Barrier Activity in *Candida albicans* Mediates Pheromone Degradation and Promotes Mating

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Mating in *Candida albicans* and *Saccharomyces cerevisiae* is regulated by the secretion of peptide pheromones that initiate the mating process. An important regulator of pheromone activity in *S. cerevisiae* is barrier activity, involving an extracellular aspartyl protease encoded by the *BAR1* gene that degrades the alpha pheromone. We have characterized an equivalent barrier activity in *C. albicans* and demonstrate that the loss of *C. albicans* *BAR1* activity results in opaque a cells exhibiting hypersensitivity to alpha pheromone. Hypersensitivity to pheromone is clearly seen in halo assays; in response to alpha pheromone, a lawn of *C. albicans* Δ*bar1* mutant cells produces a marked zone in which cell growth is inhibited, whereas wild-type strains fail to show halo formation. *C. albicans* mutants lacking *BAR1* also exhibit a striking mating defect in a cells, but not in α cells, due to overstimulation of the response to alpha pheromone. The block to mating occurs prior to cell fusion, as very few mating zygotes were observed in mixes of Δ*bar1* a and α cells. Finally, in a barrier assay using a highly pheromone-sensitive strain, we were able to demonstrate that barrier activity in *C. albicans* is dependent on *Bar1p*. These studies reveal that a barrier activity to alpha pheromone exists in *C. albicans* and that the activity is analogous to that caused by *Bar1p* in *S. cerevisiae.*

*Candida albicans* a and α cells secrete peptide mating pheromones (α-factor and α-factor, respectively) that act on cells of the opposite mating type and induce physiological changes that precede cell fusion (4, 9, 29, 36, 41). These changes include arrest of the cell in the G1 phase of the cell cycle and the formation of mating projections that seek out a partner of the opposite mating type for cell fusion (3, 52). The pathway controlling the response to mating pheromones in *C. albicans* shows many similarities with that of the related yeast *Saccharomyces cerevisiae*. Both fungi use a conserved mitogen-activated protein (MAP) kinase cascade to transduce the mating signal from a pheromone receptor-coupled G protein to a transcriptional response in the nucleus (6, 31). The final step in the signaling cascade leads to the activation of the transcription factor Ste12p (Cph1p in *C. albicans*) and the resulting expression of many pheromone-responsive genes (for reviews of mating in *C. albicans* and *S. cerevisiae*, see references 2, 14, 27, 33, and 46).

Despite many similarities between the regulation of mating in *C. albicans* and *S. cerevisiae*, there are also a number of important differences. Prominent among these is the requirement in *C. albicans* for cells to undergo a heritable but reversible transition from the white phase to the opaque phase for efficient mating (36). The white-opaque transition is a form of phenotypic switching, and only in the opaque phase do *C. albicans* cells respond strongly to pheromone and undergo mating (3, 4, 28, 29, 36, 41). White-opaque switching is regulated by the α1-α2 heterodimer, so only mating-competent cells, i.e., a or α cells, are able to switch to the opaque phase (36). The white-opaque switch involves differential regulation of a large number of genes, including several that are implicated in mating (26).

White-opaque switching may have evolved to enable efficient mating in the in vivo niche of *C. albicans*, i.e., a mammalian host (17, 23). Unlike *S. cerevisiae*, which is commonly found growing in the soil or on the surfaces of fruit, *C. albicans* is a natural commensal of humans and an opportunistic pathogen, with the potential to cause both mucosal and systemic infections (13). Mating of *C. albicans* cells has been shown to occur in vivo, using animal models of systemic infection and skin infection and, more recently, a gastrointestinal tract model of commensal growth (12, 21, 24). White-opaque switching and the mating response have also been implicated in increasing the efficiency of formation of *C. albicans* biofilms (8). The in vivo role of white-opaque switching and mating in *C. albicans* is still under investigation, however, particularly given the apparent absence of a meiotic pathway to complete a true sexual mating cycle (2, 23, 33, 46).

The response of *C. albicans* cells to mating pheromone is dependent not only on the white or opaque phase of the cell but also on nutritional conditions (3). Under optimal medium conditions, the pheromone response of opaque cells of *C. albicans* involves the differential regulation of more than 200 genes, and relatively few of these genes overlap with those regulated by pheromone in *S. cerevisiae* (3). Even under optimal conditions, however, wild-type *C. albicans* cells fail to undergo a complete cell cycle arrest in the presence of pheromone (3, 10, 41). In contrast, *S. cerevisiae* cells responding to pheromone efficiently arrest their growth in the G1 phase of the cell cycle, and this is often visualized by the formation of halos of growth inhibition in a lawn of responding cells (35). *C.
C. albicans strains do not exhibit clear areas of growth inhibition in the halo assay, although mutant strains that are hypersensitive to pheromone can produce halos, demonstrating more efficient cell cycle arrest. An example of a hypersensitive C. albicans strain is one lacking the SST2 gene, which encodes a regulator of G protein signaling (10). Disruption of SST2 resulted in heightened signaling via the MAP kinase cascade, and opaque cells exhibited increased sensitivity to alpha pheromone and enhanced growth inhibition in the halo assay.

