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Authors

Jones, Stephen K
Clarke, Starlynn C
Craik, Charles S
[et al.](#)

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Evolutionary Selection on Barrier Activity: Bar1 Is an Aspartyl Protease with Novel Substrate Specificity

Stephen K. Jones, Jr.,^{a*} Starlynn C. Clarke,^b Charles S. Craik,^b Richard J. Bennett^a

Department of Molecular Microbiology and Immunology, Brown University, Providence, Rhode Island, USA^a; Department of Pharmaceutical Chemistry, University of California—San Francisco, San Francisco, California, USA^b

* Present address: Stephen K. Jones, Jr., Department of Molecular Biosciences, University of Texas—Austin, Austin, Texas, USA.

ABSTRACT Peptide-based pheromones are used throughout the fungal kingdom for coordinating sexual responses between mating partners. Here, we address the properties and function of Bar1, an aspartyl protease that acts as a “barrier” and antagonist to pheromone signaling in multiple species. *Candida albicans* Bar1 was purified and shown to exhibit preferential cleavage of native α pheromone over pheromones from related fungal species. This result establishes that protease substrate specificity co-evolved along with changes in its pheromone target. Pheromone cleavage by Bar1 occurred between residues Thr-5 and Asn-6 in the middle of the tridecapeptide sequence. Surprisingly, proteolytic activity was independent of the amino acid residues present at the scissile bond and instead relied on residues at the C terminus of α pheromone. Unlike most aspartyl proteases, Bar1 also exhibited a near-neutral pH optimum and was resistant to the class-wide inhibitor pepstatin A. In addition, genetic analysis was performed on *C. albicans* *BAR1* and demonstrated that the protease not only regulates endogenous pheromone signaling but also can limit interspecies pheromone signaling. We discuss these findings and propose that the unusual substrate specificity of Bar1 is a consequence of its coevolution with the α pheromone receptor Ste2 for their shared peptide target.

IMPORTANCE Pheromones are important for intraspecies communication across the tree of life. In the fungal kingdom, extracellular proteases play a key role in antagonizing pheromone signaling in multiple species. This study examines the properties and function of *Candida albicans* Bar1, an aspartyl protease that cleaves and thereby inactivates α pheromone. We demonstrate that Bar1 plays important roles in regulating both intra- and interspecies pheromone signaling. The fungal protease shows preferential activity on the endogenous pheromone, but, surprisingly, cleavage activity is dependent on amino acid residues distal to the scissile bond. We propose that the unusual substrate specificity of Bar1 is a direct result of coevolution with Ste2, the receptor for α pheromone, for recognition of the same peptide target. The novel specificity of Bar1 reveals the complex forces shaping the evolution of mating pathways in fungi and uncovers a protease with potentially important applications in the biotechnology industry.

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Address correspondence to Richard J. Bennett, richard_bennett@brown.edu.

Pheromone signaling involves the secretion of species-specific chemicals to coordinate cell behavior. Fungi have been intensively studied for their use of sexual pheromones to regulate intercellular signaling and conjugation. In ascomycetes and basidiomycetes, pheromones are peptides or lipopeptides that are secreted into the extracellular milieu and induce morphological and transcriptional responses in target cells. In both of these fungal lineages, mating specificity is determined by the sets of pheromones and pheromone receptors expressed by different cell types (1–6).

In the model ascomycete *Saccharomyces cerevisiae*, **a** cells secrete **a** pheromone and express the α pheromone receptor Ste2, while α cells secrete α pheromone and express the **a** pheromone receptor Ste3 (7). Pheromone signaling between the 2 cell types activates a conserved mitogen-activated protein kinase (MAPK) signaling cascade, leading to the induction of mating genes and the

formation of mating projections or “shmoo” (8–10). The pheromones produced by *S. cerevisiae* **a** and α cells show distinct physical properties and are secreted by different mechanisms; α pheromone is an unmodified peptide that is secreted by the traditional secretory pathway, whereas **a**-factor is farnesylated and carboxymethylated, and transport requires a specific transmembrane translocator (11, 12). The use of both modified and unmodified pheromones is conserved across the ascomycetes, whereas basidiomycetes utilize only lipid-modified pheromones (5, 13).

Candida albicans is a human fungal pathogen related to *S. cerevisiae*, although these species are as divergent as humans and fish (14). Similar to *S. cerevisiae*, mating in *C. albicans* involves pheromone signaling between **a** and α cells (15–18). However, sexual competency in *C. albicans* is dependent on cells undergoing a phenotypic switch from the conventional “white” state to the alternative “opaque” state (19, 20). These two states show marked

differences in morphology, metabolism, and signaling, including distinct responses to pheromone (21–23). The **a** and α pheromones are encoded by *MFa* and *MF α* genes, respectively, and pheromone signaling between opposite-sex opaque cells leads to the formation of mating projections and conjugation, producing tetraploid **a**/ α cells. Although mating incompetent, *C. albicans* white cells can respond to pheromones secreted by opaque cells of the opposite mating type. However, rather than forming mating projections, pheromone-treated white cells adhere to inert surfaces and undergo biofilm formation (24–26).

In addition to the secretion of pheromones, yeast cells also produce degradative enzymes that target mating pheromones for destruction. In both *S. cerevisiae* and *C. albicans*, **a** cells secrete an aspartyl protease, Bar1, that acts as a “barrier” to α pheromone signaling by inactivating it (27–30). Bar1 is produced by **a** cells and sharpens the gradient of α pheromone produced by α cells, thereby facilitating chemotropism and mating between **a** and α partners (31–33). In addition to increasing mating efficiency, Bar1 promotes higher growth rates in subpopulations of **a** cells exposed to α pheromone by overcoming pheromone-induced cell cycle arrest (34). Interestingly, loss of Bar1 in *C. albicans* results in transitioning from a heterothallic to a homothallic (selfing) mode of sexual reproduction. *C. albicans* **a** cells secrete low levels of α pheromone (in addition to **a** pheromone), and loss of Bar1 causes positive feedback of α pheromone on these cell types, triggering same-sex **a**-**a** mating (35). The presence of Bar1 therefore prevents autocrine signaling by α pheromone in *C. albicans* **a** cells, whereas the high level of α pheromone produced by α cells is sufficient to override Bar1 activity during **a**- α mating (30, 35). Interestingly, the distantly related archiascomycete *Schizosaccharomyces pombe* produces a secreted carboxypeptidase, Sxa2, that acts to degrade its own α pheromone-like peptide (36, 37). This indicates that distinct pheromone-degrading proteases have evolved in different ascomycete lineages and supports the assertion that these proteases play key roles in pheromone signaling.

C. albicans Bar1 contains two Asp-(Thr/Ser)-Gly catalytic motifs, as found in other aspartyl proteases, and shares significant homology with well-characterized members of this class (38, 39). Aspartyl proteases have been referred to as acidic proteases, given that they are often optimally active at pH 3 to 5, and most are susceptible to inhibition by the aspartyl protease inhibitor pepstatin A (40). However, *S. cerevisiae* Bar1 has been shown to have several unusual characteristics for an aspartyl protease, including a pH optimum of 6.5 and resistance to pepstatin A (28, 39). In contrast, the biochemical properties of purified *C. albicans* Bar1 have not been examined, nor has its specificity for endogenous α pheromone.

In this work, we investigate the biochemical properties of *C. albicans* Bar1 and reveal that the protease is highly selective in cleaving the endogenous α pheromone. Interestingly, substrate cleavage occurs in the middle of the pheromone, but specificity is largely determined by amino acids present at the C terminus of the sequence. As such, this substrate specificity is highly unusual for an endoprotease and may have arisen due to constraints on Bar1 activity imposed by the pheromone receptor Ste2, which must also recognize α pheromone. We also examine the role of *BAR1* in intra- and interspecies pheromone signaling in *C. albicans*. These experiments reveal that in addition to modulating endogenous α pheromone activity, *C. albicans* Bar1 also restricts signaling in response to pheromones secreted by related *Candida* species. To-

gether, our studies reveal that *C. albicans* Bar1 is an aspartyl protease with novel substrate specificity that acts to regulate both intra- and interspecies signaling events and is a potentially invaluable tool with applications in biotechnology.

