* strain, CAI-4, derived from a clinical strain, SC5314 (5). Because use of the Ura-blaster cassette requires *ura3* auxotrophy, its use is limited to laboratory strains and precludes the study of clinical isolates.

Recently Lay et al. (16) found that many strains harboring genetic lesions induced by the Ura-blaster technique showed variable OMP decarboxylase activities, usually reduced compared to the wild-type progenitor strain SC5314 (16). Variation in the levels of OMP decarboxylase may cause problems in studies of virulence (13). It was suggested that position effects (15) can reduce the levels of OMP decarboxylase activity to less than wild-type levels. This finding calls into question previous studies in which decreased virulence is attributed to disruption of the targeted gene, although some laboratories have not been able to repeat the variation in OMP dehydrogenase (W. A. Fonzi et al., personal communication). Lay et al. (16) called for the development of a different selectable marker, one that does not influence virulence.

A variety of selectable markers have been used in fungi. However, until now no dominant selectable marker has been found to be useful in *Candida*. There are two major reasons for this: (i) *Candida* species in general, and *C. albicans* in particular, have been found to be naturally resistant to most selectable markers available, including hygromycin, benomyl, cycloheximide, mitomycin C, and tunicamycin (B. Magee, personal

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

Strain(s)	Parent strain	Genotype	Reference(s) or source
FC18	Clinical isolate of *C. albicans*	Wild type	
WO-1	Clinical isolate of *C. albicans*	Wild type	23
SC5314	Clinical isolate of *C. albicans*	Wild type	5
CAI-4	SC5314	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	5
1006		*ura3*/*ura3 arg4*/*arg4 ser*/*ser lys1*/*lys1 IMH3*^{r}	9
16F, R1b, R3b, 36	Clinical isolates of *C. dublinensis*	Wild type	26, 27
660, 678	Clinical isolate of *C. tropicalis*	Wild type	Scherer strain collection
669	Clinical isolate of *C. pseudotropicalis*	Wild type	Scherer strain collection
688	Clinical isolate of *C. parapsilosis*	Wild type	Scherer strain collection
653	Clinical isolate of *C. krusei*	Wild type	Scherer strain collection

communication); and (ii) at least 11 *Candida* species read the CUG codon as serine instead of leucine (21), rendering potential transgenic resistance genes and/or reporter genes nonfunctional in these yeasts (24). Mycophenolic acid (MPA) resistance (Mpa^r), conferred by a mutation of the *IMH3* gene, which encodes IMP dehydrogenase, is a selectable phenotype that has been successfully used in *C. albicans* for selection in spheroplast fusion (9) and, more recently, as a reporter for virulence gene activation in vivo (26) or as a selectable marker if used in high copy number (14). This gene was cloned by Kohler et al. (14) from strain SS and found to be 2,908 bp in size. Herein we report on the isolation, sequence, and use of the dominant *IMH3*^{r} allele, which confers Mpa^r in both *C. albicans* and *C. tropicalis*. Finally, we demonstrate that the ectopic integration of a single copy of the *IMH3*^{r} allele is sufficient to confer Mpa^r and can be used as a selectable marker for gene disruption.

MATERIALS AND METHODS

Strains and culture conditions. Strains used in this study and their relevant genotypes are described in Table 1. *C. albicans* strains were maintained in YEPD medium (1% yeast extract, 2% Bacto Peptone, 2% dextrose, 1.5% plant agar (Sigma, St. Louis, Mo.). 5-fluoro-orotic acid (5-FOA) medium contains 0.42% g of yeast nitrogen base without amino acids, 1.2% dextrose, and 1.5% agar, with 60 mg of uridine and 0.6 g of 5-FOA dissolved in dimethyl sulfoxide added to the cooled medium. For selection and testing of Mpa^r, we used minimal medium (mm) consisting of 0.67% yeast nitrogen base without amino acids, 2% dextrose, and concentrations of MPA (Sigma) in ethanol ranging from 1 to 10 μg/ml.

Construction of p3408. The following oligomers were designed corresponding to the published sequence of the *IMH3* gene (14), with *Xho*I (underlined) restriction sites added to the previously identified *Xba*I sites (bold underlined): B15106 (5′CTCGAGT**CTAGA**TGTTTATGATACTAAG-3′) and C15107 (5′CTCGAG**TCTAGA**ACTCAGTATATCTTCA-3′).