In S. cerevisiae, MATa mating cells also have an extracellular barrier activity that antagonizes alpha pheromone. Barrier activity involves the secretion of an aspartyl protease in a cell that acts specifically to degrade alpha pheromone and is encoded by the BARI (SST1) gene (30). The loss of barrier activity results in S. cerevisiae a cells that are hypersensitive to pheromone; cells produce significantly larger areas of growth inhibition in the halo assay but have a reduced mating efficiency (5, 19, 35, 47). In this paper, we address whether an ortholog of ScBARI exists in C. albicans and if deletion of the gene leads to a hypersensitive phenotype in opaque a cells. We provide evidence that orf19.2082 in C. albicans encodes a protein with barrier activity (C. albicans Bar1p) against alpha pheromone. In addition, we examine the regulation of other aspartyl proteases, including those encoded by the SAP (secreted aspartyl protease) family of genes, by mating pheromone.

### TABLE 1. Strains used in this study

| Strain(s)        | Genotypea | Mating type | Source       |
|------------------|-----------|-------------|--------------|
| RBY1117          | leu2/leu2 his1/his1* | a/a    | This study   |
| RBY1118          | leu2/leu2 his1/his1* | a/a    | This study   |
| RBY1119          | leu2/leu2 his1/his1* | o/o   | This study   |
| RBY1120          | leu2/leu2 his1/his1* | o/o   | This study   |
| RBY1177          | leu2::HisG gpd2::HisG HIS1* | a/a    | 3            |
| RBY1178          | leu2::HisG gpd2::HisG HIS1* | o/o   | 3            |
| RBY1179          | arg4::HisG arg4::HisG HIS1* | a/a    | 3            |
| RBY1180          | arg4::HisG arg4::HisG HIS1* | o/o   | 3            |
| RBY1167          | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | a/a    | 3            |
| RBY1197/8, RBY1218, RBY1220 | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | a/a    | This study   |
| RBY1199, RBY1223, RBY1224 | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | a/a    | This study   |
| RBY1201, RBY1202 | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | a/a    | This study   |
| RBY1203, RBY1204 | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | a/a    | This study   |
| DSY18, DSY19     | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | a/a    | This study   |
| DSY20, DSY21     | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | a/a    | This study   |
| DSY26, DSY27     | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | a/a    | This study   |
| DSY71, DSY72, DSY75 | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | o/o   | This study   |
| DSY73, DSY74     | leu2::HisG gpd2::HisG HIS1* | arg4::HisG arg4::HisG HIS1* | a/a    | 45           |
| DSY121           | ura3::imm34::ura3::imm34 sap4::HisG sap4::HisG-UAR3-HIS3 | a/a | 45           |
| DSY124           | ura3::imm34::ura3::imm34 sap4::HisG sap4::HisG-UAR3-HIS3 | a/a | 45           |
| DSY130           | ura3::imm34::ura3::imm34 sap4::HisG sap4::HisG-UAR3-HIS3 | a/a | 45           |
| DSY131           | ura3::imm34::ura3::imm34 sap4::HisG sap4::HisG-UAR3-HIS3 | a/a | 45           |
| DSY142           | ura3::imm34::ura3::imm34 sap4::HisG sap4::HisG-UAR3-HIS3 | a/a | 45           |
| DSY144           | ura3::imm34::ura3::imm34 sap4::HisG sap4::HisG-UAR3-HIS3 | a/a | 45           |
| DSY145           | ura3::imm34::ura3::imm34 sap4::HisG sap4::HisG-UAR3-HIS3 | a/a | 45           |
| 3294             | his1::his1 ura3::ura3 arg5.6/arg5.6 | a/a | 32           |
| Ca29             | his1::his1 ura3::ura3 arg5.6/arg5.6 | a/a | 10           |
| PCA034           | his1::his1 ura3::ura3::Act-FARI::URA3 arg5.6/arg5.6 | a/a | P. Côte et al., unpublished data |
| PCA202           | his1::his1 ura3::ura3::Act-FARI::URA3 arg5.6/arg5.6 | a/a | This study   |

* Strains marked with an asterisk also contain the genotype URA3::ura3::imm34 IRO1/Δiro1::imm34.

1. a: alpha
2. o: opaque
3. yps7: HIS1::yps7
4. gpd2::HisG HIS1*
pheromone. A second aspartyl protease in C. albicans, encoded by the YPS7 gene, also shows a limited ability to degrade α-factor.

**MATERIALS AND METHODS**

**Media and reagents.** Standard laboratory media were prepared as described previously (18). Spender medium contained 1.35% agar, 1% nutrient broth, 0.4% potassium phosphate, and 2% mannitol (pH 7.2). Alpha pheromone peptide (GFR1TNFGYFEPO) was synthesized by Genesyn. Synthesis.