RESULTS

Purification and characterization of *C. albicans* Bar1 protease.

To define the biochemical activity of *C. albicans* Bar1, a recombinant form of the protein was overexpressed and purified from *Pichia pastoris*. The *C. albicans* *BAR1* gene was engineered with a C-terminal hexahistidine tag and overexpressed using the Pichia-Pink expression system (see Materials and Methods). *BAR1* was expressed under the control of the *P. pastoris* AOX1 promoter and contained the *S. cerevisiae* α pheromone signal peptide sequence to ensure efficient secretion into the supernatant. Secreted Bar1 protein was collected and purified by nickel affinity chromatography (Fig. 1A) to a final yield of 6 mg/liter. The protein migrated as a broad range of high-molecular-weight bands in Western blots, suggestive of protein glycosylation. To test this, *C. albicans* Bar1 was treated with a commercial mixture of glycosylases that increased protein migration by SDS-PAGE (Fig. 1B), indicating that the recombinant protein was highly glycosylated. Aspartyl proteases often contain two catalytic aspartic acid residues that are present in conserved Asp-(Thr/Ser)-Gly motifs (39). *C. albicans* Bar1 contains two such DSG motifs at residues 56 to 58 and 232 to 234, and a mutant Bar1 protein was purified that contained an amino acid substitution (D232A) at one of the predicted catalytic aspartyl residues (see Fig. S1 in the supplemental material).

To assess the functionality of recombinant Bar1, we tested its ability to complement a *C. albicans* mutant lacking the *BAR1* gene. A quantitative readout of the *C. albicans* response to pheromone is the pheromone-induced cell death (PID) assay; a significant proportion of opaque **a** cells responding to α pheromone undergo cell death that can be measured by staining with the vitality dye propidium iodide (41). The pheromone response is dependent on Bar1 activity, as **a** cells lacking *BAR1* undergo PID at lower pheromone concentrations than wild-type Bar1⁺ cells (41). Thus, wild-type opaque **a** cells exposed to 10 μ M α pheromone showed low levels (<10%) of cell death, whereas *bar1 Δ /bar1 Δ* mutants showed ~70% cell death (Fig. 1C). Addition of recombinant Bar1 (12.7 nM) to *bar1 Δ /bar1 Δ* mutants reduced cell death to <10% of the population, whereas addition of the Bar1(D232A) mutant failed to restore wild-type levels of PID (Fig. 1C).

A halo assay was also used to verify the functionality of recombinant Bar1. In this assay, α pheromone is spotted onto a lawn of **a** cells, which are then allowed to grow to confluence. Growth is inhibited in *C. albicans* cells that respond to the exogenous pheromone, generating “halos” that indicate the strength of the pheromone response (30). Unlike the weak halos produced by wild-type opaque **a** cells, *bar1 Δ /bar1 Δ* mutants produced a more distinct halo in response to α pheromone. Addition of recombinant Bar1 reduced the clarity of the halo formed by *bar1 Δ /bar1 Δ* cells (Fig. 1D), whereas the Bar1(D232A) mutant did not show complementation. Together, these results establish that recombinant Bar1 inhibits its physiological target and that mutation of one of the catalytic aspartyl residues abolishes activity consistent with its classification as an aspartyl protease.

Biochemical characterization of *C. albicans* Bar1. To determine the kinetics of *C. albicans* Bar1 cleavage, an internally quenched (IQ) version of *C. albicans* α pheromone (GFRLTNF-

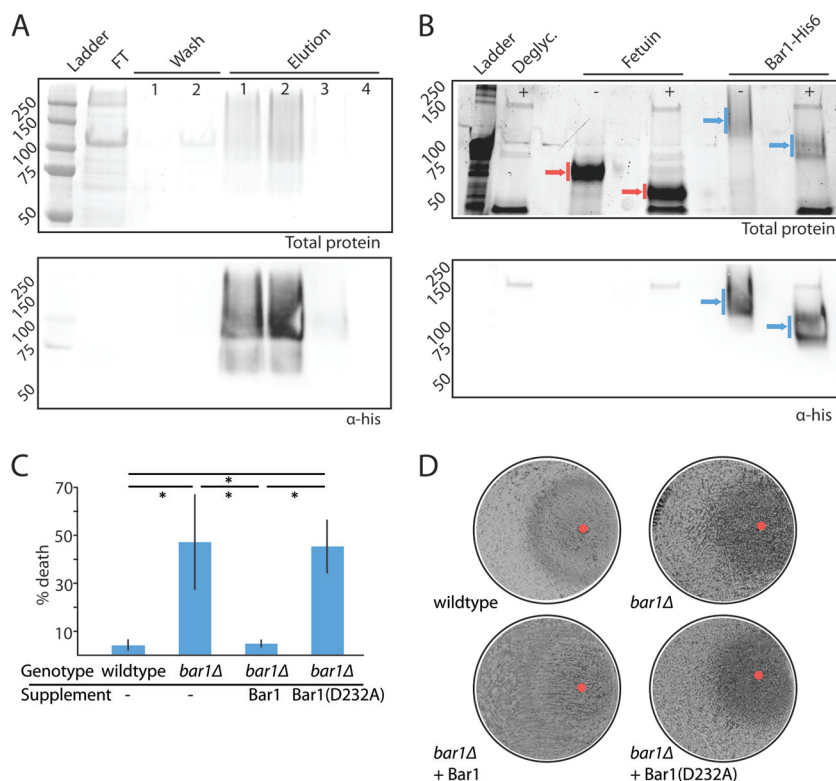


FIG 1 Purification and analysis of *C. albicans* Bar1 protease. (A) Purification of His-tagged *C. albicans* Bar1 from *Pichia pastoris* using nickel affinity chromatography. Nickel beads were washed twice (20 mM imidazole), and protein was eluted with 500 mM imidazole. FT, flowthrough. Total protein was assayed by Coomassie staining (top panel), and Bar1 was detected by Western blotting with an anti-His antibody (lower panel). (B) Protein glycosylation was assessed by treatment of Bar1 or fetuin (positive control) with a cocktail of deglycosylation (Deglyc.) enzymes. Blue arrows, Bar1; red arrows, fetuin. The presence or absence of deglycosylase enzymes is noted with $\pm$. Total protein is shown (top panel) using BioRad Stainfree indicator, and Bar1 was detected by Western blotting with an anti-His antibody (lower panel). (C) To evaluate the activity of recombinant Bar1, wild-type and *bar1Δ*/*bar1Δ* opaque α strains were treated with or without 300 nM α pheromone for 5 h, together with recombinant Bar1 or Bar1(D232A) at 127 nM. The percentage of death was analyzed by staining with propidium iodide and flow cytometry. Values are means $\pm$ standard deviations (SD). $n = 3$. *, $P < 0.05$ by t test. (D) Cell cycle arrest was analyzed by plating a lawn of opaque α cells on solid medium and spotting 20 μ g α pheromone (red circle) together with Bar1 or Bar1(D232A), followed by imaging of plates after 3 days of culture at 22°C.

GYEPG) was utilized. Quenching of the C-terminal EDANS fluorophore occurs by resonance energy transfer to the N-terminal dabcyI quencher, and upon cleavage of the peptide, the quencher is released so that fluorescence is detected. Under defined conditions, Bar1 processed α pheromone with a k_{cat}/K_m of $\sim 7.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 2A), whereas the Bar1(D232A) mutant showed no detectable cleavage activity on the IQ substrate. This rate is comparable to that of other secreted *Candida* aspartyl proteases for their respective substrates (42). Bar1 exhibited maximal activity at a pH of 6.5 and at 37°C, with the greatest activity in low-molarity solutions (Fig. 2B to D). Interestingly, the potent aspartyl protease antagonist pepstatin A did not inhibit Bar1 activity, even at 100 μ M (Fig. 2E). *S. cerevisiae* Bar1 is likewise insensitive to this compound (43). Pepstatin A acts as a transition state analog of aspartyl protease substrates (44) and may be a poor mimic of *C. albicans* α pheromone, which contains polar, rather than hydrophobic, residues at its cleavage site (see below and reference 40).