PCR. PCR amplification was carried out in a 50-μl reaction volume containing 10 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 20 μM deoxynucleoside triphosphates, 5 μM each oligonucleotide primer, 50 ng of genomic DNA, and 5 U of *Taq* polymerase (Boehringer Mannheim Biochemicals). After an initial incubation time of 94°C for 5 min, the samples were cycled 30 times as follows: 94°C for 1 min, 55°C for 1 min, and 70°C for 1 min. A 10-min extension at 72°C completed the reaction. Aliquots of the reactions were fractionated on 0.7% TAE (Tris-acetate-EDTA) agarose, and fragments were cloned using a TA cloning kit (Invitrogen, Carlsbad, Calif.) to create p3408 (derived from strain 1006), p*IMH3*C (derived from strain CAI-4), and p*IMH3*W (derived from strain WO-1). Plasmids were then sequenced by ATG Technologies (Minneapolis, Minn.). Plasmid p3408 was digested with *Xho*I and fractionated on 0.7% TAE-agarose. The 2.7-kb fragment containing the *IMH3* gene was excised and cloned into *Xho*I-linearized pABSKII to yield p3417.

Transformation and selection for Mpa^r. Two-milliliter cultures of *C. albicans* strains FC18, WO-1, and CAI-4, and *C. tropicalis* strains 660 and 678 from the Scherer strain collection (http://alces.med.umn.edu/Candida/strains), were grown overnight in YEPD broth at 30°C with shaking at 200 rpm. Samples (0.2 ml) of cells were pelleted, washed with water, recentrifuged, and resuspended in freshly made OSB (0.8 ml of 50% polyethylene glycol 8000, 0.2 ml 1 M lithium acetate, 25 mg dithiothreitol, 25 μl of carrier DNA [10 mg of sonicated salmon sperm DNA per ml]). Samples containing FC18 and WO-1 were transformed with p3408 DNA (2.5 μg) or p*IMH3*W (2.5 μg); CAI-4 was transformed with 2.5 μg of p3417 in duplicate (Table 2). Cells (including no-DNA controls) were incubated at 43.5°C for 45 min. Strains FC18 and WO-1 and one-half of the CAI-4 transformation were plated onto MM with 5 μg of MPA; the second-half of the CAI-4 transformation was plated onto MM. Plates were incubated at 30°C. Colonies appeared within 3 days. (Due to the labile nature of MPA, plates were made 24 h prior to the transformation experiment to ensure accurate levels of the drug.)

Genomic DNA isolation and analysis. For DNA isolation, 1 ml of log-phase cells grown in YEPD was centrifuged. The supernatant was removed and replaced with 0.5 ml of TENTS buffer (10 mM Tris-Cl [pH 7.5], 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% [wt/vol] sodium dodecyl sulfate), 0.2 g of acid-washed glass beads, and 0.5 ml of phenol-chloroform (1:1, vol/vol). The mixture was vortexed for 4 min and then centrifuged for 5 min. The supernatant was transferred to a new tube and precipitated with 0.1 volume of ammonium acetate and 2 volumes of 95% ethanol. The solution was centrifuged for 5 min, the supernatant was removed, and the pellet was washed with 70% ethanol and allowed to dry. The pellet was resuspended in 50 μl of Tris-EDTA.

Segregation of Mpa^r with *URA3*. Prototrophic and Mpa^r CAI-4-derived strains (*URA3*/*ura3* Mpa^r) were streaked onto MM–5 μg of MPA, MM, and YEPD and grown at 30°C for 3 days. Colonies from these plates were then picked onto plates containing 5-FOA, MM–5 μg of MPA, MM, or YEPD to test for plasmid stability. Two days later, colonies growing on 5-FOA were plated onto MM–5 μg of MPA and MM to examine segregation of Ura3 and Mpa^r.