**Strains.** The C. albicans strains used in this study are listed in Table 1. Newly constructed strains were derived from SNY152 (40) and 3294 (32). All, a and α derivatives of SNY152 were generated by growth on sorbose medium, as previously described (4, 22), to create RBV1132 (αα) and RBV1133 (αα). PCR products for targeting the BAR1 open reading frame (ORF) were generated using oligonucleotides 1 and 2 to amplify the 5′ flank of BAR1 and oligonucleotides 3 and 4 to amplify the 3′ flank of BAR1. PCR products for targeting the YPS7 ORF were similarly generated from oligonucleotides 5/6 and 7/8. Oligonucleotides 9/10 and 11/12 were used to target the SAP7 ORF, and oligonucleotides 13/14 and 15/16 were used to target the SAP9 ORF. Oligonucleotide sequences are listed in Table 2. Selectable marker sequences (Candida dubliniensis HIS1, Candida maltesa LEU2, and C. dubliniensis ARG4) were also amplified by PCR from plasmids pSNS2, pSN40, and pSN69, respectively, as described previously (40). Fusion PCR products were generated by using oligonucleotides 1 and 4 to amplify BAR1, oligonucleotides 5 and 8 to amplify YPS7, oligonucleotides 9 and 12 to amplify SAP7, and oligonucleotides 13 and 16 to amplify SAP9. These PCR primers amplified the flanking sequences for each gene together with a marker PCR product (40). The first allele of each of these genes was replaced using the LEU2 marker in strain RBV1132. Second alleles were replaced using the HIS1 marker to generate a homozygous knocked-out each of these genes. Correct integration of the PCR products was verified by PCR across the 5′ and 3′ disruption junctions, and loss of the ORF was confirmed by using PCR primers internal to each ORF. The resulting (white-phase) strains were switched to the opaque phase by being passed on SCD medium (18).

**Wild-type copies of the BAR1 and YPS7 genes were reintroduced into ∆bar1/∆bar1 and ∆yps7/∆yps7 strains, respectively. First, the wild-type genes were amplified by PCR, together with approximately 1 kb of upstream sequence. The BAR1 ORF was amplified using oligonucleotides 17 and 18, and the YPS7 ORF was amplified using oligonucleotides 19 and 20. Using the Apal and Xhol restriction sites (underlined in Table 2), the PCR products were introduced into pSFS2A (42), which contains a dominant nourseothricin resistance marker. The resulting constructs were linearized with HpaI (BAR1 gene) and AflII (YPS7 gene) and integrated into the 5′-flanking regions of their respective genes. Correct integration of the BAR1 and YPS7 genes was confirmed by PCR across the boundaries of the inserted DNAs.

Several of the strains, including the SAP1-6 and SAP9-6 mutants, were derived from previously published αα strains by selection for a or α derivatives on sorbose medium. SAP1-6 mutants were previously described (20, 45), while the mutant lacking SAP9 was published and was a gift of Bernhard Hube (Robert Koch Institute, Germany).

**Halo assays.** A lawn of C. albicans cells was formed by plating approximately 10^7 opaque cells (from an overnight culture grown in SCD medium) onto solid Spender medium. Alpha pheromone (10 mg/ml in 10% dimethyl sulfoxide [DMSO]) or a 10% DMSO control was spotted (2 μl) onto the lawn of cells. Plates were incubated at room temperature for 3 days and then photographed.

**Pheromone experiment.** Overnight cultures in SCD medium were inoculated with an opaque colony of a wild-type or ∆bar1 mutant strain. The following morning, cells from each culture were washed with water and resuspended in Spender medium to a concentration of 2 x 10^7 cells/ml. Alpha pheromone was added at different concentrations (0.01, 0.1, and 1 μg/ml) to each culture, and the cultures were incubated at 25°C for 5 h. Cells were examined using a Zeiss Axioplan 2 microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) equipped with a Hamamatsu ORCA camera (Hamamatsu Photonics, Hamamatsu City, Japan).

**Quantitative mating assays.** Quantitative mating assays were performed by modification of a previous protocol (36). Azoetotropic mating strains were grown in the opaque phase at 23°C overnight in liquid SCD medium, and approximately 2 x 10^7 cells of each strain were mixed together. The mixed strains were deposited onto 0.8-μm filters by using a Millipore vacuum sampling manifold and then placed on the surfaces of Spender medium plates for 48 h at 23 to 25°C. Cells were collected and various dilutions plated onto dropout medium to select for mating conjugants and to monitor the parent-plus-conjugant population, as previously described (36). Cells were also taken from the mating mixes, and cell morphology and zygote formation were examined by microscopy using a Zeiss Axioplan 2 microscope equipped with a Hamamatsu ORCA camera.

**QPCR assays.** Cell cultures were prepared as previously described (3). Total RNA was prepared using a hot phenol procedure. To eliminate DNA contamination, RNAs were reextracted twice with hot phenol (pH 4.3; Fisher Scientific), followed by chloroform extraction and precipitation with ethanol. RNAs were reverse transcribed with Superscript (Stratagene), and CDNs were amplified by quantitative PCR (QPCR) in an Applied Biosystems 7300 real-time PCR system. Signals from each experimental sample were normalized to signals from expression of the 36B4 gene, whose expression was not regulated by pheromone (3). Expression values were averaged for four independent experiments. Oligonucleotides for the ASP genes were selected from previously published work (7, 39, 51). Oligonucleotides 21 and 22 were used to amplify ∆bar1, and oligonucleotides 23 and 24 were used to amplify YPS7 (Table 2).

