

Interspecies pheromone signaling promotes biofilm formation and same-sex mating in *Candida albicans*

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Edited by Jasper Rine, University of California, Berkeley, CA, and approved January 3, 2011 (received for review November 17, 2010)

The opportunistic pathogen *Candida albicans* undergoes a parasexual mating cycle in which cells must switch from the conventional “white” form to the alternative “opaque” form to become mating competent. Pheromones secreted by opaque cells induce the formation of polarized mating projections and result in cell–cell conjugation. In contrast, white cells are unable to undergo mating, but can still respond to pheromone by expression of adhesion genes that promote biofilm formation. In this study, we have analyzed the dual ability of pheromones to activate mating by opaque cells and biofilm formation by white cells. We first show that there is considerable plasticity in interactions between the α pheromone and its receptor, Ste2, by analysis of analogs of the α pheromone. Significantly, substituted forms of α pheromone can induce a response in opaque cells and this is sufficient to drive same-sex a-a cell fusion and homothallic mating. In addition, pheromone analogs were able to induce adhesion and biofilm formation in white cells of *C. albicans*. Because of the observed plasticity in pheromone signaling, we subsequently tested putative pheromones from multiple *Candida* species and identified nonnative ligands that can induce self-mating and biofilm responses in *C. albicans*. Our findings demonstrate that environmental signals can initiate *C. albicans* parasexual reproduction and biofilm formation, and highlight the role of the pheromone-signaling apparatus in mediating these functions.

microbiota | pathogenesis | phenotypic switch

Many human pathogens have predominantly clonal lifestyles yet still retain the ability to undergo sexual or parasexual reproduction (1). In the case of the fungal pathogen *Candida albicans*, this species was long thought to be an obligate asexual organism, but a mating cycle has been established that shows fundamental differences from that of the related hemiascomycete, *Saccharomyces cerevisiae* (for reviews, see refs. 2–4). *C. albicans* mating occurs between a and α diploids and is dependent on a unique phenotypic switch; cells must convert from the more common white form to the alternative opaque form to undergo efficient cell conjugation (5–8). The switch between states is a stochastic, heritable event that occurs approximately once every 10,000 cell generations. White and opaque cells exhibit differential expression of more than 400 genes, with opaque cells showing higher expression of several genes that are directly implicated in mating (9, 10).

C. albicans mating is driven by sex-specific pheromones that are expressed by the two cell types; α cells secrete α pheromone and respond to a pheromone via the Ste3 receptor; a cells produce a pheromone and respond to α pheromone via the Ste2 receptor (11–13). Pheromone binding activates a conserved MAPK cascade, leading to the formation of mating projections and subsequent cell–cell conjugation (14, 15). Although only opaque cells exhibit the mating response to pheromone, white cells can also respond by undergoing increased cohesion and biofilm formation (16). Recent studies have shown that both opaque and white cells use the same conserved MAPK cascade for pheromone signaling, but whereas signaling activates the Cph1/Ste12 transcription factor in opaque cells, the Tec1 transcription factor is activated in white cells (17, 18). Curiously, pheromone signaling by white a cells also requires a specific region in the Ste2 receptor (in-

tracellular region 1) that is not necessary for signal transduction in opaque cells (19).

The α pheromone secreted by *C. albicans* cells is a 13-amino acid peptide that is produced by proteolytic processing of the *MF α* gene product (11, 13). In both *S. cerevisiae* and *C. albicans*, mature α pheromone is targeted by the Bar1 protease. This aspartyl protease is secreted by a cells and inactivates the α pheromone, thereby enabling nonmating cells to overcome pheromone-induced cell-cycle arrest (20–22). Bar1 activity may also sharpen the gradient of α pheromone, promoting accurate partner recognition (23). Recent studies in *C. albicans* have established that Bar1 also regulates a novel program of homothallic mating in this yeast. It is now apparent that opaque a cells secrete both a and α pheromones; inhibition of self-mating requires that Bar1 degrade α pheromone to prevent autocrine activation of the mating response (24). In the absence of Bar1, self-fertilization of opaque a cells can occur, leading to the formation of same-sex tetraploid a-a cells. Same-sex mating has also been described in another prominent human fungal pathogen, *Cryptococcus neoformans* (25–28). It therefore appears that flexibility in sexual programs can have significant advantages for microbial pathogens (1).

