Genetically Engineered Transvestites Reveal Novel Mating Genes in Budding Yeast

Genetically engineered transvestites reveal novel mating genes in budding yeast

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ABSTRACT

Haploid budding yeast has two mating types, defined by the alleles of the *MAT* locus, *MAT***a** and *MAT*α. Two haploid cells of opposite mating types mate by signaling to each other using reciprocal pheromones and receptors, polarizing and growing towards each other, and eventually fusing to form a single diploid cell. The pheromones and receptors are necessary and sufficient to define a mating type, but other mating type-specific proteins make mating more efficient. We examined the role of these proteins by genetically engineering "transvestite" cells that swap the pheromone, pheromone receptor, and pheromone processing factors of one mating type for another. These cells mate with each other, but their mating is inefficient. By characterizing their mating defects and examining their transcriptomes, we found Afb1 (**a**-factor barrier), a novel *MAT*α-specific protein that interferes with **a**-factor, the pheromone secreted by *MAT***a** cells. Strong pheromone secretion is essential for efficient mating, and the weak mating of transvestites can be improved by boosting their pheromone production. Synthetic biology can characterize the factors that control efficiency in biological processes. In yeast, selection for increased mating efficiency is likely to have continually boosted pheromone levels and the ability to discriminate between partners who make more and less pheromone. This discrimination comes at a cost: weak mating in situations where all potential partners make less pheromone.

INTRODUCTION

Biological processes are typically defined by the genes that are necessary and sufficient for function. However, in many cases, this minimal gene set does not encompass all the proteins involved in a process, and additional proteins promote biological efficiency. Finding these additional proteins may require the detection of subtle phenotypes, making it hard to know if all the genes involved in a process have been identified. One way to answer this question is to reengineer a pathway and ask whether the synthetic version fully mimics the natural function. Here, we show that this form of synthetic biology illuminates how cells of the budding yeast, *Saccharomyces cerevisiae,* mate efficiently.

Budding yeast can be stably maintained as haploids or diploids. Haploids mate when two cells of opposite mating types signal to each other using reciprocal pheromones and receptors, polarize and grow towards each other, and eventually fuse to form a single diploid. Yeast has two mating types, **a** and α (Figure 1A), determined by two alternative alleles at the *MAT* locus, *MAT***a** and *MAT*α, which encode different transcription factors (Herskowitz 1988). These factors regulate the expression of mating type-specific genes, many of which are involved with the production and detection of the pheromones yeast cells use to signal to one another. The pheromones (**a-** and α-factor) are detected by G-protein coupled receptors; *MAT***a** cells express **a**-factor (Betz and Duntze 1979), which is secreted through an ATP binding cassette (ABC) transporter (Ste6) (McGrath and Varshavsky 1989) and the α-factor receptor (Ste2) (Blumer *et al.* 1988; Dohlman and Thorner 2001). *MAT*α cells express α-factor (Kurjan and Herskowitz 1982; Singh *et al.* 1983) and the **a**-factor receptor (Ste3) (Hagen *et al.* 1986; Dohlman and Thorner 2001). Pheromone binding activates a signaling pathway which produces three

responses: cell polarization, cell cycle arrest in G1, and increased transcription of mating-type specific genes (Bardwell 2005).

Bender and Sprague (1989) used mutations that alter pheromone and receptor expression to show that a cell's mating type is determined by which pheromones and receptors it expresses. Although pheromone secretion and detection are the essential elements for mating, additional, mating type-specific genes make mating more efficient. One of these is the *MAT***a**-specific α-factor protease, Bar1 (Sprague Jr and Herskowitz 1981; MacKay *et al.* 1988), which helps *MAT***a** cells detect an α-factor gradient and polarize towards *MAT*α partners (Jackson and Hartwell 1990; Barkai *et al.* 1998). Yeast cells also express mating-type specific agglutinins, which help cells attach to mating partners (Cappellaro *et al.* 1991) in liquid but individually have little effect on mating efficiency on solid media (Lipke *et al.* 1989; Roy *et al.* 1991; de Nobel *et al.* 1995). Evidence for the final, characterized *MAT***a**-specific gene was produced by Bender and Sprague (1989) who noticed that cells expressing *MAT***a**-specific proteins and Ste3 were unable to mount a pheromone response. The gene responsible for this was later identified as *ASG7*, which terminates pheromone signaling after mating has occurred and allows diploid cells to escape from the G1 arrest of their parental haploid cell (Kim *et al.* 2000; Roth *et al.* 2000).

