Homothallic and heterothallic mating in the opportunistic pathogen *Candida albicans*

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Abstract

Candida albicans is the most common fungal pathogen in humans, causing both debilitating mucosal infections and potentially life-threatening systemic infections[1,2]. Until recently, *C. albicans* was thought to be strictly asexual, existing only as an obligate diploid. A cryptic mating cycle has since been uncovered in which diploid a and α cells undergo efficient cell and nuclear fusion, resulting in tetraploid a/α mating products (refs. [3–6]). Whereas mating between a and α cells has been established (heterothallism), we report here two pathways for same-sex mating (homothallism) in *C. albicans*. First, unisexual populations of a cells were found to undergo autocrine pheromone signalling and same-sex mating in the absence of the Bar1 protease. In both *C. albicans* and *Saccharomyces cerevisiae*, Bar1 is produced by a cells and inactivates mating pheromone α, typically secreted by α cells[7–10]. *C. albicans* Δbar1 a cells were shown to secrete both a and α mating pheromones; α-pheromone activated self-mating in these cells in a process dependent on Ste2, the receptor for α-pheromone. In addition, pheromone production by α cells was found to promote same-sex mating between wild-type a cells. These results establish that homothallic mating can occur in *C. albicans*, revealing the potential for genetic exchange even within unisexual populations of the organism. Furthermore, Bar1 protease has an unexpected but pivotal role in determining whether sexual reproduction can potentially be homothallic or is exclusively heterothallic. These findings also have implications for the mode of sexual reproduction in related species that propagate unisexually, and indicate a role for specialized sexual cycles in the survival and adaptation of pathogenic fungi.

The hemiascomycetous fungi are a diverse range of species that include the prototypical model yeast, *Saccharomyces cerevisiae*, as well as the prevalent human pathogen, *Candida albicans*. Sexual reproduction in both fungi is driven by the secretion of sex-specific pheromones that promote mating between partners of the opposite sex[11–13]. Pheromone gradients are sensed by G-protein-coupled cell-surface receptors that initiate the mating response[14,15]. *C. albicans* mating is thought to be exclusively heterothallic, whereas isolates of *S. cerevisiae* that express HO endonuclease are homothallic because of mating-type switching[16]. The discovery that unisexual populations of *C. albicans* can express both **a** and

α mating pheromones [17] led us to test whether autocrine pheromone signalling and homothallism can occur in this species.

C. albicans **a** cells derived from the laboratory strain SC5314 were treated with synthetic α-pheromone and the quantitative polymerase chain reaction used to compare the induction of both **a**- and α-pheromone genes (*MFa*, and *MFα*, respectively). Opaque-phase cells were used for these experiments, as these are the mating-competent form of *C. albicans* [6]. Both *MFa* (300-fold) and *MFα* (373-fold) were highly induced in response to α-pheromone, in contrast to *S. cerevisiae* where only **a**-pheromone is upregulated[18]. In both *C. albicans* and *S. cerevisiae*, an extracellular barrier activity acts to antagonize the function of α-pheromone. The activity, encoded by *BAR1*, is an aspartyl protease that cleaves α-pheromone, inactivating it[7–10]. We therefore analysed a *C. albicans* Δ*bar1* mutant to determine whether α-pheromone production has a functional role in these cells. *C. albicans* Δ*bar1* cells exhibited distinctive colony morphologies when cultured on solid media. Mutant colonies appeared increasingly wrinkled during growth on yeast peptone dextrose (YPD) medium (Fig. 1a). Cells retrieved from these colonies were highly elongated and showed similarities to cells that had undergone extended exposure to pheromone[12]. To confirm that elongated cells were undergoing a mating-type response and were not hyphal (filamentous) cells, green fluorescent protein (GFP) reporters were introduced under the control of pheromone-specific promoters. Both *FIG1* and *FUS1* genes are highly expressed in response to pheromone[17], and pFIG1-GFP and pFUS1-GFP constructs were therefore used to monitor activation of the mating program. Significantly, both *FIG1* and *FUS1* expression was induced, confirming that cells were undergoing a mating-like response, even in the absence of exogenous pheromone or a mating partner of the opposite sex (Fig. 1b). To confirm that this phenomenon was not limited to strains derived from SC5314, Δ*bar1* mutants were also generated in P37005, a natural **a**/**a** isolate of *C. albicans*, and found to exhibit similar cell and colony phenotypes (Supplementary Fig. 1).