Examination of mutations necessary to confer Mpa^r. The *IMH3*^{r} allele was separated into two subclones by PCR; each subclone contained one of the exons and part of the intron. No overlap exists between the intron sequences. Primers B15106 (above) and B17359 (5′CATATGTTAAAGAATCATCAGAGTAATAGTATAATGTGATA-3′) were used to subclone exon 1; C15107 (above) and B17360 (5′CATATGAAAATTTATTAACTTTCGTTCTACCGATA-3′) were used to subclone exon 2. *Xho*I restriction sites added to the oligonucleotides are

TABLE 2. Plasmids used in this study

Plasmid	Marker	Description	Reference
p1129	None	*ARG4* cloned from p1076 in pUC18	10
p3408	*IMH3R*	*IMH3* cloned from strain 1006	This study
pABSKII	*URA3*	pBSKII vector with *ARS2*, *URA3*, and blue/white selection	Unpublished
p3417	*URA3*/*IMH3*^{r}	*IMH3*^{r} cloned into polylinker of pABSKII	This study
p3394	*IMH3R*	*IMH3*^{r} inserted into *Msc*I-*Spe*I site of p1129	This study

underlined. The subclones were used to transform strains, selecting for Mpa^r. The exon subclones were used both individually and together.

Ectopic integration and gene disruption with *IMH3*^{r}. Plasmid p1129, containing the 3.3-kb *ARG4* gene subcloned into pUC18 (10), was digested with *Msc*I and *Spe*I and blunt ended with T4 polymerase to delete a 1.6-kb region encompassing the entire open reading frame (ORF) of *ARG4*. The *IMH3*^{r} allele was excised from p3408 by digestion with *Eco*RI and blunt ended with T4 polymerase. The fragments were ligated to create p3394. FC18 and WO-1 were transformed with p3394 as described above. Mpa^r transformants were restreaked onto MM–MPA (10 μg/ml) to confirm resistance. DNA was extracted as previously described, and digested with *Bam*HI and *Sac*I. Gels were blotted with a Zeta-probe nylon membrane as instructed by the manufacturer (Bio-Rad, La Jolla, Calif.). Radioactive probes of *IMH3* or *ARG4* were generated with a Rediprime kit (Amersham, Piscataway, N.J.).

Nucleotide sequence accession number. The *IMH*^{r} sequence reported here has been assigned GenBank accession number AF249293.

RESULTS

The *IMH3*^{r} gene from the Mpa^r strain 1006 possesses three mutations and a deletion. We performed PCR to clone the *IMH3*^{r} alleles from strains 1006 and CAI-4, using primers designed from the previously reported *IMH3* sequence (14). Both the Mpa-susceptible strain CAI-4 and the Mpa^r strain 1006 yielded a 2,704-bp fragment, as opposed to the 2,908-bp fragment expected based on the previously reported sequence (14). Sequence analysis and subsequent BLAST searches of GenBank sequences confirmed that the 2.7-kb fragment contained the *IMH3* gene and its previously identified sequence motifs, including two exons separated by an intron of 248 nucleotides (nt) (14). The DNA sequences differ at several places; however, the putative amino acid sequences encoded by the two alleles that we sequenced are identical to the published sequence, except for one conservative and two nonconservative changes that occur within the ORF in the gene from 1006: I47V, S102A, and G482D. The first two of these mutations are in exon 1, and the third mutation is in exon 2. BLAST (2) analysis was performed on the approximately 200-bp sequence in the noncoding region of the 3′ end of the second exon. This sequence is found in strain SS but is missing in the noncoding region at the 3′ end of the gene in 1006 and CAI-4. The analysis identified the missing nucleotides as a potential ϕ-like long terminal repeat (LTR) element (8); it appears to have been excised from strains 1006 and CAI-4 since these strains possess a GTAATA footprint at nt 2676 in these strains which may be indicative of excised of LTR-like elements (8).