In this work, we show that the *C. albicans* pheromone/receptor interaction exhibits considerable plasticity, and that multiple pheromone analogs are able to activate the signaling machinery. We also establish that pheromone signaling alone is sufficient to induce same-sex mating between opaque cells and the productive formation of tetraploid cells. White cells of *C. albicans* respond to pheromone analogs by undergoing increased cell–cell cohesion and biofilm formation. Moreover, pheromones from related *Candida* pathogens can induce both same-sex mating and biofilm formation in opaque and white cells of *C. albicans*, respectively. We therefore discuss the potential roles for interspecies signaling in driving adaptive events in *C. albicans* biology.

Results

Analysis of Pheromone Analogs on *C. albicans* Mating. *C. albicans* α pheromone is a 13-amino acid peptide (GFRLTINFGYFEPG) that is sensed by its cognate receptor, Ste2, on *MTLa* cells (11, 13). To determine the specificity of the α pheromone for mating signaling, we analyzed the activity of a series of di-alanine-substituted pheromone analogs (Fig. 1A) for their ability to induce a mating response in opaque cells of *C. albicans*. As shown in Fig. 1B, each of the 12 di-alanine-substituted analogs were still able to induce a morphological response in opaque a cells, as evidenced by the production of wrinkled colonies because of filament-like mating projections (Fig. 1C and Fig. S1) (24). Each of these responses was strictly dependent on the Ste2 receptor for the α pheromone, as

Author contributions: K.A. and R.J.B. designed research; K.A. performed research; K.A. and R.J.B. analyzed data; and K.A. and R.J.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1017234108/-DCSupplemental.

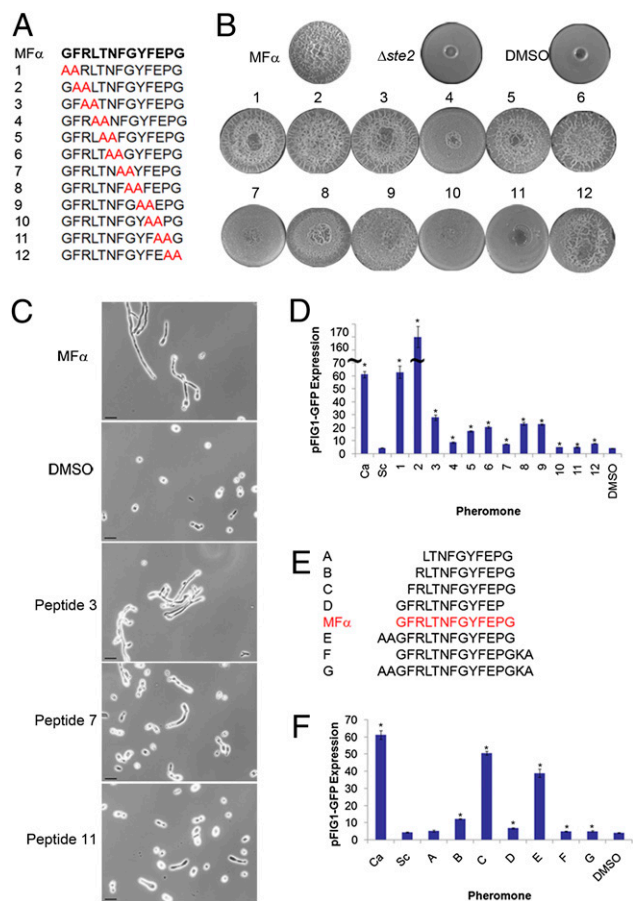


Fig. 1. Substituted pheromone peptides retain activity in *C. albicans*. (A) Sequences of di-alanine derivatives of the 13 amino acid *C. albicans* α pheromone. (B) Substituted pheromones induce a morphological change (wrinkled colonies) because of induction of mating. Colony images of opaque *C. albicans* a cells exposed to 25 μ g of the indicated pheromone daily for 3 d. (Holes in colonies indicate where pheromone was added.) (C) Cells exposed to pheromones form filament-like mating projections. Images of cells taken from the colonies shown in B. (Scale bar, 16 μ m.) (D) Quantification of mating gene expression induced by pheromone analogs using a pFIG1-GFP reporter strain (DSY700). Opaque a cells were incubated with 10 μ g/mL of peptide in Spider medium for 24 h, then analyzed for GFP fluorescence by flow cytometry. Values are mean fluorescence of the entire cell population (arbitrary units). (E) Sequences of α pheromone analogs of different lengths. (F) Quantification of mating-gene expression induced by pheromone analogs A to G. Cells treated with *C. albicans* native α pheromone (Ca), cells treated with *S. cerevisiae* α pheromone (Sc). Error bars represent SEM from a minimum of three replicate experiments. * $P < 0.05$ vs. DMSO control.

addition of synthetic peptides to Δ ste2 cells resulted in no change in phenotype (Fig. 1B).