**Barrier assays.** A thin lawn of a highly pheromone-responsive C. albicans strain, PC034 (Table 1), was spread on a SCD plate. Streaks of the specific strains to be tested were made on this lawn adjacent to a point source of α-factor to establish whether the strain could provide a barrier to the diffusion of the pheromone (19). Opaque strains used for the barrier assays were verified for their opaque form microscopically. Plates were incubated at 23°C for 24 to 48 h, and pictures of the plates were scanned at 300 dpi, using an Epson Perfection 3710 photo scanner.

**DNA microarray assays.** DNA microarray experiments were performed as previously described (3, 4, 10).

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**Table 2. Oligonucleotides used in this study**

| Name | Sequence |
|------|----------|
| 1. | TAAACACATGTTAGCACG |
| 2. | cacgccccggctacaggCCGCTAAAGTC |
| 3. | gtcacgctccctccgtGTTAACCTC |
| 4. | TGGCTGACAGTGCCTCCTC |
| 5. | AGTGTAAACCGCTTATGTC |
| 6. | cacgccccggctacaggCCGCTAAAGTC |
| 7. | gtcacgctccctccgtGTTAACCTC |
| 8. | CAGATAATAGAAATGAGAT |
| 9. | GTTCAAGATGTCCTAATGTG |
| 10. | cacgccccggctacaggCCGCTAAAGTC |
| 11. | gtcacgctccctccgtGTTAACCTC |
| 12. | AACGCTAGACAGGAGGAGGAGGAGGAG |
| 13. | CGCCAGTTGATCCCATACAA |
| 14. | cacgccccggctacaggCCGCTAAAGTC |
| 15. | gtcacgctccctccgtGTTAACCTC |
| 16. | TACATATGTTGTCCTTGGC |
| 17. | GGGGCATTAGGGCGGCGGTAGAT |
| 18. | CAGGGCGCTCGACAGGTTAGTGA |
| 19. | ACTACAGGGGCTTAAC |
| 20. | CAGGGCGCTCGACAGGTTAGTGA |
| 21. | AGCTATGTTGTCCT |
| 22. | CAGATATAGAAATGAGAT |
| 23. | GGCATATAAACCTTACAGG |
| 24. | GTTGATAGATGTTAGGAT |

*Lowercase letters indicate exogenous sequences used for fusion PCR reactions, as described previously (40). Underlined sequences are Apal and Xhol restriction sites.*
RESULTS

Identification of a putative mating pheromone barrier gene in C. albicans. S. cerevisiae MATa cells down-regulate their response to alpha pheromone in part by the induced production of a secreted aspartyl protease, termed Bar1p, that degrades the pheromone (5). The inefficient cell cycle arrest generated in C. albicans by the analogous pheromone could therefore be due to a related proteolytic function. We identified ORFs in the C. albicans genome that encode an aspartyl protease-like domain similar to that encoded by the S. cerevisiae BAR1 gene. Eighteen known or putative ORFs with sequence similarity to S. cerevisiae BAR1a were closely related to ScBAR1 in C. albicans and were up-regulated by pheromone (Table 3). Among them, the secreted aspartyl (SAP) family genes were closely related to ScSAP, with SAP9 having the greatest similarity. However, based on the S. cerevisiae model, we also predicted that the ScSAP1 ortholog would be a pheromone-induced MTLa-specific gene. In C. albicans, a-specific genes are regulated by the a2 transcription factor, which acts together with a cofactor protein, Mcm1 (49). Only one of the genes found to contain binding sites for a2/Mcm1 encodes an aspartyl-related protease; orf19.2082 thus represents a candidate BAR1 gene of C. albicans (also called SAP30, orf19.9629, or orf6.7473). We now present evidence that orf19.2082 is the C. albicans ortholog of ScSAP1, and we refer to this gene as BAR1 throughout this report. The promoter region of C. albicans BAR1 is shown in Fig. 1, which illustrates the position of the putative a2/Mcm1 binding site 203 to 223 nucleotides upstream of the ORF. The BAR1 gene was found to be expressed only in a cells by transcriptional profiling of pheromone-treated a and α cells of C. albicans (49). In the latter experiments, C. albicans BAR1 was one of only seven genes that were induced by pheromone treatment of a cells but not by pheromone treatment of α cells and was the only aspartyl protease in this group. We also confirmed that BAR1 is induced in pheromone-treated a cells by quantitative PCR (discussed below).

A potential binding site for C. albicans homolog of Ste12p (Cph1p) can also be found in the promoter region of BAR1 (Fig. 1). Ste12p mediates the transcriptional activation of pheromone-induced genes in S. cerevisiae, and Cph1p is similarly implicated in the pheromone response in C. albicans (6, 31). A potential consensus site for Ste12/Cph1p binding, TGTCTG/GA, has been identified (3, 11, 15), and a binding site is present in the promoter region of BAR1 (Fig. 1).

In the following sections, we present several lines of evidence supporting the identification of orf19.2082 as the C. albicans ortholog of S. cerevisiae BAR1. In addition, we also tested the potential role of several other C. albicans aspartyl proteases as potential barriers to mating pheromone. In particular, we include an analysis of the role of the SAP family of genes in the response of C. albicans a cells to alpha pheromone. The results indicate that C. albicans BAR1 is a highly specific protease that acts to degrade the alpha mating pheromone during the mating of a cells.