An *IMH3* allele from 1006 is able to confer Mpa^r in *C. albicans* and *C. tropicalis*. Kohler et al. (14) previously demonstrated that the insertion of *IMH3* on a high-copy-number plasmid was capable of conferring Mpa^r. We wished to determine whether overexpression of the *IMH3* allele from the already Mpa^r strain 1006 was capable of conferring resistance as well. We had preliminary evidence that 1006 was heterozygous at the *IMH3* locus (data not shown). The *IMH3*^{r} allele was cloned into pABSKII, a Bluescript-based plasmid containing both *ARS2* and *URA3* to create p3417. CAI-4 was transformed with p3417, and the transformants were selected for *URA3* prototrophy, Mpa^r (in the presence of uridine), or both. The *URA3* prototrophic colonies were restreaked onto MM–MPA (10 μg/ml) to examine Mpa^r. The Mpa^r colonies were restreaked onto MM to examine prototrophy. Cosegregation of *URA3* prototrophy and Mpa^r was 100%. Twenty-four colonies that were identified as Ura$^+$ and Mpa^r (eight from each selection protocol) were plated onto 5-FOA. The resulting 5-FOA-resistant colonies were then screened Mpa^r. None of the colonies tested was resistant, even when the concentration of MPA was lowered to 1 μg/ml. Therefore, the resistance was due to the *IMH3* allele carried on the plasmid. This allele was designated *IMH3*^{r}.

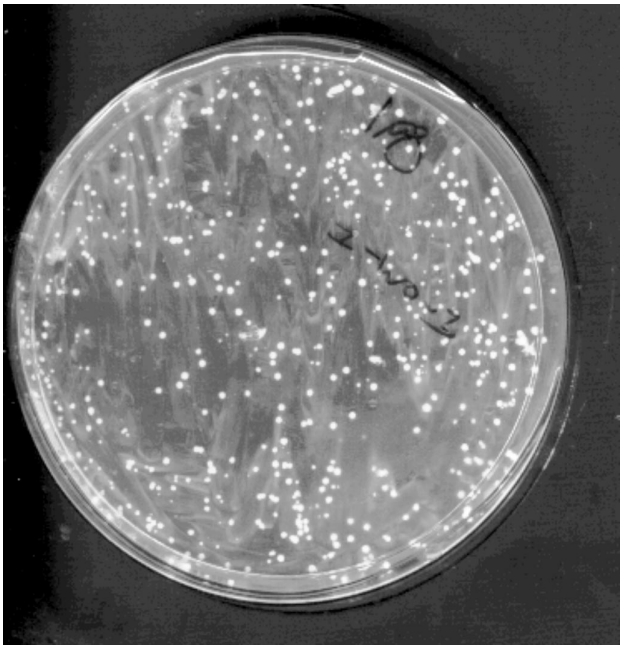

FIG. 1. Typical selection for Mpa^r after transformation with p3408. WO-1 was transformed with uncut p3408 and plated onto medium containing MPA (1 μg/ml). The plates were incubated at 30°C for 3 days. Transformants were picked and replated onto MPA at 1, 10, and 20 μg/ml. Almost all colonies grew under all conditions tested.

To determine whether single-copy integration could confer Mpa^r in other *Candida* species, the *IMH3*^{r} allele was amplified by PCR, and the product was cloned into pCR2.1 to create p3408. This plasmid, containing *IMH3*^{r}, was linearized and used to transform the clinical isolates in Table 1 (Fig. 1). Selection for Mpa^r was consistently achieved with *C. tropicalis* and *C. albicans*. Other *Candida* species tested were naturally Mpa^r at concentrations as high as 20 μg/ml under the conditions tested. Colonies of putative *C. albicans* and *C. tropicalis* transformants were restreaked onto MM–MPA (10 μg/ml) to confirm that they were Mpa^r. Resistant colonies of *C. tropicalis* were screened by PCR using the primers that were used to clone the *IMH3*^{r} allele, and a 2.7-kb fragment was obtained. The primers failed to produce a product in the untransformed control strains of *C. tropicalis* (Fig. 2).

The *IMH3*^{r} allele possesses an ARS element. Southern blot analysis of the Mpa^r strains of *C. albicans* transformed with plasmid p3408 showed that the plasmid was replicating autonomously (Fig. 3), suggesting the presence of an unidentified autonomously replicating sequence (ARS) element. Analysis of the sequence of the *IMH3*^{r} allele reveals the presence of putative ARS elements at nt 7 (5′GTTTATGATAC3′), based on similarity to the previously identified ARS consensus sequence (5′TTTATGTTT3′) (3), and at positions 101 (5′ATT TAATTTTC3′) and 358 (5′TTTTTCGCTTTTT3′), based on