Although all 12 di-alanine analogs induced the mating pathway, there were clear differences in the efficiency of the response by opaque a cells (Fig. 1B). To quantify the effectiveness of pheromone analogs at inducing mating, a GFP reporter under the control of the *FIG1* promoter was used (24) (Fig. 1D). Addition of the *C. albicans* α pheromone (10 μ g/mL) to opaque a cells induced *FIG1* expression, whereas addition of the *S. cerevisiae* α pheromone (WHLQLKPGQPMY) did not. Each of the di-alanine-substituted pheromone analogs also induced significant expression of the *FIG1* reporter over the DMSO control, although peptides 10 and 11, with substitutions at amino acids 10 to 12, were the weakest inducers of the mating response (Fig. 1D). Notably, peptide 2 induced stronger *FIG1* expression than the native *C. albicans* pheromone. A similar phenomenon

was previously described in *S. cerevisiae*, where several single alanine-substituted analogs showed increased bioactivity by more than threefold (29).

To also investigate the effect of peptide length on pheromone signaling, we used a series of peptide analogs ranging from 10 to 17 amino acids (Fig. 1E). Removal of three amino acids from the N terminus of α pheromone (peptide A) abolished *FIG1* expression, but removal of one or two amino acids from the N terminus (peptides B and C) reduced *FIG1* expression but still retained significant activity (Fig. 1F). Furthermore, the addition of two amino acids to the N terminus (peptide E) only reduced *FIG1* expression slightly. These findings support the observations with alanine-substituted pheromones that manipulation of the N terminus of the peptide has only a modest effect on mating signaling. In contrast, manipulation of the C terminus, either by deletion of one amino acid (peptide D) or by addition of two amino acids (peptides F and G) resulted in a marked reduction in mating signaling (Fig. 1F). These results indicate that there is considerable plasticity in the pheromone-receptor interaction in *C. albicans* and that the organism can respond to a variety of pheromone analogs.

Pheromone Signaling Drives Homothallic a-a Mating. One intriguing feature of *C. albicans* mating is the ability of cells to undergo primary homothallicism via same-sex mating. Opaque a cells lacking the Bar1 protease secrete α pheromone, and this initiates an autocrine feedback loop resulting in same-sex a-a mating (24, 30). To test if pheromone is sufficient to drive the homothallic program of mating, we cocultured two auxotrophic a strains in the presence of varying concentrations of synthetic pheromone, and potential a-a mating products were detected by plating onto selective media. In the absence of synthetic pheromone, no mating products were obtained. However, in the presence of the *C. albicans* α pheromone, mating products were detected after 5 d culture on Spider medium (Fig. 2). Mating products were analyzed by cell cytometry to confirm that they were tetraploid and by PCR to confirm their *MTLa* genotype (Fig. 2A and B). These results establish the ability of α pheromone to induce homothallic mating of opaque a cells. The demonstration of wild-type a cells mating in the absence of α partners confirms that activation of pheromone signaling is sufficient to drive self-fertilization in *C. albicans*.

Comparison of mating efficiencies in the presence of varying concentrations of native pheromone and pheromone analogs was revealing. First, all 12 di-alanine-substituted analogs were still able to induce homothallic mating, albeit to varying degrees (Fig. 2C). Again, peptides 10 and 11 containing substitutions near the C terminus of the peptide were the least effective at inducing conjugation, consistent with low induction of the mating response (Fig. 1D). Peptides that were of different lengths at the C terminus (peptides D, F, and G) also tended to be weak inducers of same-sex mating (Fig. 2D). Curiously, high concentrations (25 μ g/d) of the native pheromone and peptides 1, 2, and 3, resulted in fewer mating events than lower concentrations (0.5 μ g/d) of these same peptides. Similar results were seen with peptides missing amino acids at the N terminus, as peptides B and C showed more frequent mating at lower pheromone concentrations than at higher concentrations. Significantly, these peptides were also the strongest inducers of the mating program based on *FIG1* expression, and indicates that overstimulation of the pheromone-signaling pathway depresses overall mating frequencies. This finding is consistent with previous studies, where both *C. albicans* and *S. cerevisiae* mutants lacking the Bar1 protease showed a decreased frequency of a-a conjugation, presumably because of excessive stimulation of pheromone signaling (22, 31).