Bender and Sprague (1989) used mutations at *MAT* and exogenous promoters to manipulate pheromone and receptor expression. As a result, any quantitative defects in mating could reflect incorrect levels of pheromone and receptor expression or the accessory role of other genes in mating. To distinguish these possibilities we constructed “transvestite” strains: genetically engineered strains that have a wild-type allele at *MAT* but express the pheromone, pheromone receptor, and proteins responsible for secreting or processing pheromones that are normally induced by the other *MAT* allele (Figure 1B). These strains should mate well if we

have swapped all the genes required for efficient mating and expressed them at the right level. Mating defects in these engineered cells indicate the presence of additional, uncharacterized, mating type-specific proteins or incorrect expression of the known mating genes.

By studying these genetically engineered cells, we learned more about the requirements for efficient mating. *MAT***a**-playing-α cells (*MAT***a** cells that express α-factor and Ste3) mate three-fold worse than genuine *MAT*α cells. Their main defect is low α-factor secretion: increasing α-factor production makes them mate almost as well as genuine *MAT*α cells. In contrast, *MAT*α-playing-**a** cells (*MAT*α cells that express **a**-factor, Ste6, Ste2, and Bar1) mate 60-fold worse than genuine *MAT***a** cells. These transvestites have two defects: they express a novel, *MAT*α-specific **a**-factor blocker, which we named Afb1 (**a**-factor barrier), and they show a transient as opposed to a prolonged arrest when exposed to α-factor. Our manipulations reveal that mating is not robust to reduced levels of pheromone production.

MATERIALS AND METHODS

Yeast strains and culturing: Table S1 lists the strains we used. All strains were derived from the W303 wild-type background (*ade2-1 can1-100 his3-11,15 leu2-112 trp1-1 ura3-1*) using standard genetic techniques. All media were prepared as described (Sherman *et al.* 1974) and contained 2% wt/vol of glucose. Cells were grown in Synthetic Complete media (2% glucose) (SC) or Yeast Extract Peptone Dextrose (2% glucose) (YPD) at 30^{o}C in culture tubes on roller drums or on agar plates or at room temperature (25^{o}C) for timelapse microscopy. Mating assays used agar plates containing SC without adenine (SC-ade), SC without uracil (SC-ura), or SC without adenine and uracil (SC-ade-ura). Bovine serum albumin (BSA) was used to reduce

the non-specific absorption of α-factor to glass and plastic surfaces. A 10% wt/vol stock was prepared in deionized water and then diluted into media to 0.1% wt/vol. Synthetic α-factor (Biosynthesis, Lewisville, TX) was suspended in dimethyl sulfoxide (DMSO) and then diluted into either YPD + 0.1% BSA or SC + 0.1% BSA at the appropriate concentration. Yeast extract was obtained from EMD Millipore (Billerica, MA). Peptone and yeast nitrogen base were obtained from BD (Franklin Lakes, NJ). Bacto-agar was obtained from US Biological (Swampscott, MA). Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Quantitative Mating Assay: Quantitative mating assays were modified from Reid and Hartwell (1977). Briefly, cells were grown to log phase (~$5x10^6$ cells/mL). $5x10^6$ cells were harvested from each strain, mixed at a 1:1 ratio, sonicated, and filtered onto a 0.22μm nitrocellulose filter (Millipore, MA). Filters were placed on a YPD plate and incubated at 30^oC for 5 hours. To assay for the initial ratio of the haploid cells, a $2.5x10^{-5}$ dilution of the initial mating mixture was plated onto SC-ade and SC-ura plates. After 5 hours, cells were washed off the filters into 1 mL of deionized water and then plated onto SC-ade, SC-ura, and SC-ade-ura plates at appropriate dilutions to produce ~400 colonies per plate. SC dropout plates were incubated for 2 days before counting the colonies on each plate. Mating efficiencies were determined by dividing the number of colonies on the SC-ade-ura plate by the number of colonies on whichever of the SC-ade or SC-ura plates plated after the mating incubation had fewer colonies. Three technical replicates were done of each mating assay and averaged for a single biological replicate. Error bars are the standard deviation of at least 5 biological replicates. Statistical significance was determined using Student's *t*-test.