Pheromone induction of BAR1 in C. albicans. Transcriptional profiling studies of the response of C. albicans a cells to alpha pheromone revealed that under nutrient-limiting conditions, 144 genes were up-regulated by pheromone and 66 genes

![Diagram](https://via.placeholder.com/150)

**FIG. 1.** Schematic showing the promoter region of C. albicans orf19.2082 (C. albicans BAR1). The C. albicans BAR1 gene is expressed preferentially in a-type cells due to the presence of binding sites for a2 and Mcm1 proteins (49). In addition, there is a potential binding site for Cph1p (3), the C. albicans homolog of S. cerevisiae Ste12p, and this site may mediate induction of the C. albicans BAR1 gene in response to alpha pheromone.
were down-regulated (expression change of >3-fold) (3). These experiments were performed in nutrient-deficient Spider medium, as opaque cells responded robustly to pheromone under these conditions (as measured by the number of genes induced, their induction ratios, and the fraction of cells exhibiting morphological responses) (3). Included among the set of pheromone-regulated genes were a number of protease genes, including several that were highly up-regulated by pheromone, e.g., \( \text{SAP4, SAP5, and SAP6} \), and several that were repressed by pheromone, e.g., \( \text{SAP1 and SAP3} \) (3). In the same studies, the \( \text{BAR1} \) gene was one of the most induced genes, with expression increased >100-fold 4 h after exposure to pheromone. To analyze more accurately the response of \( \text{C. albicans BAR1} \) and the \( \text{SAP} \) family of genes to pheromone, we carried out QPCR to determine the changes in expression of these genes in cells responding to pheromone. These experiments were important to confirm the pattern of expression of the protease genes, since many of these genes are highly homologous and it is possible that the microarray experiments did not distinguish between closely related genes. For the PCR experiments, oligonucleotides were chosen to specifically amplify each target gene, as previously described (7, 39, 51).

QPCR was performed on opaque a cells grown in Spider medium and treated with alpha pheromone (or a DMSO control) for 4 h. Again, Spider medium was used because this medium produced an efficient response of opaque cells to pheromone, both morphologically and in transcriptional profiling experiments (3). Cells were harvested and cDNAs prepared from the cells as a template for QPCR (see Materials and Methods). Figure 2 shows the relative expression levels of several \( \text{C. albicans} \) protease genes in response to alpha pheromone. For this study, we compared expression levels of \( \text{BAR1} \), the \( \text{SAP} \) genes (\( \text{SAP1-10} \)), and \( \text{YPS7} \) (orf19.6481). The last gene is a gene of unknown function but is also predicted to encode an aspartyl protease whose \( \text{S. cerevisiae} \) homolog is encoded by \( \text{YPS7} \) (Table 3; see also www.candidagenome.org).

Several protease genes were highly regulated by alpha pheromone, as predicted from the microarray analyses. In particular, the \( \text{BAR1, SAP4, and SAP6} \) genes were highly induced in opaque a cells responding to alpha pheromone, with each gene induced >200-fold. Several other aspartyl protease genes were induced more modestly by alpha pheromone, with \( \text{SAP2, SAP5, and SAP7} \) induced 11-, 6-, and 18-fold, respectively. In contrast, \( \text{SAP1 and SAP3} \) were negatively regulated by alpha pheromone (repressed 74- and 23-fold, respectively), confirming the down-regulation of these genes observed previously in microarray and Northern analyses (3, 29).

**Deletion of the \( \text{BAR1} \) gene enhances alpha pheromone sensitivity.** In \( \text{S. cerevisiae} \), deletion of the \( \text{BAR1} \) gene results in an increased sensitivity of a-type cells to the alpha mating pheromone. This can be demonstrated by spotting alpha pheromone onto a nascent lawn of \( \text{MATa} \) cells: once the lawn has grown to maturity, a halo is observed around the spot of the pheromone due to efficient cell cycle arrest of neighboring cells. The halo assay provides a simple semiquantitative measure of pheromone activity, as the size of the halo is proportional to both the amount of pheromone present and the strength of the pheromone response in the responding mating partner (35). Thus, in \( \text{S. cerevisiae} \), deletion of the \( \text{BAR1} \) gene results in increased halo formation due to the increase in effective pheromone concentration, as no pheromone is degraded by the protease.