Two models were considered for how autocrine pheromone signalling could lead to activation of a mating response. First, **a**-pheromone secretion could signal to Ste3, the receptor for **a**-pheromone (Fig. 2a, model I). Alternatively, α-pheromone could induce mating via binding to Ste2, the receptor for α-pheromone (model II). Although not mutually exclusive, these two models were tested by construction of strains lacking different combinations of pheromone or pheromone receptor genes. Significantly, loss of either α-pheromone (MFα) or its receptor (Ste2) completely abolished the wrinkled colony appearance of Δ*bar1* **a** strains (Fig. 2b). In contrast, loss of *STE3* (encoding the receptor for **a** pheromone) had no effect on colony phenotype (Fig. 2b). Thus, autocrine signalling in **a** cells is driven by α-pheromone production and requires the presence of Ste2, the cognate receptor for α-pheromone (model II).

Autocrine pheromone signalling was also observed between neighbouring colonies of cells, as Δ*bar1* **a** cells induced a response in adjacent colonies of **a** cells. Cells from wrinkled colonies again exhibited polarized growth indicative of a mating response (data not shown). Neighbouring cells failed to respond in the absence of *STE2*, indicating that signalling required the receptor for α-pheromone (Fig. 2c, row 2). Furthermore, Δ*bar1* cells were unable to induce a response in adjacent cells if either *MFα* or *STE2* was also deleted (Fig. 2c, rows 3 and 4). The dependence of signalling on *STE2* is demonstrative of a positive feedback loop in these cells; α-pheromone secreted by Δ*bar1* cells binds to Ste2 receptors on these cells and results in further augmentation of pheromone production. Only when this feedback loop is in effect does pheromone secretion reach the levels necessary for autocrine signalling. *STE3*, encoding the receptor for **a**-pheromone, was not involved in this signalling (Fig. 2c, column 4 and row 5). Production of α-pheromone by Δ*bar1* **a** cells was also

evident in halo assays and was shown to increase biofilm formation by these cell types (see Supplementary Information).

The existence of a robust autocrine mating pathway led us to test whether *C. albicans* is capable of homothallic mating. Two different auxotrophic Δ*bar1* strains were mixed and co-incubated on Spider medium for 3 days. Cells were recovered from the mating mixture and plated onto selective medium to detect potential **a–a** mating products. A number of prototrophic mating products were obtained and analysis by PCR and flow cytometry revealed that these were stable tetraploid cells of mating type **a** (Fig. 3a, b). To compare the frequency of homothallic and heterothallic mating in *C. albicans*, several quantitative mating crosses were performed (Fig. 3c). Mating between wild-type **a** and α cells occurred efficiently (94%), whereas no mating was observed between two wild-type **a** strains (<0.00002%). Significant levels of unisexual mating were obtained, however, using two Δ*bar1* **a** strains (0.037%), an increase of more than 1,850-fold over wild-type **a–a** mating. The efficiency of unisexual mating was further increased in crosses between Δ*bar1* and wild-type **a** strains, with 0.88% of cells undergoing mating, an increase of more than 40,000-fold over that between wild-type **a** strains (Fig. 3c). In fact, Δ*bar1* **a** cells mated as efficiently with other **a** cells as with α cells (0.80%), indicating a loss in sexual preference for one mating-type partner over the other. These experiments demonstrate that mating can take place even within unisexual populations of *C. albicans* cells, and that Bar1 acts to limit these types of self-fusion events. Notably, mating between wild-type **a** cells also occurred at an appreciable frequency (0.22%) if co-cultured with α cells. In these ménage à trois matings, α cells presumably provided levels of α-pheromone that were sufficient to overcome Bar1 activity and drive **a–a** mating. Levels of α-pheromone may be augmented by autocrine pheromone signalling in **a** cells, as deletion of *MF*α in **a** cells led to a slight reduction in the frequency of ménage à trois mating (0.12%). In addition, α cells did not need to be in contact with **a** cells to drive same-sex mating (Supplementary Fig. 4). Mating between wild-type α cells (0.043%) was similarly observed when co-cultured with **a** cells (see Fig. 3).

If self-mating in unisexual populations is a direct consequence of autocrine pheromone signalling, then mutants deficient in signalling should be unable to self-mate. Both Δ*bar1*/Δ*ste2* and Δ*bar1*/Δ*mf*α **a** strains did not exhibit autocrine signalling and also showed no detectable mating with wild-type **a** strains (<0.0002%; Fig. 3c). In contrast, Δ*bar1*/Δ*ste3* **a** strains were competent for both autocrine signalling and unisexual mating (1.2%). The potential for unisexual mating is therefore dependent on the ability of cells to undergo autocrine pheromone signalling. To examine the efficiency of inbreeding and outbreeding by different *C. albicans* strains, SC5314 Δ*bar1* **a** strains were also mated with different clinical isolates (Fig. 4a). Again, Δ*bar1* strains mated with both **a** and α isolates at a similar frequency (average of 9.8% and 17.6%, respectively). In fact, mating was generally more efficient between SC5314 and clinical isolates (up to 26% **a–a** mating) than that between SC5314 strains alone. Mating zygotes could be detected in unisexual crosses and fluorescent labelling of the nuclei confirmed that karyogamy had taken place (Fig. 4b and Supplementary Fig. 5). The tetraploid products of unisexual mating were generally stable, but could be induced to undergo chromosome loss to produce diploid cells, thereby completing a parasexual mating cycle (Supplementary Fig. 7).