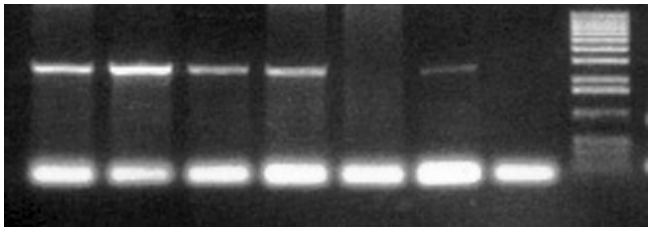

FIG. 2. Plasmid p3408 is sufficient to confer Mpa^r in clinical isolates of *C. tropicalis*. Primers used to clone the *IMH3*^{r} allele were used to detect the presence of the *IMH3*^{r} allele in *C. tropicalis* strain 678 transformed with p3408 (Lanes 1 to 6, transformants). Sequence similarity between the *IMH3* allele of *C. tropicalis* and the primers used was not sufficient to detect the 2.7-kb *IMH3* homolog in untransformed *C. tropicalis* (lane 7). Lane 8, 1.kb Plus DNA ladder (Gibco-BRL, Grand Island, N.Y.).

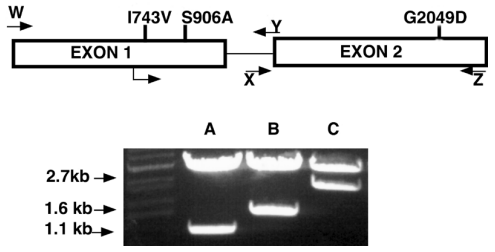

FIG. 4. Structure of the *IMH3*^{r} allele and location of primer pairs. The *IMH3* gene consists of two exons, of 1.1 and 1.6 bp, respectively, and an intron of 248 bp. The mutations which distinguish the *IMH3*^{r} allele from the wild-type gene are shown in their approximate locations. The primers which were used to amplify the exons separately are shown as arrows. Amplification of the gene was carried out with the following primers: for exon 1, B15106(W) and B17359(Y); for exon 2, C15107(Z) and B17360(X).

identified ARS sequences of *C. maltosa* (23). Although relatively stable, plasmid p3408 was lost when placed under extended nonselective conditions (data not shown).

Mutations in both exons are required to confer Mpa^r. Because the *IMH3*^{r} allele contains several sequence changes in the promoter and three mutations affecting the amino acid sequence, we wished to determine which mutations were sufficient to confer Mpa^r. We therefore used PCR to obtain fragments of the gene. A 1.1-kb fragment which included the promoter and exon 1 with half of the intron and a 1.6-kb fragment which contained the remainder of the intron and exon 2 were constructed (Fig. 4). The PCR products were used separately and together to transform FC18 and WO1 to Mpa^r. The 2.7-kb PCR product, containing the complete gene, was used as a positive control. Neither fragment alone was sufficient to confer Mpa^r, although both the positive control and cotransformation with the combined PCR fragments yielded resistant transformants (although the number of Mpa^r yeast cells were greatly reduced in cotransformation with the two exons compared to the positive control). This demonstrates that both exons are necessary for Mpa^r and that neither alone is sufficient to confer resistance.

***IMH3*^{r} is capable of conferring Mpa^r by single-copy integration.** Despite the ARS element on the fragment encoding the *IMH3*^{r} allele, we found several transformants of CAI-4 in which Mpa^r was mitotically stable. However, due to the sequence similarity of *IMH3*^{r} and the wild-type *IMH3*, it was difficult to determine whether these stable resistant cells were the result of ectopic integration, insertion, or gene conversion at the chromosomal *IMH3* locus. To determine whether the *IMH3*^{r} allele could confer resistance in a single, ectopically integrated copy, we targeted the *IMH3*^{r} allele to the *ARG4* locus (10). *IMH3*^{r} was used to replace the sequence between the *Msc*I and *Spe*I sites of p1129 (10), which contains the *ARG4* ORF (Fig. 5, top), to create p3394. Strains FC18 and WO-1 were transformed with *Eco*RI-linearized p3394 and selected for Mpa^r, and transformants were analyzed by Southern blot analysis. Blots A and B in Fig. 5 demonstrate that gene replacement with *IMH3*^{r} occurred at the *ARG4* locus in strain WO-1; similar results were observed in strain FC18 (data not shown). Of the 12 transformants tested in each strain, 100% homologous integration occurred at the *ARG4* locus. This is the first report describing the use of a dominant selectable marker for gene disruption in clinical isolates.