To test if \( \text{C. albicans} \) aspartyl protease genes modulate the sensitivity to alpha pheromone, \( \text{BAR1, YPS7, and SAP1-9} \) homozygous deletion mutants were constructed in \( \text{MTLa} \) cells. A lawn of opaque cells of each mutant strain was plated on Spider medium plates, pheromone (or a control) was spotted onto the lawn, and the cells were allowed to grow to maturity. Again, Spider medium was used for this assay to ensure a robust response of opaque a cells to alpha pheromone (3). Figure 3 shows the results of halo assays carried out with strains in which \( \text{BAR1, YPS7, or a SAP gene (SAP1-9)} \) was deleted compared to the result with a wild-type strain. The wild-type a strain showed no clear halo formation in response to alpha pheromone, confirming that \( \text{C. albicans} \) cells do not naturally undergo extended growth arrest in response to mating pheromone (3, 10, 41). In contrast, a mutant lacking the
**BAR1** gene produced a large halo around the spot of the pheromone, indicating that neighboring cells had responded to pheromone by arresting their growth (Fig. 3A). The **Δaps1-9** mutants, like the wild type, failed to produce a significant halo around the pheromone spot. The **Δaps7** strain, however, did produce a detectable halo, although the halo was smaller than that observed for **Δbar1** strains. The phenotype of multiple **Δaps7** mutants was variable, as two of four independent **Δaps7** mutants showed the halo phenotype displayed in Fig. 3, while two mutants showed no halo formation (i.e., appeared indistinguishable from the wild type). We confirmed that the halo phenotypes of both **BAR1** and **YPS7** mutants were due to the loss of the targeted gene by reintroduction of one copy of the wild-type gene to the mutant locus (Fig. 3B). These experiments are consistent with **C. albicans** **BAR1** encoding a protein with alpha pheromone-degrading activity and also indicate that
YP7, although not a pheromone-responsive gene, can also act to degrade alpha pheromone.

Deletion of the BARI gene leads to a defect in mating in C. albicans cells. To examine if the loss of C. albicans BARI or YPS7 leads to a change in mating efficiency, deletion strains lacking these genes were tested in quantitative mating experiments. Opaque a and α strains harboring different auxotrophic markers were coinoculated on Spider medium for 48 h and then plated on tester plates to determine the frequency of formation of prototrophic mating products (see Materials and Methods). Mating between wild-type strains occurred with an average mating efficiency of 15% in this assay (Fig. 4). Mating of Δyps7 strains occurred at a frequency similar to that of wild-type strains, with 19% of a and α cells forming mating products. Significantly, mating of Δbar1 a and α strains showed a greatly reduced mating efficiency, with an average mating frequency of <0.1%. To determine if the mating defect was specific to a or α cells, wild-type strains were crossed with Δbar1 mating partners. Mating of Δbar1 a strains with wild-type α strains resulted in extremely low mating efficiencies (0.01%), while Δbar1 α strains mated with wild-type a strains at normal efficiencies (22%) (Fig. 4). These mating experiments revealed that Δbar1 strains exhibit an a-specific defect in mating, with mating reduced >100-fold relative to that of wild-type a strains. Experiments with S. cerevisiae Δbar1 mutants also revealed a mating defect in a strains, although this defect was more modest (4- to 33-fold) than that observed here with C. albicans Δbar1 mutants (5, 16).

To further characterize the defect in mating, we analyzed cells taken from mixes of mating a and α cells after 48 h of incubation. Wild-type strains contained many mating zygotes, as expected from the relatively efficient mating between a and α strains (Fig. 5). In contrast, very few, if any, zygotes were visible for matings between Δbar1 strains, even though the cells exhibited long mating projections characteristic of cells responding to mating pheromones (Fig. 5). These experiments demonstrate that the block to mating occurs early in Δbar1 mutants, with cells unable to undergo fusion with a mating partner.

Deletion of BARI sensitzes opaque cells to alpha pheromone. The morphologies of opaque wild-type and Δbar1 strains were compared in the presence of alpha pheromone. Alpha pheromone induces the formation of characteristic mating projections in C. albicans, and these can be many times the length of the original mother cell (4, 28, 29, 41). Wild-type and Δbar1 opaque a cells were treated with various doses of alpha pheromone (1, 0.1, and 0.01 μg/ml), and their cellular morphology was determined after 5 h of incubation. At a high pheromone concentration (1 μg/ml), both wild-type and Δbar1 mutant cells showed a marked response to pheromone, as observed by the formation of long mating projections (Fig. 6). The Δbar1 strain also exhibited long mating projections at low concentrations of pheromone, and even at 0.01 μg/ml pheromone, it elicited mating projections that resembled those seen at 1 μg/ml (Fig. 6). In contrast, wild-type strains did not produce any significant mating projections at low concentrations of pheromone (0.1 and 0.01 μg/ml). Instead, these cells appeared to generate small mating projections that then resumed a normal budding morphology (Fig. 6).

These results are consistent with the predicted function of the BARI gene—expression of the Barlp protease leads to a breakdown of alpha pheromone, and thus, higher concentrations of pheromone are necessary to overcome proteolytic processing of the pheromone. In the absence of the Barlp protease, alpha pheromone is not degraded, and thus, smaller
amounts of pheromone can efficiently induce a mating response.