We note that each of the pheromone analogs is capable of inducing polarized mating projections, activating mating gene expression, and even productive self-mating events. The only peptide that was not active was peptide A, which is lacking the

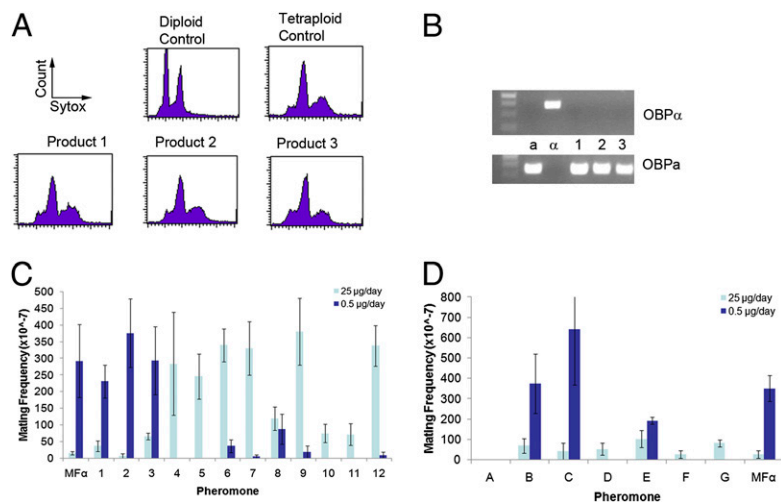


Fig. 2. Addition of α pheromone or analogs induces same-sex mating of *C. albicans* a cells. (A) Addition of pheromone to *C. albicans* opaque a cells results in cell–cell conjugation and progeny that have tetraploid DNA content. Flow cytometry plots showing DNA content of cells fixed and stained with Sytox Green. Three representative mating product strains are shown. (B) PCR confirming the mating type of a–a matings using primers directed at *OBPa* (Lower) and *OBPa* (Upper). Control a and α strains together with three pheromone-induced a–a mating products. (C) Frequency of mating induced by di-alanine-substituted pheromones. Opaque a cells from strains RBY1118 (-his) and RBY1179 (-arg) were coincubated with either 25 μ g (light bars) or 0.5 μ g (dark bars) of pheromone daily for 5 d on Spider medium. Mating products were detected by plating cells on selective medium (-his, -arg), as described (24). (D) Frequency of mating induced by pheromones of different lengths. Error bars represent SEM from a minimum of three experiments.

first three residues at the N terminus. Substitutions at position 11 of the α pheromone (in peptides 10 and 11) also had a very significant affect on pheromone activity, and reduced mating signaling to a larger extent than that seen with the corresponding substitution in *S. cerevisiae* (29). Overall, these data indicate that an important region for bioactivity exists near position 11, but the first three amino acids in particular do not contain critical amino acid contacts for signaling through *C. albicans* Ste2.

Pheromone Analogs Increase Cell Adhesion by White Cells of *C. albicans*.

Opaque cells are the mating-competent form of *C. albicans*, and yet recent experiments demonstrate that a productive pheromone-signaling pathway also operates in white cells. Work by Daniels et al. showed that white cells exhibit increased adhesion in the presence of pheromone, promoting attachment to each other and to synthetic surfaces and resulting in biofilm formation (16). Interestingly, the white response is dependent on a specific domain in Ste2, intracellular region 1, which is dispensable for pheromone signaling in opaque cells (19).

We tested the ability of white cells to form biofilms in response to pheromone analogs by measuring adherence to plastic (17–19). As shown in Fig. 3, white a cells did not adhere to plastic in the absence of pheromone, whereas addition of the *C. albicans* α pheromone resulted in efficient formation of a cohesive film. Comparison of the di-alanine peptide analogs revealed that all of the analogs were capable of increasing biofilm response by white cells. Similar to the native α pheromone, peptide analogs 1, 2, and 3 induced the strongest adherence to plastic and did so even at low concentrations (Fig. 3 and Fig. S2). The majority of the remaining analogs induced strong biofilm formation when used at higher concentrations (10 μ g/mL) (Fig. 3 A and B) but not at lower concentrations (0.2 μ g/mL) (Fig. S2). In contrast, peptides 4, 10, and 11 induced only weak adherence compared with the native pheromone. Overall, the results with white cells show strong parallels to those obtained with opaque cells, including the fact that peptides 10 and 11 are the weakest inducers of a cellular response.