Our findings have implications for sexual reproduction in *C. albicans* and related species, as they reveal a mechanism for same-sex mating in hemiascomycetes. In *C. albicans*, heterothallic mating between **a** and α cells can occur, yet a predominantly clonal population structure suggests that outbreeding is rare[19]. The discovery of self-mating indicates that homothallism could act to create a burst of genetic diversity by increasing recombination within unisexual populations. Completion of the parasexual cycle could promote allele

shuffling or increase karyotypic variation within the diploid genome. Furthermore, isolates of some related species are thought to be exclusively homothallic. For example, isolates of *Lodderomyces elongisporus* are able to form asci from identical cells, yet the genome apparently encodes only one pheromone gene (*MFα*) and one receptor gene (*STE2*)[20]. Discovery of autocrine signalling and self-mating reveals a mechanism by which such species could reproduce sexually despite the absence of mating partners of the opposite sex.

Homothallism has been well characterized in *S. cerevisiae* where it is a direct result of mating-type switching. *C. albicans* and related species are unable to undergo mating-type switching, yet we demonstrate two potential pathways by which self-mating can occur. First, inhibition of Bar1 activity promotes autocrine signalling and mating within unisexual populations of *C. albicans*. We hypothesize that certain niches in the mammalian host will favour self-fertilization by downregulating Bar1 activity. Second, we show that same-sex mating can occur because of pheromone signalling in conventional **a**–α mixes. In this case, pheromone secretion by even a limiting number of α cells can promote **a**–**a** mating (Supplementary Table 3). In addition, certain combinations of *C. albicans* **a** and α strains are incompatible for heterothallic mating as nuclei fail to undergo karyogamy[21]. Our results show that pheromones produced during contact between two such 'infertile' strains could still result in self-mating within sexes.

The program of same-sex mating in *C. albicans* shows unexpected similarities to that in other human fungal pathogens. In the basidio-mycetous fungus *Cryptococcus neoformans* mating between **a** and α isolates can occur, yet clinical and environmental isolates are over-whelmingly of the α mating type. Same-sex mating between α isolates of *C. neoformans* has been observed both in the laboratory and in the wild, and is thought to be the predominant mode of sexual reproduction in this organism[22–25]. *Pneumocystis carinii* is another fungal pathogen predicted to undergo a modified sexual cycle involving cells of the same mating type[26]. It thus seems that autocrine pheromone signalling and homothallism may be traits shared by a diverse group of medically relevant fungi. These pathogens have retained the ability to undergo sexual reproduction and yet have evolved modifications to these programs that support their survival and propagation. Elucidating the respective mechanisms of homothallism and heterothallism will therefore be critical to the understanding of these species as human pathogens.

METHODS SUMMARY

C. albicans strains were derived from SC5314 except where otherwise noted. Mutant strains were created using either the PCR fusion or *SAT1* flipper methods described previously[27,28]. For a complete list of strains and primers see Supplementary Tables 1 and 2. Media were made as described previously[10]. All *C. albicans* cells used in this study were in the opaque phase as this is the mating-competent form of the organism[6]. Quantitative PCR was performed on **a** cells that had been treated with $10 \ \mu g \ ml^{-1}$ α-pheromone for 4 h in Spider medium, as described previously[10,29]. For analysis of colony morphology, patches of *C. albicans* cells (~0.5 cm^2) were spaced 0.75 cm apart on YPD medium and imaged after 4 days growth at 30 °C using a Zeiss Stemi 2000-C stereoscope with an Infinity 1 camera. Cells were examined using a Zeiss Axioplan 2 microscope equipped with a Hamamatsu-ORCA camera. Mating frequencies were determined by mixing 2×10^7 cells of differently marked auxotrophic strains on Spider medium and incubating at room temperature for 3 days. Cells were then resuspended in water and plated onto selective media to determine frequency of mating products[10]. For analysis of **a**–**a** mating in the presence of α cells, 2×10^7 cells of each of three strains were mixed and incubated on Spider medium (two differently marked **a** strains and an α strain negative for both markers). Cells were recovered and analysed as above. The same strategy (two differently marked α strains and an **a** strain

negative for both markers) was applied to detect α–α mating. Flow cytometry to determine cell ploidy was performed by staining DNA with Sytox Green and analysis on a FACSCalibur[4]. PCR to determine mating type was performed using primers directed at *OBPa*/α as described previously[30].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Samaranayake LP, et al. Fungal infections associated with HIV infection. Oral Dis 2002;8 Suppl. 2:151–160. [PubMed: 12164650]