Additive effect of mutations in BAR1 and GPA2 on pheromone response in C. albicans. In C. albicans, Gpr1 is a G protein-coupled receptor that acts together with Gpa2, encoding a Go subunit, in signaling environmental cues to the cell (34, 37, 44). Previously, we showed that nutritional signals play an important part in the efficiency of the response of C. albicans to pheromone and identified Gpa2 as a component of the nutrient-sensing pathway (3). C. albicans α cells lacking Gpa2 showed an enhanced response to alpha pheromone and resulted in Δgpa2 mutants forming a distinct zone of arrested growth in the halo assay (Fig. 7). The halo formed by Δgpa2 mutants was smaller than that formed by Δbar1 mutants (Fig. 7). To see if mutations in GPA2 and BAR1 are additive, we generated double mutants in which both genes were deleted from α-type mating cells and tested their response to alpha pheromone in the halo assay. While both Δgpa2 and Δbar1 strains produced halos, the double mutant strain produced a larger halo than either single mutant alone (Fig. 7). In addition, the halo formed by the Δgpa2 Δbar1 strain appeared less cloudy than that in either the Δgpa2 or Δbar1 single mutant, indicating more efficient cell cycle arrest. This result is consistent with Bar1p and Gpa2p being involved in different steps in the pheromone response; Bar1p is involved in degradation of the pheromone, while Gpa2p is involved in a nutrient signaling pathway that modulates the response to mating pheromone. The data also show that modulation of both pathways is necessary for efficient growth arrest in response to pheromone in C. albicans.

Demonstration of barrier activity by Bar1p. We used a strategy similar to the S. cerevisiae barrier assay to confirm the existence of barrier activity in C. albicans (19). C. albicans opaque cells in which Far1p was overexpressed were used for these experiments, as these cells efficiently arrest their growth in response to α-factor (P. Côté et al., unpublished data). Briefly, a lawn of Far1p-overexpressing opaque cells was used to detect whether a test strain could block the diffusion of α-factor. If the tested strain can secrete, either constitutively or in response to pheromone, a protein with pheromone-degrading activity, then it will generate a barrier to the diffusion of a source of alpha pheromone placed next to it, and the lawn of sensitive cells on the other side of this barrier will be able to grow. We tested barrier activity in a wild-type strain (3294) and in the super-pheromone-sensitive Δsst2 strain (Ca29) (10). As shown in Fig. 8, we observed a block in halo front formation beyond the streak of opaque wild-type cells. For the Δsst2 strain and its enhanced pheromone response, we observed growth of the lawn even on the pheromone spot side of the barrier, consistent with a zone of pheromone inactivation extending on both sides of the test streak (Fig. 8). Significantly, the deletion of BAR1 in strain PCa202 completely abolished the barrier activity observed in the parental wild-type strain (Fig. 8). White-phase cells of each of the tested strains did not produce observable barrier activity (data not shown). This experiment demonstrates that C. albicans cells secrete a protein with barrier activity to alpha pheromone and that this protein is encoded by BAR1.

DISCUSSION

We have identified a gene encoding a protein with barrier activity to mating pheromone in C. albicans (orf19.2082) and now designate this gene BAR1, the ortholog of S. cerevisiae BAR1. Characterization of the C. albicans BAR1 gene demonstrated that it has several properties similar to those of S. cerevisiae BAR1, including the following. (i) BAR1 is expressed only in α-type mating cells of C. albicans (49). (ii) The BAR1...
gene is induced by alpha pheromone (3, 49; this study). (iii) Strains lacking the \textit{BAR1} gene are hypersensitive to pheromone and generate distinct areas of arrested cell growth in halo assays. (iv) \textit{MTLa bar1} strains exhibit a significant defect in mating with wild-type \textit{a} strains. (v) Bar1p is responsible for the barrier activity that limits alpha pheromone diffusion. \textit{S. cerevisiae} \textit{bar1} strains have been extremely useful to the community, and together with \textit{S. cerevisiae} alpha pheromone, they have been used extensively for efficient synchronization of the cell cycle. It is envisaged that \textit{bar1} strains will prove similarly beneficial for cell cycle studies with \textit{C. albicans}.

Comparisons between mating in \textit{S. cerevisiae} and that in \textit{C. albicans} have been stimulating, as these organisms share many features in the mating pathway, but at the same time, studies have revealed important differences in the regulation of their mating behavior. For example, whereas both fungi use a con-

![FIG. 6. Morphological response of \textit{C. albicans} \textit{bar1} strains to low concentrations of alpha pheromone. Wild-type and \textit{bar1} strains were incubated with various concentrations of alpha pheromone in Spider medium for 5 h and then analyzed by microscopy. Wild-type cells (RBY1117) responded strongly to high concentrations of alpha pheromone (1 \textmu g/ml) but failed to exhibit long mating projections at lower concentrations of pheromone. In contrast, \textit{bar1} cells (RBY1197) showed a strong morphological response to pheromone even at concentrations as low as 0.01 \textmu g/ml.]

![FIG. 7. Halo formation in \textit{C. albicans} \textit{gpa2}, \textit{bar1}, and \textit{gpa2 bar1} strains. Both \textit{gpa2} (3) and \textit{bar1} (this work) strains exhibit areas of growth arrest in response to alpha pheromone in halo assays. To see if this phenotype is additive, a double mutant strain lacking both \textit{GPA2} and \textit{BAR1} was constructed and tested in the halo assay. Pheromone was spotted on the center right of each plate, and a DMSO control was spotted on the center left of the plate. The \textit{gpa2} strain (RBY1166) and the \textit{bar1} strain (RBY1197) produced cloudy halos, but the double mutant \textit{gpa2 bar1} strain (DSY18) produced a large halo that was relatively clear of background cell growth. Small colonies observed within the halo are due to the presence of a few (unresponsive) white cells within the largely opaque population.]
Similar results were seen with hypersensitive strains; halos appear clear due to efficient cell cycle arrest (35). mutants the halos appeared cloudy, whereas for pressuring the pheromone (10). Clearer halos are formed by strains overexpressing the pheromone signal.