Cleavage of α pheromone peptides by *C. albicans* Bar1. To determine the position at which Bar1 cleaves *C. albicans* α pheromone, we coinubated recombinant Bar1 with synthetic α pheromone and analyzed the digestion products by liquid chromatography-mass-spectroscopy (LC-MS [see Materials and Methods]). A single cleavage event was detected at the bond be-

tween threonine 5 and asparagine 6 (Fig. 3A). In contrast, no cleavage products were generated by the catalytically inactive Bar1(D232A) protein (data not shown), confirming that we were monitoring Bar1 activity and not that of a contaminating protease.

C. albicans cells were previously shown to respond to several pheromones from related *Candida* species (41), leading us to test Bar1 activity on each of these peptides using LC-MS (Fig. 3B and C). Bar1 cleaved both *Candida dubliniensis* and *Candida tropicalis* α pheromones, and the former, which differs by only two residues from *C. albicans* α pheromone, was cleaved with similar efficiency to the native substrate. As with *C. albicans* α pheromone, both *C. dubliniensis* and *C. tropicalis* α pheromones were cleaved between residues 5 and 6 (Thr-Asn and Thr-Arg, respectively).

To provide a more sensitive and quantitative analysis of Bar1 activity, IQ peptides were compared representing *C. albicans*, *C. tropicalis*, and *C. parapsilosis* α pheromones. Bar1 cleaved each of the three IQ peptides, although the cleavage rates of *C. tropicalis* and *C. parapsilosis* α pheromones were significantly lower (by ~ 10 -fold) than that of the *C. albicans* α pheromone (Fig. 3C). Together, these results indicate that Bar1 can cleave pheromones besides *C. albicans* α pheromone, although the endogenous pheromone is, by far, the preferred substrate.

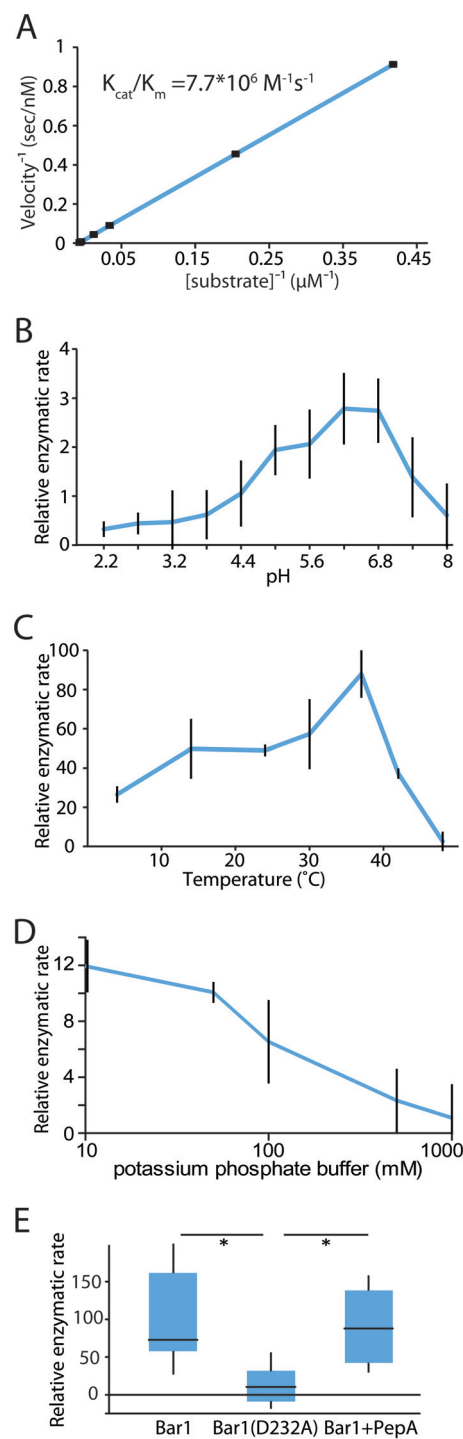


FIG 2 Characterization of *C. albicans* Bar1 protease activity. Bar1 activity was determined using an internally quenched peptide corresponding to the native α pheromone sequence of *C. albicans*, GFRLTNFGYFEPG. (A) Activity was plotted using a Lineweaver-Burk plot to determine enzyme kinetics. (B) pH dependence, (C) temperature dependence, and (D) osmolarity dependence of Bar1 activity. Relative enzymatic rate is the amount of fluorescence per unit of time. The default conditions were 37°C at pH 6.5. Data show means $\pm$ SD. (E) Enzymatic activities of Bar1, Bar1(D232A), or Bar1 together with 100 μ M pepstatin A were determined by incubation with the internally quenched *C. albicans* α pheromone peptide substrate for 90 min at 37°C at pH 6.5. A Tukey box plot is shown. $n \geq 5$. *, $P < 0.05$ by Mann-Whitney U test.

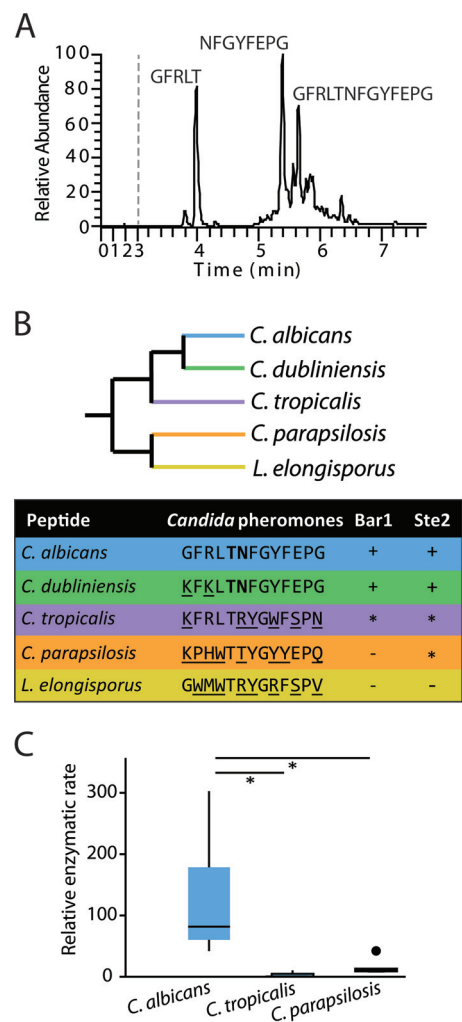


FIG 3 Analysis of *C. albicans* Bar1 protease activity on α pheromones from multiple *Candida* species. (A) Cleavage of *C. albicans* α pheromone by *C. albicans* Bar1 protease. Bar1 and α pheromone were coinubated for 1 h, and the products were analyzed by LC-MS. Mass spectrometry identified two major products (GFRLT and NFGYFEPG), as well as uncleaved pheromone (GFRLTNFGYFEPG). (B) An unrooted, phylogenetic tree of multiple *Candida* clade species is shown, as well as the activity of Bar1 on α pheromone peptides from each species. The detection of specific degradation products after 1 h of coinubation is indicated by “+” in the Bar1 column. “-” indicates no products were detected, while “*” indicates products were formed only after an extended (24-h) incubation. Boldface residues indicate amino acids adjacent to the cleavage site. The Ste2 column indicates whether the pheromone induced a robust response (+), a weak response (*), or no response (-) in *C. albicans* MTL α cells. Ste2 data are presented as adapted from Alby and Bennett (49) (see Fig. S2 in the supplemental material). (C) Bar1 activities were compared on *C. albicans*, *C. tropicalis*, and *C. parapsilosis* α pheromones using internally quenched (IQ) peptide substrates. Purified Bar1 was incubated with α pheromones for 90 min at 37°C. Relative enzymatic rate is the amount of fluorescence per unit of time due to cleavage of the fluorophore-conjugated peptide. Shown is a Tukey box plot with outliers noted by a large dot. $n \geq 5$. *, $P < 0.05$ by Mann-Whitney U test.