2. Wisplinghoff H, et al. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis 2004;39:309–317. [PubMed: 15306996]

3. Hull CM, Johnson AD. Identification of a mating type-like locus in the asexual pathogenic yeast *Candida albicans*. Science 1999;285:1271–1275. [PubMed: 10455055]

4. Hull CM, Raisner RM, Johnson AD. Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. Science 2000;289:307–310. [PubMed: 10894780]

5. Magee BB, Magee PT. Induction of mating in *Candida albicans* by construction of *MTL***a** and *MTL*α strains. Science 2000;289:310–313. [PubMed: 10894781]

6. Miller MG, Johnson AD. White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. Cell 2002;110:293–302. [PubMed: 12176317]

7. Hicks JB, Herskowitz I. Evidence for a new diffusible element of mating pheromones in yeast. Nature 1976;260:246–248. [PubMed: 768777]

8. MacKay VL, et al. The *Saccharomyces cerevisiae BAR1* gene encodes an exported protein with homology to pepsin. Proc. Natl Acad. Sci. USA 1988;85:55–59. [PubMed: 3124102]

9. Manney TR. Expression of the *BAR1* gene in *Saccharomyces cerevisiae*: induction by the α mating pheromone of an activity associated with a secreted protein. J. Bacteriol 1983;155:291–301. [PubMed: 6345506]

10. Schaefer D, Cote P, Whiteway M, Bennett RJ. Barrier activity in *Candida albicans* mediates pheromone degradation and promotes mating. Eukaryot. Cell 2007;6:907–918. [PubMed: 17416895]

11. Bennett RJ, Uhl MA, Miller MG, Johnson AD. Identification and characterization of a *Candida albicans* mating pheromone. Mol. Cell. Biol 2003;23:8189–8201. [PubMed: 14585977]

12. Lockhart SR, Daniels KJ, Zhao R, Wessels D, Soll DR. Cell biology of mating in *Candida albicans*. Eukaryot. Cell 2003;2:49–61. [PubMed: 12582122]

13. Panwar SL, Legrand M, Dignard D, Whiteway M, Magee PT. *MF*α*1*, the gene encoding the α mating pheromone of *Candida albicans*. Eukaryot. Cell 2003;2:1350–1360. [PubMed: 14665468]

14. Jackson CL, Hartwell LH. Courtship in *Saccharomyces cerevisiae*: an early cell-cell interaction during mating. Mol. Cell. Biol 1990;10:2202–2213. [PubMed: 2183023]

15. Segall JE. Polarization of yeast cells in spatial gradients of α mating factor. Proc. Natl Acad. Sci. USA 1993;90:8332–8336. [PubMed: 8397402]

16. Hall, MN.; Linder, P. The Early Days of Yeast Genetics. Cold Spring Harbor Laboratory Press; 1993.

17. Bennett RJ, Johnson AD. The role of nutrient regulation and the Gpa2 protein in the mating pheromone response of *C. albicans*. Mol. Microbiol 2006;62:100–119. [PubMed: 16987174]

18. Roberts CJ, et al. Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. Science 2000;287:873–880. [PubMed: 10657304]

19. Odds FC, et al. Molecular phylogenetics of *Candida albicans*. Eukaryot. Cell 2007;6:1041–1052. [PubMed: 17416899]

20. Butler G, et al. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature 2009;459:657–662. [PubMed: 19465905]

21. Bennett RJ, Miller MG, Chua PR, Maxon ME, Johnson AD. Nuclear fusion occurs during mating in *Candida albicans* and is dependent on the *KAR3* gene. Mol. Microbiol 2005;55:1046–1059. [PubMed: 15686553]

22. Lin X, Hull CM, Heitman J. Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans*. Nature 2005;434:1017–1021. [PubMed: 15846346]

23. Fraser JA, et al. Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. Nature 2005;437:1360–1364. [PubMed: 16222245]

24. Lin X, et al. Diploids in the *Cryptococcus neoformans* serotype A population homozygous for the α mating type originate via unisexual mating. PLoS Pathog 2009;5:e1000283. [PubMed: 19180236]

25. Bui T, Lin X, Malik R, Heitman J, Carter D. Isolates of *Cryptococcus neoformans* from infected animals reveal genetic exchange in unisexual, α mating type populations. Eukaryot. Cell 2008;7:1771–1780. [PubMed: 18552280]