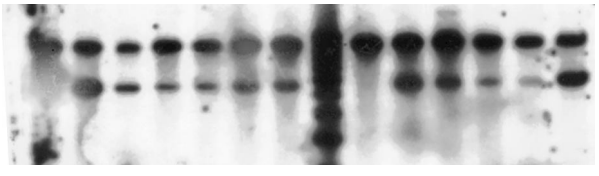

FIG. 3. The *IMH3*^{r} allele possesses an ARS. FC18 and WO-1 were transformed with uncut p3408 and selected on MPA (1 μg/ml). Mpa^r transformants were replated onto MPA at 1, 5, 10, and 20 μg/ml. Colonies were picked from the second set of plates and grown overnight in YEPD. DNA was extracted, digested with *Hin*dIII, subjected to gel electrophoresis, and blotted. Blots were hybridized with a radiolabeled *IMH3* probe. The bands at 11.0 kb are the genomic copies. The 6.7-kb bands correspond to the size of the linearized p3408 plasmid.

DISCUSSION

C. albicans is the most common fungal pathogen of humans, yet research into its pathogenesis has been greatly hampered by the fact that it is both asexual and diploid. Before the advent of molecular genetics and then genomics, parasexual genetics through spheroplast fusion was the only tool available for genetic analysis (12, 19, 22). Goshorn and Scherer (9) isolated strain 1006, an Mpa^r isolate, for use in spheroplast fusions with prototrophic clinical isolates. Their work showed that the Mpa^r phenotype, probably mediated through an altered *IMH3* gene, could be exploited as a dominant selectable marker. We have identified and cloned the dominant *IMH3*^{r} allele and demonstrated that it confers Mpa^r. We present molecular proof that *IMH3*^{r} is sufficient to confer Mpa^r since resistance cosegregates with uridine prototrophy when the *IMH3*^{r} allele is carried on an autonomously replicating plasmid that bears a *URA3* marker as well. The *IMH3*^{r} allele contains three mutations. Two of these mutations, a nonconservative mutation that results in S102A and a conservative change that results in I47V, occur in exon 1. A second nonconservative change G482D, occurs in exon 2, and both exons (and hence at least two of the mutations) are required to confer Mpa^r. This is consistent with the findings of Goshorn and Scherer (9) that spontaneous resistance to MPA is a low-frequency occurrence and that Mpa^r is a stable phenotype. Finally, we used *IMH3*^{r} to disrupt