Analysis of the cleavage specificity of *C. albicans* Bar1. To further determine the substrate specificity of *C. albicans* Bar1, a series of pheromone-like peptides were incubated with recombinant Bar1, and the products were analyzed by LC-MS. The peptides included scanning di-alanine substitutions across the α

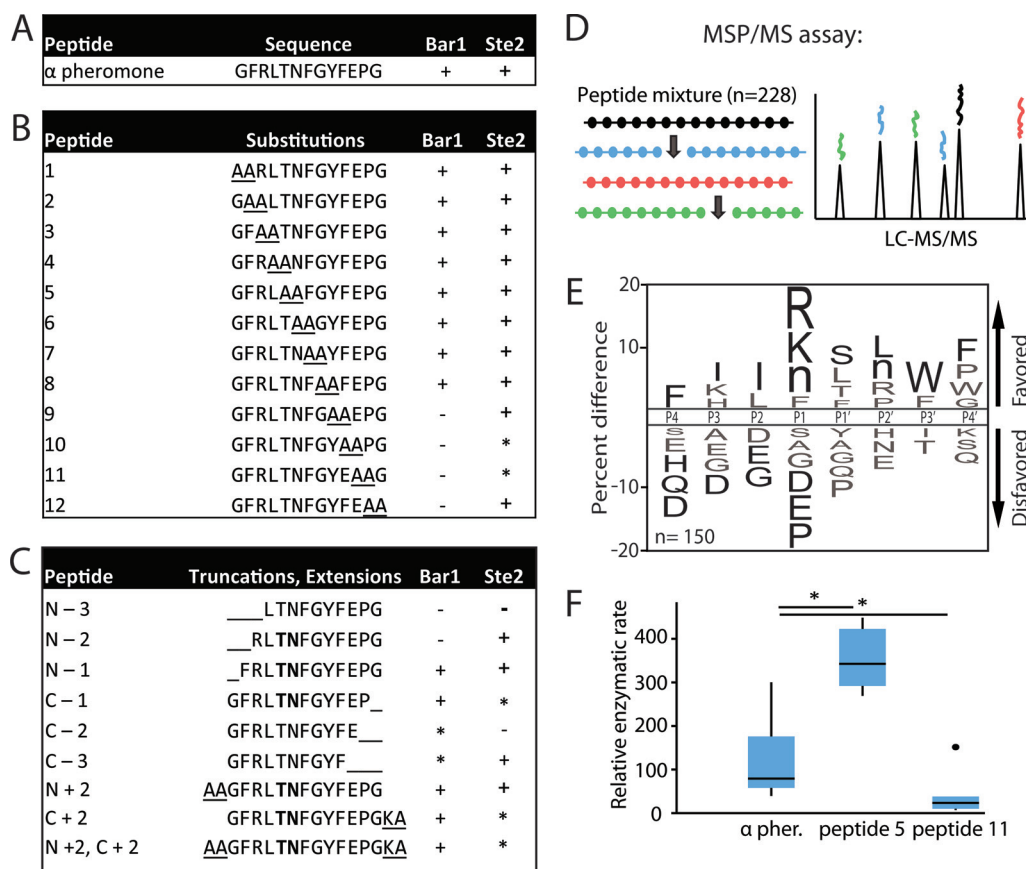


FIG 4 *C. albicans* Bar1 degradation of α pheromone analogs. Recombinant Bar1 was incubated for 1 h at 37°C with the indicated peptides, and the products were analyzed by mass spectrometry. (A) *C. albicans* α pheromone; (B) di-alanine substitutions within the *C. albicans* α pheromone sequence; (C) peptides corresponding to truncated or extended α pheromone. The detection of specific degradation products after 1 h of incubation is indicated by “+” in the Bar1 column. “-” indicates no products were detected, while “*” indicates products were formed only after extended (24-h) incubation. Boldface residues indicate amino acids flanking the cleavage site. The Ste2 column indicates whether the pheromone induced a robust response (+), a weak response (*), or no response (-) in *C. albicans* MTLA cells using published data in conjunction with data in Fig. S2 in the supplemental material (49). (D) Overview of the multiplex substrate profiling by mass spectrometry (MSP-MS) assay. *C. albicans* Bar1 was coinubated with 228 unique dodecapptides, and cleavage products identified using mass spectrometry. (E) MSP-MS data were used to generate an iceLogo identifying amino acids that were enriched or selected against in Bar1 cleavage sites. The percentage of difference is the difference in amino acid frequency surrounding the cleavage sites relative to the frequency of amino acids surrounding all peptide bonds in the library ($n = 2,964$). Residues above the midline are favored, while those below the midline are disfavored. Residues colored black significantly influence Bar1 activity ($P < 0.05$), whereas residues colored gray did not reach significance. Methionines were replaced with norleucines in the peptide library and are represented as “n.” (F) Enzymatic activity of Bar1 on substituted forms of α pheromone. Internally quenched peptides included α pheromone with alanine substitutions at positions P1 and P1’ or positions P6’ and P7’ corresponding to peptides 5 and 11 in panel B. The relative enzymatic rate is the amount of fluorescence per unit of time due to cleavage of the fluorophore-conjugated peptide. Rates were normalized to the Bar1(D232A) mutant control. Shown is a Tukey box plot with outliers noted by a large dot. $n \geq 5$. *, $P < 0.05$ by Mann-Whitney U test.

pheromone sequence (Fig. 4A and B), as well as N- and C-terminal truncations or extensions of the native sequence (Fig. 4C). Surprisingly, Bar1 cleaved most alanine-substituted peptides, even those with substitutions immediately flanking the scissile bond. However, substitution of several C-terminal residues (residues 10 to 13) effectively blocked cleavage (Fig. 4B). In each case, proteolysis occurred at the same position as in the native peptide (i.e., between the residues at positions 5 and 6). We found that Bar1 did not efficiently degrade peptides in which either the two most N-terminal or C-terminal residues were absent (see peptides N-2 and C-2 in Fig. 4C). In comparison, *S. cerevisiae* Bar1 shows similar length requirements, as this protease requires 4 amino acids or more on either side of the scissile bond for activity (39).

To generate an unbiased profile of protease substrate specificity, Bar1 was tested using a multiplex substrate profiling by mass

spectrometry (MSP-MS) approach (45). For this experiment, a physiochemically diverse library of 228 tetradecapeptides was incubated with Bar1 and the products were analyzed at multiple time points by LC-tandem MS (MS/MS) (Fig. 4D and E). Two amino acids positioned close to the scissile bond (usually within positions P4-P4’) are often sufficient for recognition and cleavage by most proteases, so the library of peptides was designed to contain all combinations of neighbor and near-neighbor amino acid pairs (45). Based on the set of cleavage sites identified in the peptide library, a consensus cleavage motif for Bar1 was generated using iceLogo (46), highlighting substrate specificity on either side of the scissile bond. Notably, the iceLogo motif for Bar1 (Fig. 4E) showed little similarity to the sequence of *C. albicans* α pheromone. The exception was at the P4 position, where a phenylalanine is present in α pheromone, and this amino acid was signifi-

cantly favored in the MSP-MS assay (Fig. 4E). Taken together, these results suggest that Bar1 recognition of α pheromone is largely independent of the amino acids present near the scissile bond. This contrasts with most other endoproteases, where the majority of substrate selectivity originates from residues at, or very close to, the scissile bond (40, 45, 47, 48).

We further explored the specificity of *C. albicans* Bar1 using IQ versions of peptides 5 and 11 (Fig. 4B). These peptides have substituted the amino acid residues immediately flanking the scissile bond (P1 and P1' [peptide IQ-5]) or those within the C-terminal region (P6' and P7' [peptide IQ-11]) with alanine residues. Cleavage of peptide IQ-5 was found to be more efficient than that of native α pheromone, whereas cleavage of peptide IQ-11 was significantly lower than that of the natural substrate (Fig. 4F). These experiments further establish that amino acid residues at the C terminus of α pheromone are important for efficient cleavage, whereas those on either side of the scissile bond can both be substituted without negatively affecting Bar1 activity.