Analysis of pheromone analogs of different lengths also revealed that white and opaque cells exhibit similar responses. For example, removal of three amino acids from the N-terminal re-

gion of the α pheromone (peptide A) abolished induction of adherence to plastic, whereas other manipulations at the N terminus of the pheromone did not negatively affect white cell signaling (see peptides B, C, and E) (Fig. 3 and Fig. S2). Analogs that were shorter or longer at the C terminus were also able to induce weak adherence to plastic (peptides D, F, and G) (Fig. 3 and Fig. S2). These results indicate that pheromone analogs that induce signaling in opaque cells are also able to do so in white cells. Multiple peptides are therefore able to activate the pheromone-signaling cascade in *C. albicans*, either inducing the mating response in opaque cells or biofilm formation in white cells.

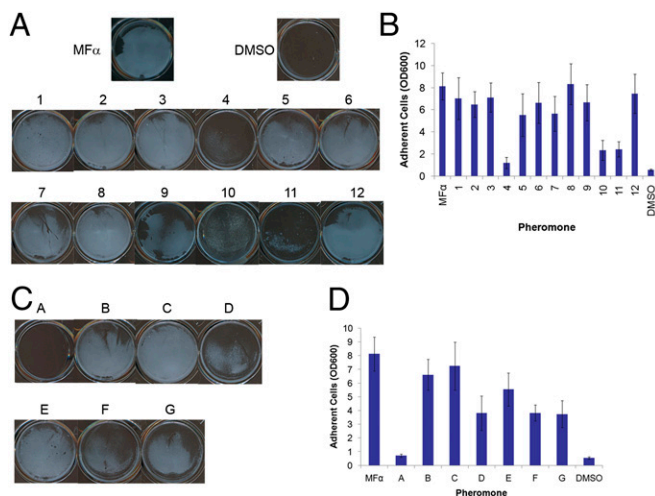


Fig. 3. Pheromone analogs induce adhesion in *C. albicans* white cells. (A) Images of white cells adhering to plastic. *C. albicans* white P37005 a cells were grown in Lee's medium for 24 h in the presence of di-alanine pheromone analogs (10 μ g). Nonadherent cells were removed by washing and pictures of the wells taken. (B) Quantification of adherent white cells shown in A. (C) Images of adherent white cells after exposure to 10 μ g of pheromones A–G for 24 h. (D) Quantification of adherent white cells shown in C. Error bars represent SEM from a minimum of three experiments.

Interspecies Pheromone Signaling Can Induce Biofilm Formation and Homothallic Mating in *C. albicans*. *C. albicans* coexists with a number of yeast and bacterial species as part of the human microbiota. The discovery that alternative pheromone analogs can successfully signal to *C. albicans* a cells suggested that pheromones from different *Candida* species might induce responses in *C. albicans*. With the availability of genome sequences for multiple *Candida* species and conservation of pheromone-processing proteins (32), we identified potential α pheromones for several hemiascomycete species (Fig. 4A; for details on pheromone identification, see Fig. S3).

The response of *C. albicans* opaque a cells to each synthetic pheromone was first monitored by examination of colony morphology (Fig. 4B) and by pFIG1-GFP expression (Fig. 4C). Notably, even highly divergent pheromones from species such as *Candida parapsilosis* and *Lodderomyces elongisporus* were able to induce a mating response in *C. albicans*, as evidenced by both morphological changes and FIG1 expression. These responses were specific, as α pheromones from more divergent species, including *Candida lusitanae*, *Kluyveromyces lactis*, and *S. cerevisiae*, did not induce mating (Fig. 4C). Furthermore, none of the peptides were able to induce a mating response in $\Delta ste2$ cells. Although the responses to *Candida dubliniensis* and *C. parapsilosis* pheromones was relatively strong, *Candida tropicalis* and *L. elongisporus* peptides were less efficient at inducing FIG1 ex-

pression (Fig. 4C). Significantly, the alternative pheromones were also successful at inducing same-sex mating of *C. albicans* cells. Thus, *C. dubliniensis* and *C. parapsilosis* pheromones, and to a lesser extent *C. tropicalis* and *L. elongisporus* pheromones, could induce fusion of opaque a cells and formation of stable a-a tetraploid cells (Fig. 4D). These results support our conclusion that activation of the mating program alone is sufficient to drive homothallic mating of *C. albicans* cells.