26. Smulian AG, Sesterhenn T, Tanaka R, Cushion MT. The *ste3* pheromone receptor gene of *Pneumocystis carinii* is surrounded by a cluster of signal transduction genes. Genetics 2001;157:991–1002. [PubMed: 11238389]

27. Noble SM, Johnson AD. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. Eukaryot. Cell 2005;4:298–309. [PubMed: 15701792]

28. Reuβ O, Vik A, Kolter R, Morschhauser J. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene 2004;341:119–127. [PubMed: 15474295]

29. Guthrie, C.; Fink, GR. Guide to Yeast Genetics and Molecular Biology. Academic Press; 1991.

30. Lockhart SR, et al. In *Candida albicans*, white-opaque switchers are homozygous for mating type. Genetics 2002;162:737–745. [PubMed: 12399384]
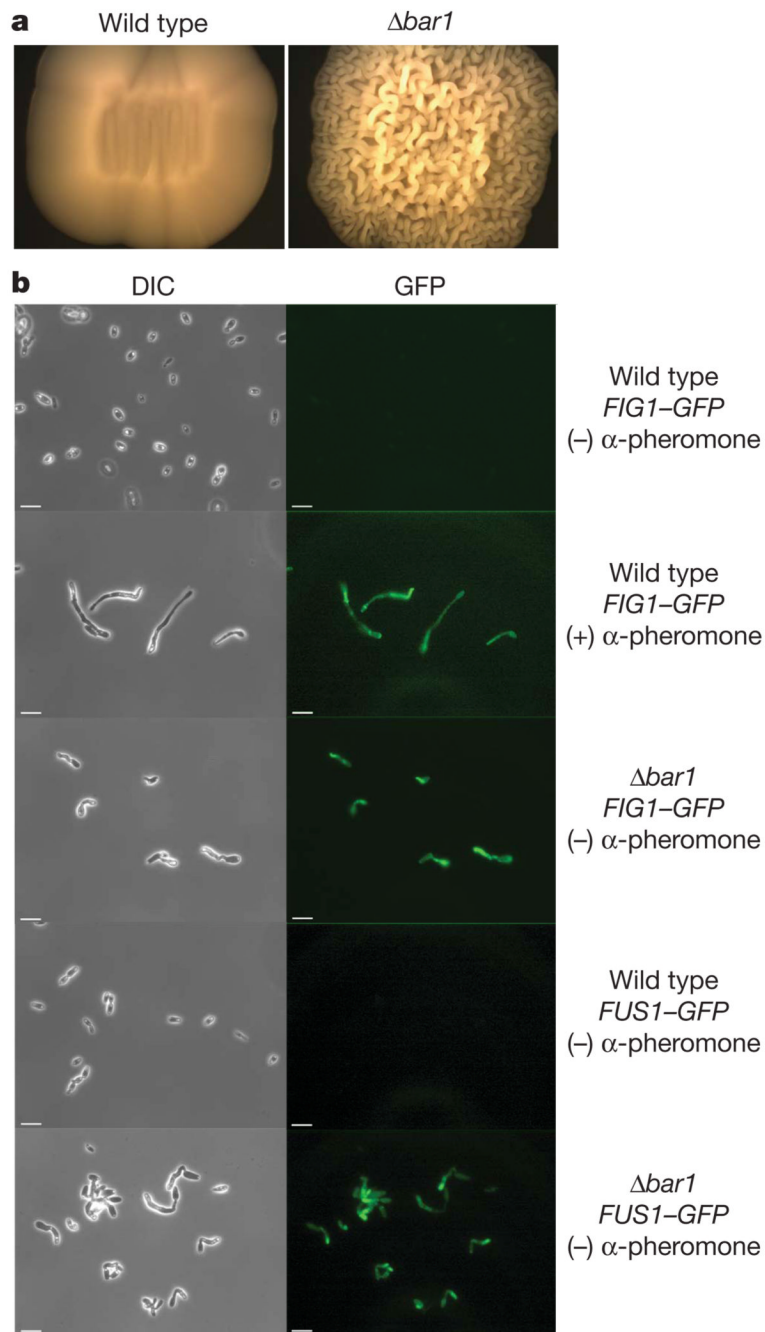
Figure 1. Deletion of *BAR1* results in an autocrine mating response
a, Patches of wild-type and Δ*bar1* **a** cells after 4 days growth on YPD medium. **b**, Mating response of wild-type and Δ*bar1* **a** cells with and without α-pheromone in Spider medium for 36 h. Differential interference contrast (DIC, left) and GFP fluorescence (GFP, right) images are shown. Induction of the mating response was monitored using GFP reporters under the control of the mating-response genes *FIG1* or *FUS1*. All *C. albicans* cells used in this study were in the opaque phase as this is the mating-competent form of the organism[6,30]. Scale bars, 16 µm.
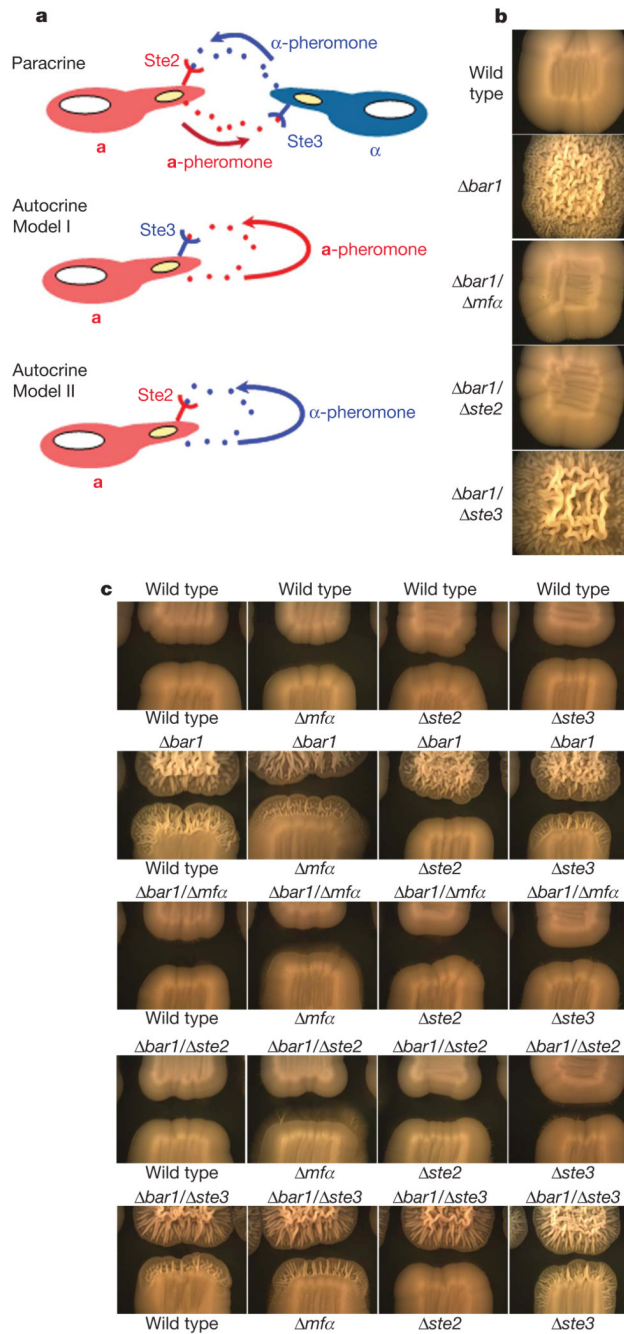
Figure 2. Autocrine signalling in *C. albicans* a cells