Pheromone signaling in *C. albicans* is mediated by α pheromone binding to the Ste2 receptor on the surface of **a** cells. We therefore compared the specificity of *C. albicans* Bar1 with that of Ste2 by examining the ability of different pheromones to activate a cellular response in *C. albicans* **a** cells (Fig. 4B) (49). Interestingly, Bar1 substrate specificity mirrored that of Ste2: both *C. albicans* activities were negatively affected by substitution of amino acid residues at the C terminus of α pheromone but not by residues in the middle of the peptide (see Fig. 4B and C). Furthermore, a significant correlation was found between the pheromone peptides that activate Ste2 and those that are efficiently cleaved by Bar1 ($P = 0.00061$, Pearson's $\rho = 0.51$, $n = 27$), suggesting that Bar1 and Ste2 are dependent on the same residues of α pheromone for activity.

Contribution of Bar1 to pheromone signaling in *C. albicans*. *C. albicans* **a** and α cells can exist in two alternative phenotypic states: white and opaque. Mating involves pheromone signaling between cells in the opaque (mating-competent) state, but white cells also respond to pheromone by becoming adhesive and forming biofilms (Fig. 5A) (24–26). In *C. albicans* **a** cells, signaling often occurs in response to α pheromone (encoded by the *MF α* gene) produced by opaque α cells, but it can also be activated by an autocrine loop resulting from α pheromone secreted by opaque **a** cells (35).

To determine the function of *C. albicans* Bar1 in both white and opaque cells, we first examined Bar1 levels secreted by cells in the two phenotypic states. A custom antibody was raised against *C. albicans* Bar1, and Western blots were performed on conditioned media from cultures of white and opaque **a** cells. Unstimulated cells of both types did not produce Bar1, whereas opaque cells secreted detectable levels of Bar1 when stimulated with α pheromone for 5 h (Fig. 5B). These data are consistent with RNA profiling experiments, as *BAR1* is highly induced (>200-fold) upon pheromone challenge in opaque cells (50).

Next, to test the contributions of Bar1 and autocrine pheromone signaling to cellular phenotypes, we constructed *bar1 Δ /bar1 Δ* and *bar1 Δ /bar1 Δ mfa Δ /mfa Δ* mutants in *C. albicans* **a** cells. Pheromone signaling in opaque cells was detected using the PID assay described previously, whereas white-cell responses were quantified by measuring pheromone-induced cell adherence to a plastic substrate (24, 25). Deletion of the *BAR1* gene resulted in heightened sensitivity of opaque cells to exogenous α pheromone,

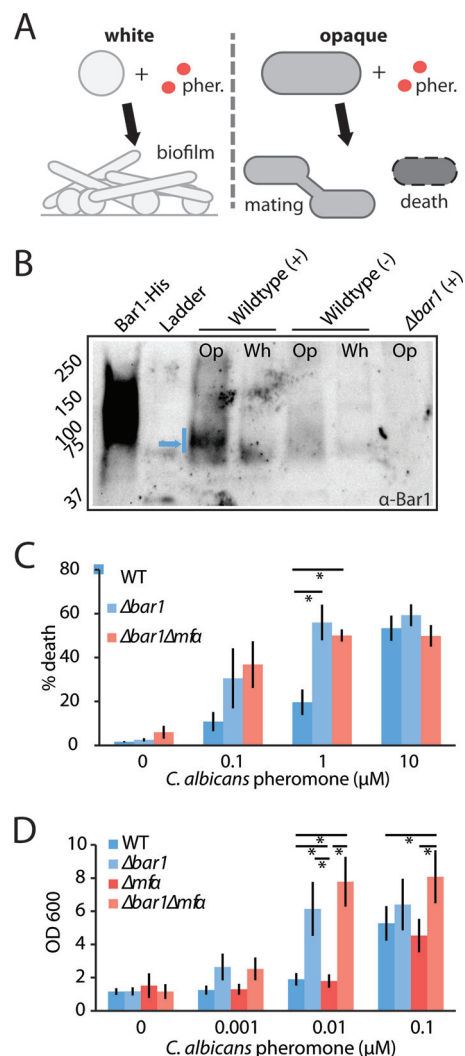


FIG 5 Analysis of the role of *C. albicans* *BAR1* on the pheromone response in white and opaque *MTLa* cells. *C. albicans* wild-type, *bar1 Δ /bar1 Δ* , and *bar1 Δ /bar1 Δ mfa Δ /mfa Δ* *MTLa* strains were compared for their responses to α pheromone. (A) Schematic indicating pheromone (pher.) responses of *C. albicans* white and opaque **a** cells. White cells become adherent and form biofilms, whereas a fraction of opaque cells experience cell death. (B) Secretion of Bar1 by white (Wh) or opaque (Op) *MTLa* cells. Cells were exposed to a vehicle control (–) or 0.3 μ M α pheromone (+) for 5 h, and Bar1 protein was detected from the supernatant via Western blotting using an anti-Bar1 antibody. Recombinant Bar1-His protein is used as a positive control, and *bar1 Δ /bar1 Δ* opaque cells are used as a negative control. Blue arrow, dominant Bar1 band. (C) Evaluation of gene function in opaque *MTLa* cells. A pheromone-induced death (PID) assay was used to assess the response of opaque **a** cells to α pheromone. Cells were treated with the indicated amount of α pheromone for 5 h and stained with propidium iodide, and the percentage of death was determined by flow cytometry. Error bars indicate standard errors (SE). (D) Evaluation of gene function in white *MTLa* cells. A biofilm assay was used to assess the response of white cells to α pheromone. Cells were incubated with α pheromone for 2 days, and adherent cells were quantified using absorbance at 600 nm. Error bars indicate SD. *, $P < 0.05$ by *t* test.

in agreement with previous studies (30). Thus, a large proportion of *bar1 Δ /bar1 Δ* cells underwent cell death at pheromone concentrations as low as 0.1 μ M, while wild-type cells required >1 μ M of pheromone to elicit a similar level of cell death (Fig. 5C). Deletion of the *MF α* gene (encoding α pheromone) prevents autocrine