We also examined the potential for interspecies signaling to activate biofilm formation by white cells of *C. albicans*. As expected, pheromones from multiple *Candida* species were able to induce adhesion of *C. albicans* white a cells to plastic (Fig. 4E and F). Again, the efficiency of biofilm formation varied depending on the pheromone, but showed parallels to the response in opaque cells. Thus, pheromone-induction of biofilm formation decreased in the order *C. dubliniensis* > *C. parapsilosis* > *L. elongisporus* > *C. tropicalis*. Biofilm formation was not induced by *S. cerevisiae* pheromone or from any other species that was tested. These results demonstrate that a divergent set of pheromone sequences from different *Candida* species are able to signal to *C. albicans* and induce a biological response in both white and opaque cells.

Discussion

Plasticity of the *Candida* Pheromone-Receptor Interaction. In this work, we investigated the specificity of the ligand-receptor interaction that regulates the pheromone-sensing pathway of *C. albicans*. We demonstrate that there is considerable plasticity in the interaction between the Ste2 receptor and its pheromone ligand. Thus, substitution of any of the 13 amino acids that make up the cognate pheromone can occur without a complete loss of signaling activity. Substitutions at the C terminus of the pheromone sequence had the most negative effect on signaling through Ste2. In contrast, di-alanine substitutions at the N terminus had only modest effects on bioactivity, and in some cases even resulted in increased signaling through Ste2. There was also considerable flexibility in signaling via analogs of different lengths. Substitution of one or two amino acids from the N terminus, or addition of two amino acid residues to the N terminus, did not greatly affect pheromone performance. Modifications to the C terminus were tolerated less favorably, as the addition or subtraction of amino acids had a significant effect on bioactivity, although functionality was still maintained.

Studies on *S. cerevisiae* Ste2 have shown that this receptor can also tolerate substitutions in its ligand. In this case, single alanine substitutions revealed that no side chain is absolutely required for activity (29, 33). In general, alanine analogs resulted in one order of magnitude variation in bioactivity, and included analogs with both increased and decreased activity. A genetic screen for agonists and antagonists of Ste2 also demonstrated flexibility in ligand recognition, although the vast majority of agonists differed, at most, at only 3 of the 13 amino acid residues in the native α pheromone (34). Notably, pheromone bioactivity did not correlate with binding affinity of the ligand for the receptor, and this may represent a requirement for ligand-induced receptor isomerization for signaling activity (33).

Pheromone Signaling Drives Same-Sex Homothallic Mating in *C. albicans*. In contrast to *S. cerevisiae*, we demonstrate that the addition of α pheromone to *C. albicans* cells not only activates pheromone signaling but results in productive same-sex fusion of opaque cells. Self-fertilization required Ste2, confirming that activation of the canonical pheromone signaling pathway was required for mating. These results therefore establish that pheromone signaling is sufficient to drive the program of homothallic self-mating in *C. albicans* (24, 30). Same-sex fertilization due to pheromone alone has, to our knowledge, not been observed in other hemiascomycete yeast. In contrast, in the filamentous ascomycete *Ustilago maydis*, addition of mating pheromone was