a, Model of conventional paracrine signalling between **a** and α cells and two models for autocrine signalling. Note that 'autocrine' signalling is used here to refer to signalling within populations of **a** cells. **b**, *C. albicans* **a** cells after 4 days growth on YPD medium. Whereas Δ*bar1* strains showed a highly wrinkled morphology, reconstituted strains in which the *BAR1* gene had been reintegrated did not show wrinkling (not shown). **c**, Demonstration of autocrine signalling among different populations of **a** cells after 4 days growth on YPD medium. Wrinkling at the edge of a test colony is indicative of autocrine signalling.
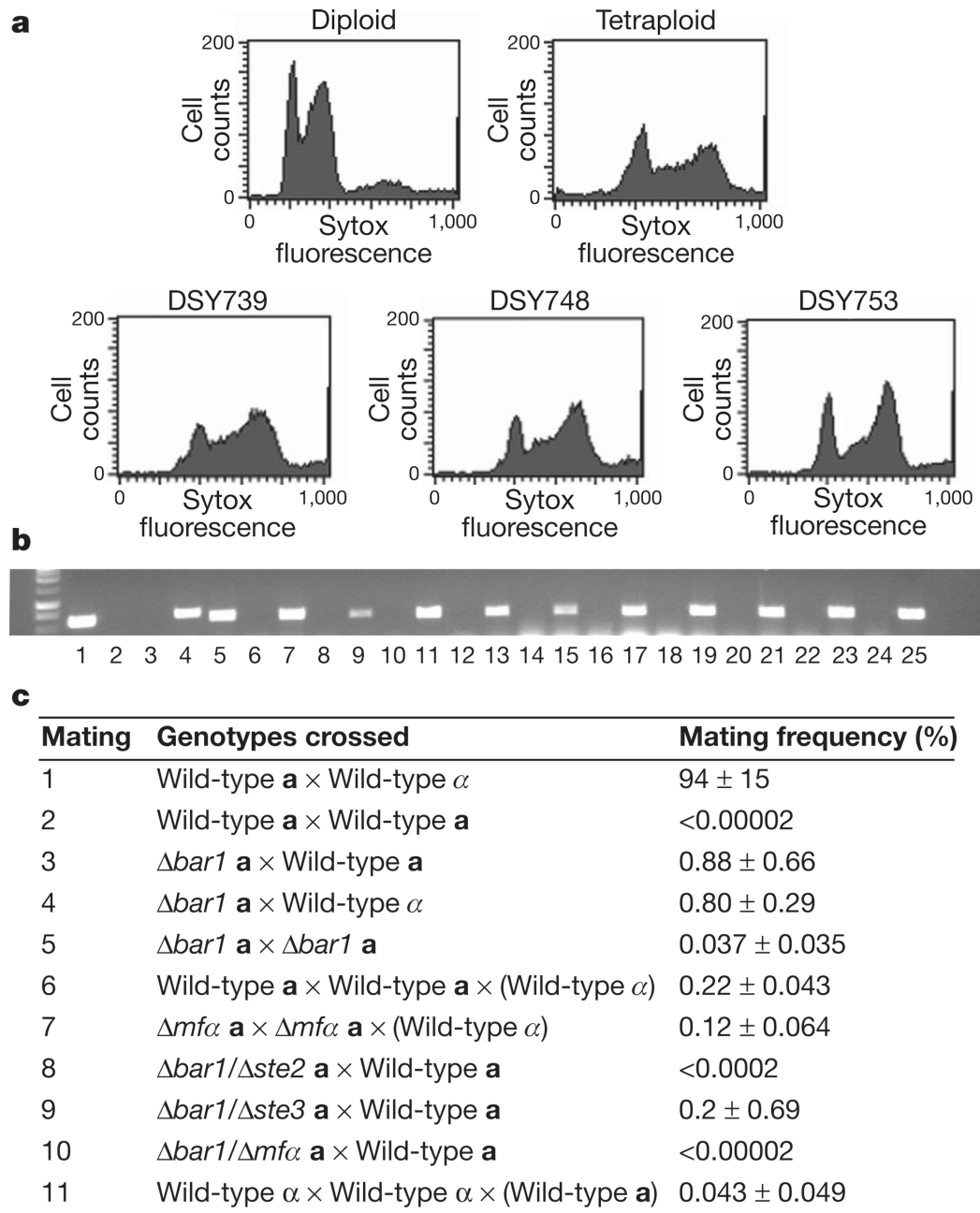
Figure 3. Homothallic mating in *C. albicans*