C. albicans BAR1

The gene may play a role in some of the observed differences in pheromone responses between fungal species. S. cerevisiae BAR1+ strains undergo efficient cell cycle arrest in response to pheromone, whereas C. albicans BAR1− strains do not (3, 10, 41). These differences may be due in part to quantitative differences in the levels of BAR1 activity between these two organisms. However, other mechanisms also influence the response of a cells to pheromone, as even for C. albicans Δbar1 mutants the halos appeared cloudy, whereas for S. cerevisiae strains, halos appear clear due to efficient cell cycle arrest (35). Similar results were seen with hypersensitive SST2 mutants of C. albicans; in this case, Δsst2 strains formed halos, but they were cloudy due to some cells failing to respond efficiently to pheromone (10). Clearer halos are formed by strains overexpressing the C. albicans ortholog of the S. cerevisiae cyclin-dependent kinase inhibitor Far1p (Côte et al., unpublished data) or in C. albicans strains lacking both Bar1p and the Gα protein Gpa2p (3). Gpa2p functions in a nutrient-sensing pathway to regulate the response to pheromone in C. albicans, and Δgpa2 mutants respond more strongly to pheromone both transcriptionally and morphologically. Since the nutrient-sensing pathway does not regulate the pheromone response in S. cerevisiae, this may be one reason why it is easier to obtain pheromone-induced growth arrest in S. cerevisiae than in C. albicans.

Deletion of the BAR1 gene led to a large, >100-fold decrease in the mating efficiency of C. albicans a cells. In S. cerevisiae, Δbar1 mutants also exhibit a decrease in mating efficiency, although the decrease is more modest (varying from 4-fold to 33-fold) (5, 16). The decrease in mating efficiency is thought to be a direct result of overstimulation of the mating pathway. Thus, shutting off the response to pheromone not only is required for cells that have failed to find a mating partner but is also a necessary step in the normal mating process. Similar defects in mating were observed in Δsst2 mutants of C. albicans, as these also lead to overstimulation of cells responding to pheromone (10).

In addition to the identification of BAR1 in C. albicans, we noted that mutations in a second gene, YPS7, resulted in increased sensitivity of a cells to pheromone. The YPS7 gene is predicted to encode an aspartyl protease, although the phenotypes of different Δyps7 strains varied. For some Δyps7 mutants, no halo was formed, while others produced a distinct halo, albeit smaller than the halo formed by Δbar1 strains. For Δyps7 mutants that displayed a halo phenotype, the phenotype was suppressed by reintroduction of one copy of the YPS7
gene, confirming that the phenotype was due to the loss of YPS7 function. Deletion of the YPS7 gene did not affect mating frequencies in C. albicans. At present, the reason for the variable phenotype of Δyps7 strains is not known. One possibility is that the expression of other pheromone-degrading proteases is up-regulated in some mutants and thus can occasionally compensate for the loss of YPS7. The epigenetic regulation of proteases in C. albicans has not been demonstrated, but it is not surprising to find that this fungal pathogen has mechanisms by which the expression of some aspartyl proteases can compensate for that of another.

Despite the identification of alpha pheromone processing activities in C. albicans BARI and YPS7, the majority of aspartyl protease genes tested (SAP1-9) did not influence the response of a cells to pheromone. These results confirm that the degradation of alpha pheromone requires targeting of the pheromone by an aspartyl protease with appropriate specificity. It also raises the question of the function of the other SAP genes in mating in C. albicans. Five members of the SAP family of genes are induced by pheromone (SAP2, SAP4, SAP5, SAP6, and SAP7), while two SAP genes are repressed by pheromone (SAP1 and SAP3). In addition, four of the SAP genes are also regulated by white-opaque switching: SAP1, SAP2, SAP3, and SAP4 are upregulated in the opaque (mating-competent) state (26, 38, 48, 50). There appear to be two main possibilities for the role of the SAP genes in mating, as follows: (i) they play an intrinsic role in mating, although the function of these genes in mating is not yet understood; or (ii) their function is related to where mating occurs in vivo, and they act to promote mating in this in vivo niche. Future experiments will be directed toward determining the role of the SAP genes in mating and testing if epigenetic regulation of these genes occurs in C. albicans.

In summary, we have shown that C. albicans MTLa cells possess a barrier activity in response to alpha mating pheromone. We have identified the ortholog of S. cerevisiae BARI in C. albicans and characterized the activity of the barrier protease. The identification of C. albicans BARI provides a further similarity between the mating processes of S. cerevisiae and C. albicans and should assist in studies requiring synchronization of the cell cycle. The regulation of SAP genes by white-opaque switching and alpha protease suggests that other aspartyl proteases also play a significant, but as yet undetermined, role in C. albicans mating.

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