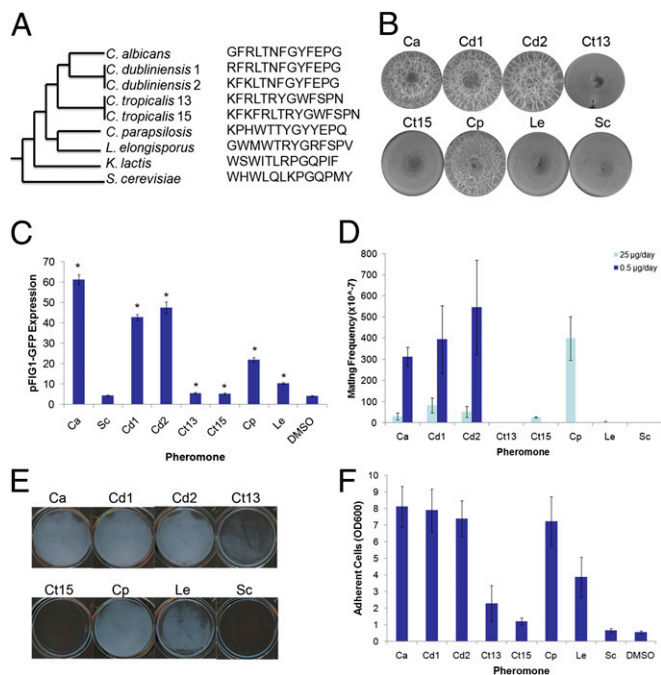


Fig. 4. Pheromones from other *Candida* species induce same-sex mating and adherence in *C. albicans*. (A) Phylogenetic tree showing relationship between *Candida* species and their predicted α pheromones (not to scale). Two potential α pheromones are shown for *C. dubliniensis* and *C. tropicalis*. (B) Pheromones from multiple other *Candida* species induce a morphological change in *C. albicans* opaque a cells. Images of colonies incubated with 25 μ g of pheromone daily for 3 d on Spider medium. (C) Quantification of FIG1 gene expression induced by 10 μ g/mL pheromone for 24 h in Spider medium (arbitrary units). (D) Pheromone analogs induce *C. albicans* a-a conjugation. Quantification of mating products after 5 d culture on Spider medium in the presence of 25 or 0.5 μ g pheromone per day. (E) Alternative pheromones induce adherence of *C. albicans* white cells. Images of adherent P37005 white cells grown in 12-well plastic dishes in Lee's medium and exposed to 10 μ g of pheromone for 24 h. (F) Quantification of adherent white cells shown in E. Error bars represent SEM from a minimum of three experiments. * $P < 0.05$ vs. DMSO control.

shown to induce conjugation tube formation and fusion of cells from the same mating type (35). In addition, pheromones have been implicated in homothallic mating in other ascomycete species. For example, in *Aspergillus nidulans*, activation of pheromone signaling is central to self-fertility and expression of pheromones and receptors in a single cell type drives this process (36, 37). Programming of pheromone signaling pathways therefore plays a key role in determining whether mating is homothallic or heterothallic in fungi. In the case of *C. albicans*, both modes of reproduction are possible in **a** cells. Heterothallic **a**- α mating is favored in the presence of α cells, but homothallic mating can occur in $\Delta bar1$ cells (24) or, as we now show, in wild-type cells in the presence of pheromones from other *Candida* species.

Biofilm Formation due to Pheromone Signaling in *C. albicans* White Cells. Recent studies have revealed that *C. albicans* white cells, although infertile, can respond to pheromone by expressing genes involved in biofilm formation (16–18). In contrast to pheromone signaling in opaque cells that targets the Ste12/Cph1 transcription factor, signaling in white cells results in Tec1 activity and up-regulation of adhesion genes (17). A particular region of the Ste2 receptor, intracellular domain 1 (located between transmembrane domains 1 and 2) was implicated in the pheromone response of white cells but not opaque cells, which may be indicative of unique interactions between Ste2 and the downstream signaling machinery in white cells (19). In this work, we show that pheromone analogs that induce mating in opaque cells also stimulate biofilm formation in white cells. In general, a strong correlation was observed between analog activity in white and opaque cells, indicating ligand-Ste2 interactions are similar in the two cell types. One exception was peptide 4, which contained alanine residues at positions 4 and 5; this peptide produced virtually no biofilm in white cells, yet was still relatively active in inducing mating in opaque cells. Overall, these results demonstrate that white and opaque cells recognize pheromone analogs with a similar specificity, although some minor differences may exist between pheromone signaling in the two cell types.

Cross-Species Signaling Drives *C. albicans* Self-Mating and Biofilm Formation. The most revealing aspect of the current work is the demonstration that the pheromone-signaling machinery in *C. albicans* can respond to signals other than the cognate ligand. *C. albicans* is a commensal species of warm-blooded hosts, where it is exposed to a variety of stimuli from the host and other microbes. The ability of *C. albicans* to adapt allows it to thrive in multiple niches in the human body and is a major reason for the success of this species as an opportunistic pathogen. The plasticity inherent in the ligand-Ste2 interaction led us to test *C. albicans* responses to pheromones from species with which it coexists. We show that pheromones from several related species, including *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, and *L. elongisporus*, induce mating and same-sex conjugation in *C. albicans* opaque cells and biofilm formation in *C. albicans* white cells. Multiple *Candida* species (e.g., *C. albicans*, *C. parapsilosis*, and *C. tropicalis*) have been shown to coinhabit the same niches (38–41), while *L. elongisporus* was recently identified from bloodstream infections (42), confirming that these species can encounter one another in nature.

Early studies in *Saccharomyces* established that pheromones from different yeast species can act interspecifically. For example, Hisatomi et al. (43) showed that synthetic pheromones from *S. cerevisiae*, *Saccharomyces kluyveri*, and *Saccharomyces exiguus* could all act upon one another. These peptides were highly homologous, with *S. kluyveri* and *S. exiguus* differing at only four or five amino acid residues, respectively, with the *S. cerevisiae* pheromone. In contrast, two of the peptides active on *C. albicans* (*C. parapsilosis* and *L. elongisporus* pheromones) show much greater divergence, differing at 8 of the 13 amino acid residues in the *C. albicans* sequence (Fig. 4). Moreover, whereas interspecies signaling events

were futile in *Saccharomyces*, we emphasize that pheromone signaling results in productive homothallic mating events in *C. albicans*, as well as increased biofilm formation in white cells.