a, Flow cytometric analysis indicating the tetraploid content of progeny isolated from **a–a** matings. The *x*-axis of each graph (Sytox) represents a linear scale of fluorescence and the *y*-axis (Cell counts) a linear scale of cell number. For analysis of additional same-sex mating products see Supplementary Fig. 6. **b**, PCR confirming the mating type of **a–a** mating products using primers directed at *OBPa* and *OBPα*. Odd-numbered lanes are *OBPa* reactions, whereas even-numbered lanes are *OBPα* reactions. Lanes 1, 2 and 3, 4 are control reactions for **a** and α cells, respectively. **c**, Table of mating frequencies for different genetic crosses. Values are listed as mean frequency ± s.d.
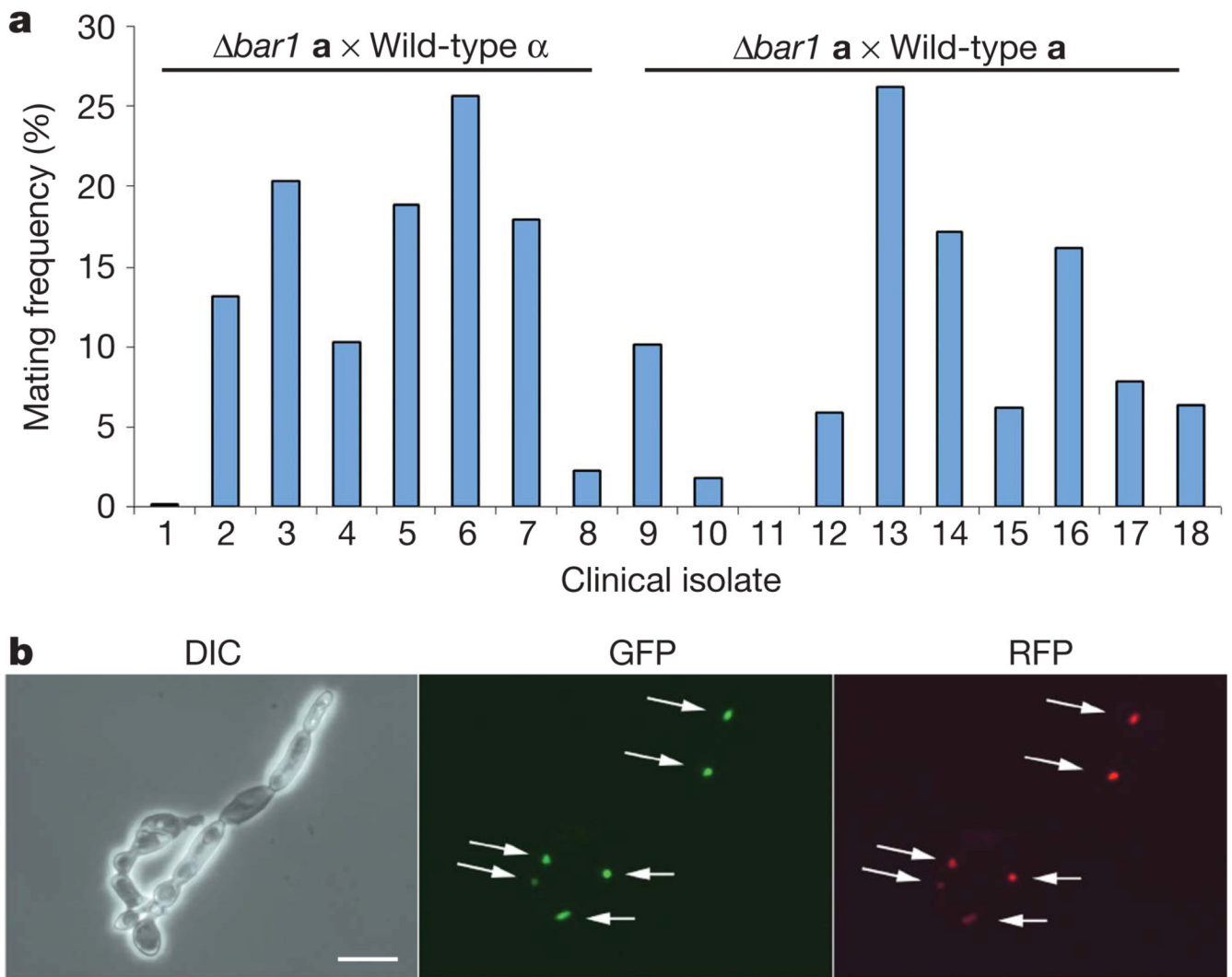
The table for part **c**:

Mating	Genotypes crossed	Mating frequency (%)
1	Wild-type **a** × Wild-type α	94 ± 15
2	Wild-type **a** × Wild-type **a**	<0.00002
3	Δ*bar1* **a** × Wild-type **a**	0.88 ± 0.66
4	Δ*bar1* **a** × Wild-type α	0.80 ± 0.29
5	Δ*bar1* **a** × Δ*bar1* **a**	0.037 ± 0.035
6	Wild-type **a** × Wild-type **a** × (Wild-type α)	0.22 ± 0.043
7	Δ*mfα* **a** × Δ*mfα* **a** × (Wild-type α)	0.12 ± 0.064
8	Δ*bar1*/Δ*ste2* **a** × Wild-type **a**	<0.0002
9	Δ*bar1*/Δ*ste3* **a** × Wild-type **a**	0.2 ± 0.69
10	Δ*bar1*/Δ*mfα* **a** × Wild-type **a**	<0.00002
11	Wild-type α × Wild-type α × (Wild-type **a**)	0.043 ± 0.049

Figure 4. Homothallic mating across clades in *C. albicans*
a, Mating frequencies of an SC5314 Δ*bar1* **a** cell crossed with naturally occurring isolates from different clades of *C. albicans*. 1–8 are crosses between Δ*bar1* **a** cells and clinical α cells. 9–18 are crosses between Δ*bar1* **a** cells and clinical **a** cells. For a complete list of strains see Methods. **b**, Mating zygote formed between a Δ*bar1* **a** cell derived from SC5314 (DSY906, expressing *HTB–RFP*; histone H2B gene–red fluorescent protein) and a clinical **a** cell derived from IHEM16614 (DSY908, expressing *HTB–YFP*). Arrows indicate fused nuclei expressing both fluorescent proteins. DIC (left), YFP fluorescence (observed using the GFP channel, middle) and RFP fluorescence (right) images are shown. Scale bar, 15 µm.