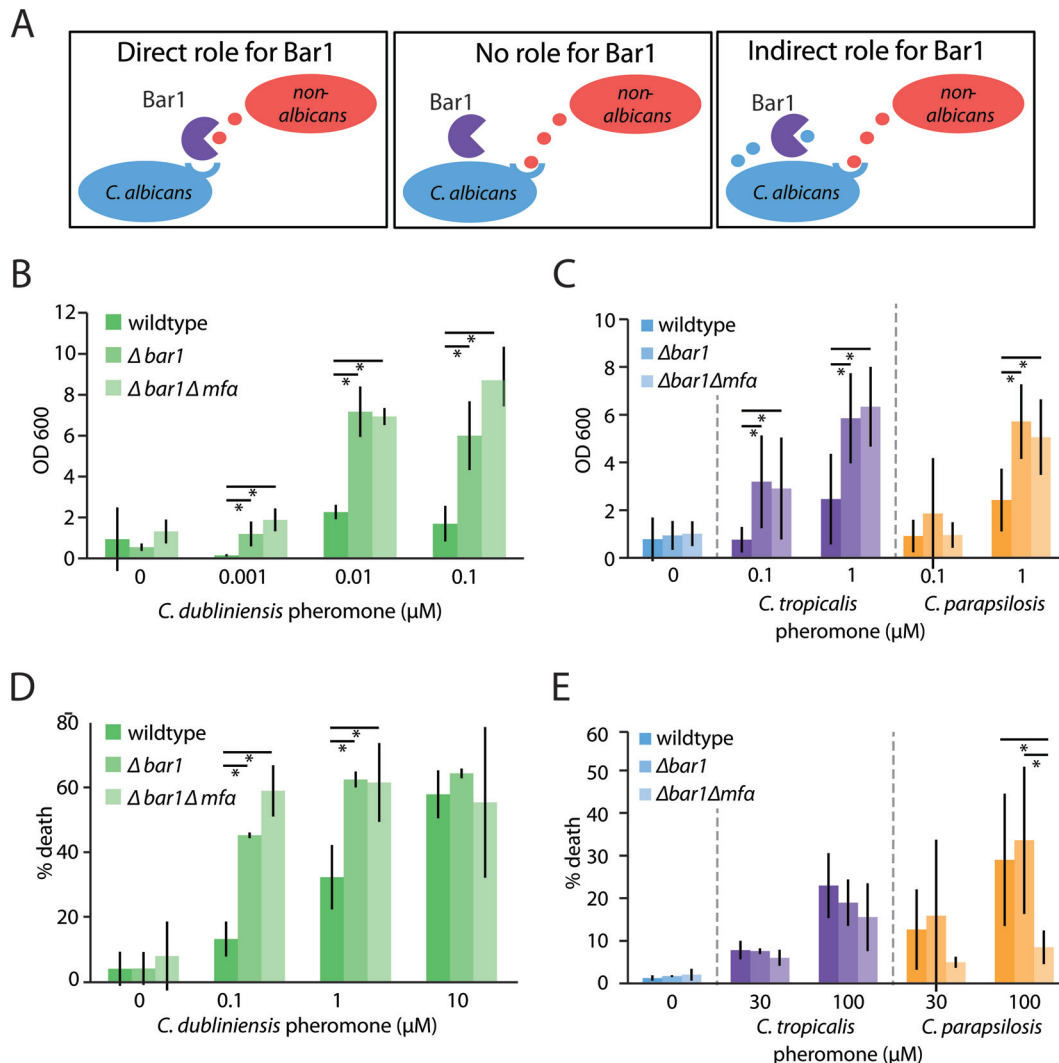


FIG 6 Role of *C. albicans* BARI in interspecies pheromone signaling. (A) Three alternative models for how *C. albicans* Bar1 may affect interspecies signaling between *Candida* species. (i) Bar1 may affect signaling through direct recognition and degradation of nonnative pheromones, (ii) it may have no effect on interspecies signaling events, or (iii) it may play an indirect role in signaling due to degradation of *C. albicans* α pheromone (and blocking of autocrine signaling) following receptor activation with a nonnative pheromone. *C. albicans* wild-type, *bar1*Δ/*bar1*Δ, and *bar1*Δ/*bar1*Δ *mfa*Δ/*mfa*Δ MTLα white cells were incubated with *C. dubliniensis* (B), *C. tropicalis* (C), or *C. parapsilosis* (D) α pheromones in a biofilm assay. After 2 days, adherent cells were quantified by spectrometry at 600 nm. Mean ± SD. *, $P < 0.05$ by *t* test. (E) *C. albicans* wild-type, *bar1*Δ/*bar1*Δ, and *bar1*Δ/*bar1*Δ *mfa*Δ/*mfa*Δ MTLα opaque cells were treated with *C. tropicalis* or *C. parapsilosis* α pheromones. The pheromone response was quantified by determining the percentage of cell death after 5 h of pheromone exposure by flow cytometry. Mean ± SD. *, $P < 0.05$.

signaling, and yet *bar1*Δ/*bar1*Δ *mfa*Δ/*mfa*Δ cells showed similar levels of PID to *bar1*Δ/*bar1*Δ mutants (Fig. 5C). This result indicates that endogenous α pheromone production and autocrine signaling have a negligible effect on the pheromone response under these conditions. Similar results were obtained with white cells; loss of *BARI* increased the sensitivity of white cells to exogenous α pheromone, and deletion of the *MFA* gene, either singly or in combination with *BARI*, did not show a significant effect on pheromone-induced adherence (Fig. 5D).

Bar1 regulation of interspecies signaling in *C. albicans*. *C. albicans* a cells have been shown to respond to α pheromones from related *Candida* species, with opaque cells induced to undergo same-sex mating, whereas white cells exhibit increased biofilm formation (49). These results led us to ask two questions. First, can

Bar1 regulate interspecies signaling by degradation of α pheromones secreted by other species? Second, does secretion of endogenous α pheromone from *C. albicans* a cells enhance interspecies signaling by activating autocrine signaling? There are three scenarios for how pheromone signaling could be influenced by *BARI* and *MFA* in *C. albicans* a cells. (i) Signaling between species is unaffected by Bar1 or endogenous α pheromone produced by a cells. (ii) Bar1 degrades non-*albicans* pheromones, thereby directly restricting interspecies pheromone signaling. (iii) Bar1 does not influence interspecies signaling directly but restricts it by degrading endogenous α pheromone produced by a cells, thereby reducing autocrine signaling (Fig. 6A).

Deletion of the *BARI* gene in *C. albicans* white a cells led to a heightened response to *C. dubliniensis*, *C. tropicalis*, and *C. parap-*

silosis α pheromones (Fig. 6B and C). This is consistent with the biochemical activity of Bar1, which was capable of degrading each of these three pheromones *in vitro* (Fig. 3B and C). Sensitivity to *C. dubliniensis* pheromone was considerably higher than that to *C. tropicalis* and *C. parapsilosis* pheromones, consistent with the close sequence homology of *C. albicans* and *C. dubliniensis* pheromones (Fig. 3B). In contrast to *BAR1*, deletion of the *MF α* gene did not affect the response to the three non-*albicans* pheromones (Fig. 6B and C). This indicates that Bar1 directly affects the response to these pheromones, similar to the result with *C. albicans* α pheromone (compare Fig. 5C with 6B and C).

Analogous experiments were performed using *C. albicans* opaque **a** cells to determine the contributions of *BAR1* and *MF α* to interspecies signaling in this cell type (Fig. 6D and E). *C. dubliniensis* α pheromone produced a similar response to the native *C. albicans* α pheromone in opaque cells, and deletion of *MF α* did not affect the outcome (Fig. 6D). In contrast, deletion of both *MF α* and *BAR1* genes depressed the response of *C. albicans* opaque **a** cells to *C. parapsilosis* α pheromone, indicating that autocrine signaling significantly affects the response to this pheromone (Fig. 6E). Taken together, these results recapitulate our biochemical findings that Bar1 acts most effectively on *C. albicans* α pheromone but also has the capacity to degrade pheromones from other *Candida* species, thereby limiting interspecies signaling.

DISCUSSION

Mating in fungi is choreographed by pheromone signaling between cell partners. In this work, we addressed the biochemical properties and function of *C. albicans* Bar1, an aspartyl protease secreted by **a** cells that degrades α pheromone. We show that Bar1 cleaves endogenous α pheromone between threonine 5 and asparagine 6, thereby inactivating the 13-amino-acid peptide. This protease exhibits several features common to secreted aspartyl proteases, including extensive glycosylation and two conserved Asp-(Thr/Ser)-Gly motifs (39). Mutation of one of the putative active site aspartyl residues, Asp-232, was sufficient to block Bar1 activity. However, Bar1 also displays unusual properties for an aspartyl protease, including a near-neutral pH optimum and resistance to pepstatin A, a potent aspartyl protease antagonist. In addition to Bar1, *C. albicans* contains a set of 10 SAP (secreted aspartyl protease) genes, several of which are implicated in pathogenesis (38, 51–53). Most *C. albicans* Sap proteases exhibit an acidic pH optimum characteristic of other aspartyl proteases (54, 55), although Sap9 and Sap10 exhibit optimal activity between pH 6 and 7 (56). In addition, the majority of *C. albicans* Saps are inhibited by pepstatin A, with Sap7 being the lone exception (54, 56–58). Notably, *S. cerevisiae* Bar1 is also an unusual aspartyl protease in that this enzyme displays a near-neutral pH optimum and is not inhibited by pepstatin A (28, 29, 38, 39). It therefore appears that *S. cerevisiae* Bar1 (ScBar1) and *C. albicans* Bar1 (CaBar1) (as well as certain Sap proteins) share unusual traits for aspartyl proteases, and this is despite the extensive evolutionary divergence between *S. cerevisiae* and *C. albicans* (14).