We propose that these studies extend the conditions under which sex and sex-related processes act in *Candida* and other fungal species. Our discovery that alternative pheromones can activate the program of homothallic mating indicates that mating could be more frequent than previously anticipated. Thus, even in the absence of mating partners (and in a *BARI*⁺ strain background) activation of the mating program can occur because of environmental telesensing. Ligand signaling can drive *C. albicans* same-sex mating, generating tetraploid cells that are competent to undergo the parasexual program of chromosome reduction, and resulting in diploid and aneuploid forms of the species (44, 45). Given the complex niche that *C. albicans* inhabits, we speculate that pheromone-like signals from the host or other microbiota could also activate the pheromone-sensing machinery and drive homothallicism. Ongoing studies into the human microbiome will therefore be potentially revealing in identifying novel ligands that could stimulate *C. albicans* mating pathways.

Taken together, our findings show that diverse signals activate *C. albicans* pheromone signaling, and that the downstream consequences of signaling include both homothallicism and biofilm formation. These results further the conditions under which the sexual machinery is active, and we speculate that interspecies signaling events will similarly direct mating in other fungi. It will therefore be important to examine all aspects of *C. albicans*' mating cycle in the context of its complex environment in the mammalian host. Based on our findings, it will be particularly exciting to examine other constituents of the host microbiota to determine what ligands are recognized by *C. albicans* and can activate (or inhibit) its sexual biology.

Materials and Methods

Media and Reagents. Media was prepared as described previously (46–48). Peptides were synthesized by Genemed Synthesis. Peptides were resuspended at a concentration of 10 mg/mL in 10% DMSO and used at the indicated concentrations.

Pheromone Response Assays. Overnight cultures of opaque cells from strain RBY1118 (*a/a*) were grown in SCD medium at room temperature. Cells were washed with water and resuspended in Spider medium (48) at a concentration of 2×10^9 cells/mL. Twenty microliters of this culture was added to Spider plates and incubated at room temperature. Next, 25 μ g of pheromone was added daily for 3 d; colonies were imaged using a Zeiss Stemi 2000-C stereoscope with an Infinity 1 camera. Cells were taken from colonies and examined using a Zeiss Axioplan 2 microscope equipped with a Hamamatsu-ORCA camera. For quantification of the response to pheromone treatment, a GFP reporter under the control of the *FIG1* promoter was used (24). Opaque cells of DSY700 (*pFIG1-GFP*) were grown overnight at room temperature in SCD medium, cells washed, and resuspended in Spider medium. Then, 6×10^7 cells were added to 3 mL of fresh Spider medium, 10 μ g/mL pheromone added, and cells grown for 24 h at room temperature. Cells were added to SCD medium, sonicated and fluorescence measured in the FL1 channel on a Becton Dickinson FACSCalibur.

Quantitative Mating Assays. Overnight cultures of opaque strains with different auxotrophic markers (RBY1118 and RBY1179) (22) were grown in SCD medium at room temperature. Cells were washed with water and resuspended in Spider medium at a concentration of 1×10^9 cells/mL. Ten microliters of each culture was mixed and plated on a nitrocellulose filter on Spider plates. Pheromone was added daily at the indicated concentration for 4 d. On day 5, cells were removed from the filter, resuspended in water, and sonicated to disperse clumps. Cells were then plated on selective media and analyzed for the formation of mating products, as previously described (24).

Biofilm Assays. Adherence assays were performed essentially as described (16, 18) with the following modifications. Overnight cultures of white cells from strain P37005 were grown in YPD at room temperature. Cells were washed with PBS (phosphate-buffered saline) pH 7.4 and resuspended in Lee's medium to a concentration of 5×10^7 cells/mL. One milliliter of cells was then

added to 12-well culture dishes (Costar, Corning Inc.) to which the indicated amount of pheromone was added. Plates were left at room temperature overnight, wells were gently washed with PBS, and plates scanned using a HP Scanjet G3010. Adherent cells were also scraped off the well and quantified at OD₆₀₀ using a spectrophotometer.

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ACKNOWLEDGMENTS. Work in the laboratory of K.A. and R.J.B. was supported by National Institutes of Health Grants R21AI081560, AI081560, and AI081704 (to R.J.B.) and F31DE019752 (to K.A.). R.J.B. also holds an Investigator in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund.