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

JANNA BECKERMAN, HIROJI CHIBANA, JOSHUA TURNER, AND P. T. MAGEE*

Department of Genetics, Cell Biology, and Development, University of Minnesota, St. Paul, Minnesota 55108

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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* 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* 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 autonomously replicating sequence elements that permit autonomous replication in the promoter region of this gene. Finally, we found the excision of a 6-bp type long terminal repeat element outside the IMH3 open reading frame of the gene in some strains. We used the IMH3* 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 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...
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 |  |
| SC5314    | Clinical isolate of C. albicans | Wild type |  |
| CAI-4     | SC5314         | Δura3::imm434/Δura3::imm434 |  |
| 1006      |               | ura3/ura3 arg4/arg4 ser7 ser7 his1/hs1 IMH3R |  |
| 16F, R1b, R3b, 36 | Clinical isolates of C. dublinensis | Wild type |  |
| 660, 678  | Clinical isolate of C. tropicalis | Wild type |  |
| 669       | Clinical isolate of C. pseudotropicalis | Wild type |  |
| 688       | Clinical isolate of C. parapsilosis | Wild type |  |
| 653       | Clinical isolate of C. krusei | Wild type |  |

TABLE 2. Plasmids used in this study

| Plasmid | Marker | Description | Reference |
|---------|--------|-------------|-----------|
| p1129   | None   | ARG4 cloned from p1076 in pUC18 |  |
| p3408   | IMH3R  | IMH3 cloned from strain 1006 |  |
| pABSKII | URA3   | pBSKII vector with AR32, URA3, and blue/white selection | Unpublished |
| p3417   | URA3/IMH3R | IMH3R cloned into polylinker of pABSKII | This study |
| p3394   | IMH3R  | IMH3R inserted into MacI-SpeI site of p1129 | This study |
RESULTS

The IMH3* gene from the Mpa strain 1006 possesses three mutations and a deletion. We performed PCR to clone the IMH3 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 Mpastrain 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 due to the presence of uridine. The primers failed to produce a product in the untransformed C. tropicalis. Other Candida species tested were naturally Mpar resistant colonies were then screened Mpar. 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*.

To determine whether single-copy integration could confer Mpa* in other Candida species, the IMH3* allele was amplified by PCR, and the product was cloned into pCR2.1 to create p3408. This plasmid, containing IMH3*, was linearized and used to transform the clinical isolates in Table 1 (Fig. 1). Selection for Mpa was consistently achieved with C. tropicalis and C. albicans. Other Candida species tested were naturally Mpa 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*. Resistant colonies of C. tropicalis were screened by PCR using the primers that were used to clone the IMH3* 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* allele possesses an ARS element. Southern blot analysis of the Mpa 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* allele revealed the presence of putative ARS elements at nt 7 (5'TTATAGTACA3'), based on similarity to the previously identified ARS consensus sequence (5'TTATAGTACA3'), and at positions 101 (5'ATT TAATTTC3') and 358 (5'TTTTTCGCTTTT3'), based on
FIG. 2. Plasmid p3408 is sufficient to confer Mpa\(^{+}\) 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.).

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\(^{+}\). 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\(^{+}\). 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 Mpar. The 2.7-kb PCR product, containing the complete gene, was used as a positive control. Neither fragment alone was sufficient to confer Mpa\(^{+}\), although both the positive control and cotransformation with the combined PCR fragments yielded resistant transformants (although the number of Mpa\(^{+}\) 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\(^{+}\) and that neither alone is sufficient to confer resistance.

**IMH3** is capable of conferring Mpa\(^{+}\) 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\(^{+}\) 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 MscI and SpeI sites of p1129 (10), which contains the ARG4 ORF (Fig. 5, top), to create p3394. Strains FC18 and WO-1 were transformed with EcoRI-linearized p3394 and selected for Mpa\(^{+}\), 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.

**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\(^{+}\) isolate, for use in spheroplast fusions with prototrophic clinical isolates. Their work showed that the Mpa\(^{+}\) 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\(^{+}\). We present molecular proof that IMH3\(^{r}\) is sufficient to confer Mpa\(^{+}\) 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\(^{+}\). This is consistent with the findings of Goshorn and Scherer (9) that spontaneous resistance to MPA is a low-frequency occurrence and that Mpa\(^{+}\) 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 allele to create p3394. SnaBI-linearized p3394 was used to transform both FC18 and WO-1. Transformants were selected on the basis of MpaR. (Bottom) DNA from p3394-transformed WO-1 was extracted and digested with BamHI and NsiI, electrophoresed, and blotted. Blots were hybridized with a radiolabeled probe of p1129 (ARG4) (A) or IMH3 (B). These blots demonstrate that the IMH3 allele integrated ectopically at the ARG4 locus. They further demonstrate that one copy of the IMH3 allele is sufficient to confer MpaR.
one allele at the ARG4 locus in two clinical isolates, thus demonstrating that a single allele is sufficient to confer Mpa' 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-1, 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 URA4 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' 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' 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' in order to determine whether they confer Mpa'.

Another interesting feature of the IMH3' 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'GTGTTAGATAC3'), based on similarity to the previously identified C. albicans ARS consensus sequence of 5'TTTAGTTT3') (3), and at positions 101 (5'ATTTAAATTCT3') and 358 (5'TTTTCTGTTTTT3'), 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' 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' 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' 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' 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' 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' allele would be ideal for haploinsufficiency studies similar to those performed on diploid Saccharomyces cerevisiae (7). Work to examine the usefulness of the IMH3' allele in a similar study for C. albicans, using strain SC5314, is under way.

Although the IMH3' 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'. A construct containing the ORF of IMH3' 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' 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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