A comparative analysis of *C. albicans* Bar1 cleavage of α pheromones from multiple *Candida* species revealed that Bar1 preferentially cleaved *C. albicans* α pheromone, whereas α pheromones from closely related species (including *C. tropicalis* and *C. parapsilosis*) were also cleaved, albeit at a much reduced rate. Bar1 did not cleave pheromones from more evolutionarily distant fungal species, and unlike most *C. albicans* Sap proteases (58), showed no

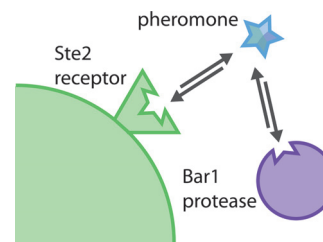


FIG 7 Model of interactions between Bar1, Ste2, and α pheromone. Both Bar1 and Ste2 recognize the same or overlapping regions of α pheromone, thereby facilitating coevolution of their substrate specificities together with pheromone divergence during speciation.

detectable activity on bovine serum albumin even when tested at high protein concentrations (data not shown). Thus, *C. albicans* Bar1 has evolved high substrate specificity toward the endogenous α pheromone. Studies on ScBar1 also suggest a species-specific function, as *S. cerevisiae* cells were unable to cleave *C. albicans* α pheromone (59). Together, these experiments establish that Bar1 has evolved selectivity toward its native pheromone in diverse fungal lineages.

An unexpected feature of *C. albicans* Bar1 cleavage activity was its dependence on amino acid residues located distal to the scissile bond. The specificity of most endoproteases is guided by amino acids located at, or very close to, the scissile bond (45, 48). In contrast, Bar1 cleaved α pheromone by recognition of amino acid residues at the C terminus of the peptide. Thus, alanine substitutions at positions 10 to 13 of α pheromone (P4' to P7') inhibited cleavage, whereas substitutions at the scissile bond did not (Fig. 4B and F). The unusual specificity of Bar1 was further supported by an unbiased analysis of activity using MSP-MS, which identifies amino acids close to the scissile bond that are enriched or de-enriched in substrates (45). The amino acids favored on either side of the scissile bond by MSP-MS analysis had little similarity to those present in *C. albicans* α pheromone. Together, these studies demonstrate that the cleavage specificity of CaBar1 for α pheromone is mediated by residues distal to the scissile bond rather than amino acids close to the site of cleavage.

Why might *C. albicans* Bar1 have evolved specificity for residues away from the scissile bond? We show that the specificity of CaBar1 exhibits a striking similarity to that of the *C. albicans* Ste2 receptor for α pheromone. A previous study revealed that substitution of residues at the C terminus of α pheromone (particularly residues 11 and 12) resulted in the largest reduction in pheromone signaling by *C. albicans* **a** cells (see the “Ste2” column in Fig. 4B and C) (49). Thus, both *C. albicans* Bar1 and Ste2 are critically dependent on the same C-terminal residues of α pheromone for activity. We therefore propose that there has been coevolution of these factors due to their dependence for the same peptide target. In particular, as receptor-pheromone interactions diverged between species, Bar1 specificity for α pheromone would have had to evolve in parallel to retain activity. By having Bar1 and Ste2 recognize the same residues in α pheromone, this would have facilitated coevolution of these factors together with their peptide target during speciation (Fig. 7). Studies have also addressed the interaction between *S. cerevisiae* Ste2 and α pheromone and have shown that ScSte2 makes multiple contacts with α pheromone, although residues near the C terminus of the pheromone (residues 10 to 13) play the most important role in receptor binding (60–

63). Thus, in both *C. albicans* and *S. cerevisiae*, the carboxyl-terminal residues of α pheromone are critical for receptor function. It will now be of interest to examine pheromone-receptor and pheromone-Bar1 interactions across multiple species to further address how these factors coevolved and if the C terminus of α pheromone has a conserved role in mediating interactions with both of its protein targets across diverse species.

To complement the biochemical analysis of *C. albicans* Bar1, we performed genetic experiments to define the role of this protease in regulating intra- and interspecies pheromone signaling. Loss of *BAR1* sensitized *C. albicans* white and opaque **a** cells to native α pheromone, indicating functional secretion of the Bar1 enzyme by both cell types. Deletion of the *BAR1* gene also sensitized *C. albicans* **a** cells to α pheromones from *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis*, indicating that Bar1 effectively inhibits signaling to pheromones produced by closely related species. This could be important in nature, as *C. albicans* is often foundocolonizing the oral cavity with multiple other *Candida* clade species (64).

We also addressed whether autocrine pheromone production contributes to interspecies signaling, as *C. albicans* **a** cells have been shown to secrete α pheromone in addition to the conventional **a** pheromone (35). To test this, the *MF α* gene (encoding α pheromone) was deleted in the presence or absence of *BAR1*, and **a** cells were challenged with α pheromones. In most cases, loss of *MF α* did not affect the response of *C. albicans* **a** cells to other species' pheromones. However, the response to *C. parapsilosis* α pheromone was reduced upon deletion of both *BAR1* and *MF α* genes, indicating that autocrine signaling enhanced interspecies signaling to this pheromone (Fig. 6E). These results are the first to demonstrate that Bar1 acts to restrict interspecies pheromone signaling and also show a role for endogenously produced pheromone in enhancing the response to a pheromone from a related species.

Finally, we note that the unusual specificity of *C. albicans* Bar1 makes it an attractive candidate for bioengineering a protease with specialized properties. There are a number of potential technical applications for a protease whose specificity is dependent on residues distal to the scissile bond, including the ability to completely remove affinity tags from purified proteins without leaving behind the cleavage site residues. In principle, this is analogous to restriction enzymes (e.g., type III restriction enzymes) that cleave the DNA at a short distance from their recognition sequence. In fact, enteropeptidase is a serine protease whose specificity determinants are located on the C-terminal side of the scissile bond, so that N-terminal tags can be removed, leaving the product with a native N terminus (48). *C. albicans* Bar1 could be engineered to perform a similar function for the complete removal of C-terminal tags from recombinant proteins. Bar1 can be highly overexpressed, shows strong substrate selectivity, and is active at neutral pH, all of which are properties that would favor its use in many applications. Further analysis of Bar1 homologs from *Saccharomycotina* species could also help identify the residues in Bar1 that are responsible for determining its substrate specificity, as Bar1 orthologs appear to be under strong selection to evolve in parallel with their pheromone substrates. Such experiments could shed light on the precise mechanism by which α pheromone is recognized and also allow further refinement of the proteolytic activity for important commercial applications.

MATERIALS AND METHODS

Media. All media were prepared using previously described methods (65, 66). Spider medium contained 1% nutrient broth, 0.4% potassium phosphate, and 2% mannitol (pH 7.2). Solid media were made with 1.35% agar.

Strain and plasmid construction. The strains used here are listed in Table S1 in the supplemental material. To overexpress *C. albicans* Bar1 protein, PCR was used to generate a DNA product containing the entire open reading frame (ORF) of *BAR1* from *C. albicans* genomic DNA. The primers used added a 6 $\times$ His (His₆) tag to the end of the ORF and restriction endonuclease cut sites. The PCR product was then cloned into the pPink α -HC plasmid under the *AOX1* promoter (Life Technologies) to yield the plasmid pBar1-His. Site-directed mutagenesis via the QuikChange II kit (Agilent Technologies) was used to mutate position +695 from adenine to cytosine to yield plasmid pBar1(D232A)-His, which contains amino acid substitution D232A in one of the two predicted active sites of the Bar1 protein. Both plasmids were transformed into *PichiaPink* strains to generate strains CAY794 and CAY800.

To construct the *C. albicans* *MF α* and *BAR1* deletion strains, plasmids pRB13 and pRB35, respectively, were used with the previously described *SAT1*-flipper method (67). Briefly, regions flanking the ORF of the gene of interest were PCR amplified together with oligonucleotides containing the *ApaI*/*XhoI* (5' flank) and *SacII*/*SacI* (3' flank) restriction sites. The products were cloned into plasmid pSFS2a. The resulting plasmids were digested with *ApaI* and *SacI* and transformed into *C. albicans*. Correct integration was confirmed by PCR, and the *SAT1* marker was excised by growth on maltose medium to induce the FLP recombinase (67). Transformations were then repeated to replace the second copy of the gene of interest. To fix cells in the opaque state, strains were transformed with plasmid pRB99 (*pACT1-WOR1*) to constitutively express *WOR1* (41).