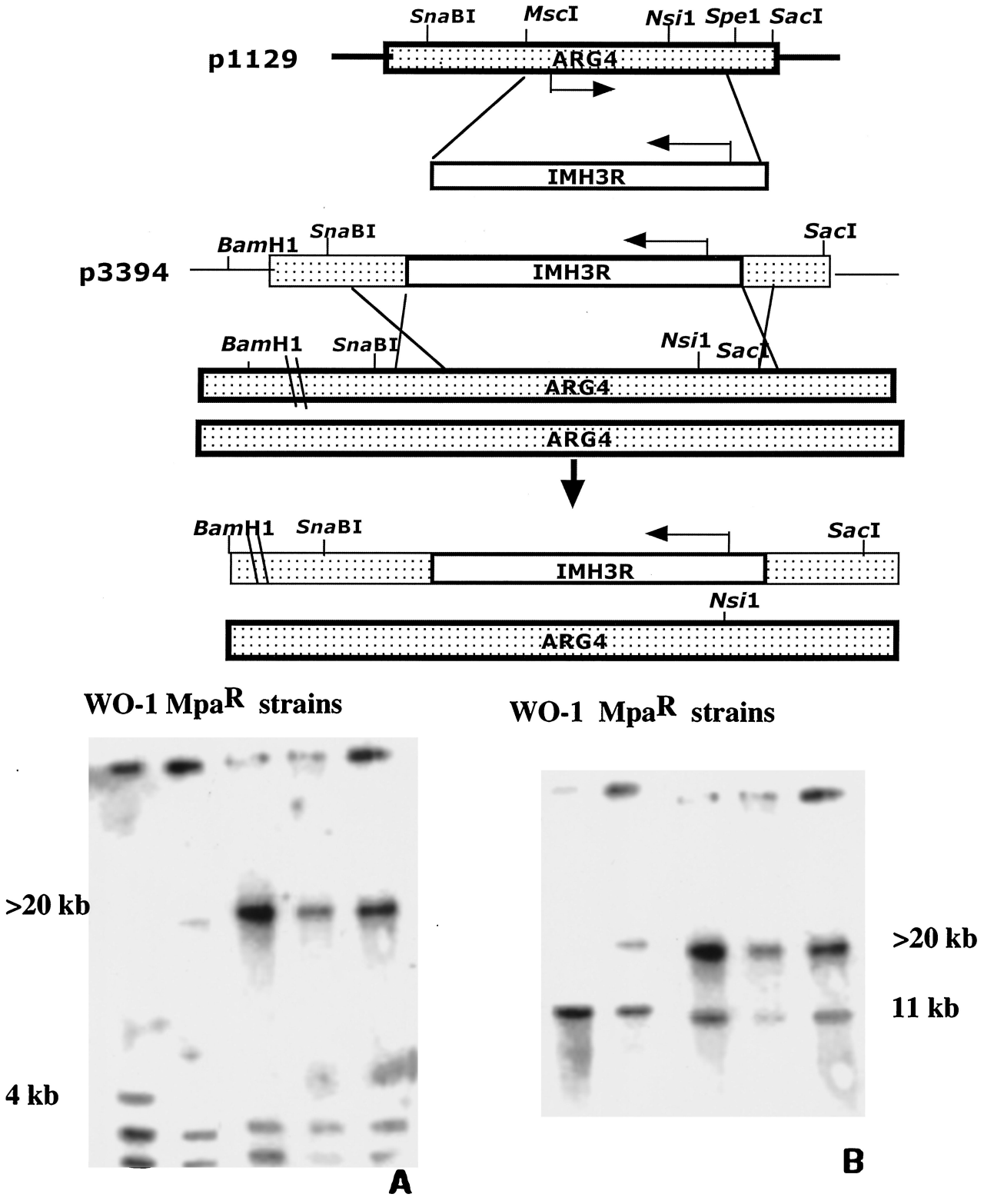

FIG. 5. Disruption of one allele of *ARG4* with *IMH3*. (Top) A 1.6-kb fragment of the *ARG4* ORF in plasmid p1129 was replaced with the *IMH3*^{r} allele to create p3394. *Sna*BI-linearized p3394 was used to transform both FC18 and WO-1. Transformants were selected on the basis of Mpa^r. (Bottom) DNA from p3394-transformed WO-1 was extracted and digested with *Bam*HI and *Nsi*I, electrophoresed, and blotted. Blots were hybridized with a radiolabeled probe of p1129 (*ARG4*) (A) or *IMH3* (B). These blots demonstrate that the *IMH3*^{r} allele integrated ectopically at the *ARG4* locus. They further demonstrate that one copy of the *IMH3*^{r} allele is sufficient to confer Mpa^r.

one allele at the *ARG4* locus in two clinical isolates, thus demonstrating that a single allele is sufficient to confer Mpa^r and that this selection strategy can be used with clinical isolates.

Due to the absence of dominant selectable markers, most gene disruption experiments in *C. albicans* use the Ura-blaster cassette and CAI-4, a Ura$^-$ (hence avirulent) mutant. Although extremely useful, the Ura-blaster technique has drawbacks. In addition to the issues recently raised about the effect (if any) of reintroduction of *URA3* at different loci (16), the requirement for *URA* auxotrophy precludes the molecular manipulation of clinical isolates that may have unique phenotypes such as drug resistance (20, 30) and phenotypic switching (25).

In principle, the *IMH3*^{r} allele is ideal for gene disruption in prototrophic strains. It confers resistance to an antibiotic to which most, if not all, clinical isolates are sensitive. Furthermore, it is a single gene of less than 3.0 kb and is easy to insert into the gene to be disrupted. Finally, spontaneous resistance is extremely rare. However, there are two virtually identical alleles in the normal *C. albicans* genome. Thus, *IMH3*^{r} preferentially integrates at the *IMH3* locus with a very high frequency. This high frequency of homologous integration precludes its use in oligonucleotide-mediated gene disruption approaches (data not shown). To obviate this problem, we are currently examining the *IMH3* genes from several fungi which appear to be naturally Mpa^r in order to determine whether they confer Mpa^r.

Another interesting feature of the *IMH3*^{r} allele is the fact that it replicates autonomously in relatively low copy number and contains several ARS-like elements. We identified three ARS elements: at nt 7 (5′GTTTATGATAC3′), based on similarity to the previously identified *C. albicans* ARS consensus sequence of (5′TTTATGTTT3′) (3), and at positions 101 (5′A TTTAATTTTC3′) and 358 (5′TTTTTCGCTTTTT3′), based on the ARS element identified in *C. maltosa* (23). Furthermore, this plasmid did not appear to multimerize like other ARS element-bearing plasmids of *C. albicans* (data not shown). We are currently comparing this ARS element to other previously identified ARS elements in the hope of developing a better ARS plasmid for use in *Candida* studies.

This marker can be used to transform *C. tropicalis* as well as *C. albicans*. In our hands, *C. dubliniensis*, *C. parapsilosis*, and *C. krusei* were naturally Mpa^r under the conditions described here; inconsistent results were obtained upon transforming *C. glabrata* with our construct. Recently Staib et al. (27) described the transformation of *C. dubliniensis* using electroporation to introduce another Mpa^r allele of *IMH3* into a *C. dubliniensis* strain. We have no explanation for the discrepancy except that the heterogeneity of clinical isolates of asexual fungi may mean that no generalizations can be made about the sensitivity or resistance to MPA of a species as a whole. It will be interesting to determine whether the *IMH3*^{r} allele that they used (27) is the same as the one we have isolated and whether the difference in results is attributable to differences in transformation techniques. Previously, Kohler et al. (14) found that *IMH3* is constitutively expressed under conditions requiring the biosynthesis of purine, such as the minimal medium described here. For this reason, more complete medium represses biosynthesis of the *IMH3* gene and prevents selection. Further support for this phenomenon is provided by Goshorn and Scherer (9), who found that exogenous application of guanine to minimal medium acts as a competitive inhibitor of MPA (a guanine analog) and prevents selection.

The *IMH3*^{r} allele can be used for purposes other than gene disruption. A similar allele, coupled with the FLP recombination system, has been used for in vivo expression studies to demonstrate the expression of particular genes in vivo (26). The *IMH3*^{r} allele should also be useful in studies of population biology, since it will allow one to mark a particular strain so that it can be followed in a mixed population. This application may very well be useful for in vitro and in vivo competition studies. Finally, the *IMH3*^{r} allele would be ideal for haploinsufficiency studies similar to those performed on diploid *Saccharomyces cerevisiae* (7). Work to examine the usefulness of the *IMH3*^{r} allele in a similar study for *C. albicans*, using strain SC5314, is under way.

Although the *IMH3*^{r} allele is a dominant selectable marker and will confer a selectable phenotype when only one allele is present, it is not sufficient by itself to achieve the goal of gene disruption in prototrophic clinical isolates, since in principle both genes need to be inactivated to achieve the deficient phenotype. Although a haploinsufficient phenotype has often been seen for *Candida* genes, and a single selectable marker is sufficient to test for haploinsufficiency (6), two dominant selectable markers are needed for complete gene disruption. There are two ways to achieve this: either one can find a second marker, or one can disrupt the first allele with the marker under a regulatable promoter, so that specific conditions render the transformant sensitive once more to the selection. We are currently examining other potential markers for *C. albicans*. Additionally, we are constructing an inducible promoter fusion of *IMH3*^{r}. A construct containing the ORF of *IMH3*^{r} fused to an inducible promoter would be used to disrupt one allele of a gene with selection under conditions that induce promoter-driven expression. For the second disruption, a construct containing the *IMH3*^{r} gene under the control of its own promoter would be used, permitting sequential selection with a single marker. We are currently developing and testing this approach.

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