***C. albicans* Bar1 protein expression and purification.** *P. pastoris* strains CAY794 and CAY800 were grown in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base [YNB], 0.0004% biotin, 1% glycerol) for 2 days at 28°C, and then cells were concentrated and switched to BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.0004% biotin, 0.5% methanol) to induce expression of Bar1 for 2 days at 28°C. The methanol concentration was maintained by further addition of methanol after 24 h. Cells were centrifuged, and the supernatant was collected, followed by 40-fold concentration using an Amicon cell (EMD Millipore, Darmstadt, Germany). The cell was also used to perform buffer exchange into Ni-nitrilotriacetic acid (NTA) binding buffer (300 mM NaCl, 50 mM NaH₂PO₄, 10 mM imidazole, pH 8.0). The resulting solution was batch bound to HisPur Ni-NTA resin (Thermo, Fisher Scientific, Waltham, MA) and then washed twice (300 mM NaCl, 50 mM NaH₂PO₄, 20 mM imidazole, pH 8.0), and the product was eluted (300 mM NaCl, 50 mM NaH₂PO₄, 500 mM imidazole, pH 8.0). The product was dialyzed into storage buffer (phosphate-buffered saline [PBS], 20% glycerol). The protein concentration was determined using a Bradford protein assay (Thermo Scientific).

Analysis of peptide cleavage by liquid chromatography-mass spectrometry. Lyophilized peptides (Lifetein LLC) were dissolved in 10% dimethyl sulfoxide (DMSO), mixed with 127 nM *C. albicans* Bar1 or Bar1(D232A) protein in 50 μ M potassium phosphate solution (pH 6.5) for 1 h at 37°C, and then passed through a centrifugal filtration unit to separate peptide fragments from the peptidase. Samples were analyzed using a Shimadzu high-performance liquid chromatograph and Thermo LCQ Deca XP Max ion trap mass spectrometer system.

MSP-MS. MSP-MS assays were carried out as described previously (45). Briefly, ~60 nM Bar1, Bar1(D232A), or matched no-enzyme control were assayed against a diverse library of 228 tetradecapeptides pooled at 500 nM in 10 mM KH₂PO₄ (pH 6.5) and incubated at 37°C. After 60, 240, and 1,200 min, 30 μ l of assay mixture was removed, quenched with 7.5 μ l 2% formic acid, and frozen. Prior to mass spectrometry acquisition, peptide samples were desalted using Millipore C₁₈ ZipTips and rehydrated in

0.2% formic acid. LC-MS/MS data were acquired using a Thermo Scientific LTQ-Orbitrap mass spectrometer, which was equipped with a Thermo Scientific EASY-Spray ion source, an EASY-Spray PepMap C₁₈ column (3 μ M, 100 Å), and a Waters nanoACQUITY ultraperformance liquid chromatography (UPLC) system. Mass spectrometry peak lists were generated using PAVA, and data were searched against the 228-member peptide library using Protein Prospector software v.5.12.4 (UCSF). Protein Prospector score thresholds were selected with a minimum protein score of 15 and minimum peptide score of 10. Maximum expectation values of 0.01 and 0.05 were used for protein and peptide matches, respectively. Peptides corresponding to cleavage products were imported into iceLogo to generate substrate specificity profiles as described previously (45). Octapeptides corresponding to P4-P4' were used as the positive data set, and octapeptides corresponding to all possible cleavages in the MSP-MS library ($n = 2,964$) were used as the negative data set.

Protein characterization. To determine Bar1 levels in culture, *C. albicans* strains were grown at 22°C overnight in synthetic complete dextrose (SCD) medium, and cells were washed. Next, 2×10^8 cells were grown for 5 h in 10 ml SCD medium with or without 0.3 μ M synthetic α pheromone. The supernatant and cells were separated by centrifugation, and 40 μ l of each was prepared for SDS-PAGE in Laemmli sample buffer. Samples were analyzed by SDS-PAGE and Western blotting. For anti-His blots, a 1:5,000 dilution of Sigma's horseradish peroxidase (HRP)-conjugated monoclonal anti-hexahistidine (anti-His₆) antibody (A7058) was used according to manufacturer's directions. For anti-Bar1 blots, a 1:1,000 dilution of sera containing a polyclonal anti-Bar1 antibody designed by New England Peptides (project 2095, raised against epitopes Ac-YFINETIRSNDWKC-amide and Ac-CSDDKITSVTNPQ-amide) was used, followed by a 1:10,000 dilution of goat anti-rabbit Ig-HRP (Jackson ImmunoResearch). Proteins were visualized using a chemiluminescent substrate (Pierce) and a Chemidoc XRS+ system (Bio-Rad).

For assessment of glycosylation of *C. albicans* Bar1, a protein deglycosylation assay was performed on the purified Bar1 protein according to the manufacturer's directions (New England Biolabs P6039S), and followed by SDS-PAGE and Western blotting as described above. Assessment of the signal peptide sequence of Bar1 was completed using SignalP-4.1 prediction (<http://www.cbs.dtu.dk/services/SignalP/>).

IQ peptide assays. IQ peptides were produced by LifeTein, LLC (Hillsborough, NJ) with the dabyl at the N terminus and EDANS at the C terminus. Unless otherwise noted, all experiments were done at 37°C in 50 μ M potassium phosphate buffer, pH 6.5, with a substrate concentration of 50 μ M and 60 nM recombinant Bar1 protease or Bar1(D232A) mutant protease. The protease and substrate were combined in a 96-well plate immediately before beginning the experiment, and then fluorescence emission was measured over 90 min using a BioTek Synergy HT plate reader (Winooski, VT). For experiments containing inhibitors, the inhibitor was added to the protease before being combined with the substrate. Each experimental replicate was an average of three technical replicates. Values were normalized to the Bar1(D232A) mutant control when noted. Rates were determined over the period where substrate was in excess of the enzyme.

Pheromone-induced cell death. *C. albicans* opaque a cells derived from strain AM2003 (41) were grown in SCD medium overnight at 22°C in a rotating drum. Cultures were washed with water and then resuspended in Spider medium at 2×10^7 cells/ml, and α pheromone was added at the indicated concentration. After 5 h of incubation at 22°C in a rotating drum, 500 μ l of culture was removed and washed with water. Then the cells were resuspended in SCD medium containing 1 μ g/ml propidium iodide for 15 min. Cells were washed again, resuspended in SCD medium, and analyzed by flow cytometry on a FACSCalibur cell sorter (BD Biosciences). At least 10,000 events were analyzed for fluorescence in channel FL3. For halo assays, 10^5 opaque cells were plated onto Spider medium. Twenty micrograms of α pheromone was spotted onto

the center of the plate, and the plates were incubated at 22°C for 3 days, after which images were acquired.

Pheromone-induced adherence assay. *C. albicans* white a cells derived from strain P37005 (68) were grown overnight in Spider medium at 22°C on a rotating drum. Cells were washed with water and then administered to each well of a 12-well plate containing 1 ml of Lee's plus glucose medium at a concentration of 4×10^7 cells/ml. Pheromones were added at the indicated concentration, and then plates were incubated at 22°C for 2 days with no shaking. Each well was washed gently 3 times with water to remove nonadherent cells. Adherent cells were scraped and resuspended in 1 ml of water, and the optical density of each sample was determined at 600 nm on a Nanodrop 2000c (Thermo Scientific).

Statistical analysis. Statistics were analyzed using the PAST software package (<http://folk.uio.no/ohammer/past/>). Using the Anderson-Darling method, data sets were tested for normality, and parametric tests were used when appropriate and possible. Tests between samples were performed using the Student's *t* test, Tukey's test, or Mann-Whitney test, as indicated in the figure legends. Asterisks indicate a *P* value of 0.05 or less, unless otherwise noted. In order to correlate Bar1 cleavage of the tested peptides and activation of Ste2 signaling by the same peptides, the efficiency of Bar1 cleavage was first binned into three groups (products detected by HPLC-MS after 1 h of incubation with Bar1 or after 24 h of incubation, or products were not detected even after 24 h of incubation). Next, activation of Ste2 signaling was compared using data from published pheromone-induced biofilm assays (49) and binned into three groups (similar or better response than with the wild-type α pheromone, a lower but detectable response, or no response). The binned data were then correlated using Spearman's rho.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01604-15/-/DCSupplemental>.

Figure S1, PDF file, 0.1 MB.

Figure S2, PDF file, 0.02 MB.

Table S1, XLS file, 0.1 MB.

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