Bioassay for α-factor production: The bioassay for α-factor production was modified from Gonçalves-Sá and Murray (2011). For details see File S1.

Shmooing index: Cells were grown to log phase (~$5x10^6$ cells/mL), washed into YPD + 0.1% BSA with various concentrations of synthetic α-factor added at $5x10^5$ cells/mL, and then incubated at 30°C on a roller drum for 2 hours. After incubation, the cells were sonicated, fixed using 60% ethanol at -20°C, and resuspended into 20% glycerol in phosphate buffered saline (PBS). Culture tubes were BSA-coated by incubating overnight at 4°C with PBS + 2% BSA. The PBS + 2% BSA was poured out immediately prior to the use of the culture tube. At least 200 cells were counted to determine the percentage of cells shmooing. Error bars are standard deviations. Statistical significance was determined using Student's *t*-test.

Microscopy: Microscopy was done at room temperature using a Nikon Ti-E inverted microscope with either a 20x Plan Apo VC 0.75NC air lens or a 60x Plan Apo VC 1.4NA oil lens, and images were acquired with a Photometrics CoolSNAP HQ camera (Roper Scientific, AZ). Timelapse photography was done using Metamorph 7.7 (Molecular Devices, CA). For details see File S2.

Halo Assay: Halo assays were modified from Sprague (1991). Cells whose **a**-factor production was to be measured were grown to saturation in YPD at 30°C. For halo assays on individual strains, $4.5x10^8$ cells of each strain were pelleted and resuspended in 20µL of deionized water. For halo assays on cell mixtures, cells were mixed at a 1:8 ratio (*MAT***a**

wildtype:cell type of interest) with a final cell count of $4.5x10^8$. Cells were pelleted and resuspended in 20µL of deionized water. 10µL of each strain or strain mix was spotted onto YPD plates and incubated overnight (~24 hours) at 30°C. Supersensitive *MATα sst2Δ* cells grown to stationary phase were then sprayed over the cell spots using a martini atomizer (Item 900432, Oenophilia, Hillsborough, NC). Plates were incubated overnight (~18 hours) at 30°C, and pictures were taken using a Panasonic (Secaucus, NJ) Lumix DMC-TZ5 camera.

RNA isolation and sequencing: Cells were grown to log phase ($5x10^6$ cells/mL) in YPD + 0.1% BSA at 30°C. 10 mL of the culture was harvested by spinning at 4°C, washed in 1 mL RNase-free ice-cold water, pelleted, and flash frozen in dry ice. 10nM α-factor was added to the remaining culture, incubated for 2 hours at 30°C, and harvested in the same manner. RNA was isolated as described by Collart and Oliveiro (2001) and dissolved in 1mM sodium citrate, pH 6.4. RNase-free chemicals were obtained from Invitrogen (Carlsbad, CA) except for chloroform, which was obtained from VWR (Radnor, PA).

RNA libraries were prepared using the Illumina TruSeq kit (www.illumina.com) and sequenced using an Illumina HiSeq 2000 with 50 base pair, single end reads with 89x mean coverage across the genome.

Sequence analysis: To analyze the sequencing data, the RNA sequences were aligned to the S288C reference genome r64 (downloaded from the Saccharomyces Genome Database www.yeastgenome.org) using TopHat (Trapnell *et al.* 2009). We then used Cufflinks (Trapnell *et al.* 2010) to look for genes with significantly different levels of gene expression between *MAT***a** *bar1Δ* cells and *MAT*α-playing-**a** P_{BAR1}-*BAR1* cells. Significant differences in expression

were identified using the default setting in Cufflinks, which tests the observed log-fold-change in gene expression against the null hypothesis of no difference between the two samples with a false discovery rate of 0.05 (Trapnell *et al.* 2010). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and are accessible through GEO Series accession number GSE49372 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49372).

RESULTS

Transvestite cells can mate: To find genes required for efficient mating, we constructed two types of transvestite strains. *MAT***a**-playing-α cells are *MAT***a** cells that have been engineered to produce α-factor and the **a**-factor receptor by replacing the open reading frame of *STE2* with *STE3* and the open reading frames of the two **a**-factor genes with those of the two α-factor genes (replacing *MFA1* with *MFα1* and *MFA2* with *MFα2*) (Figure 1B). We also deleted *BAR1*, which encodes the α-factor protease (Sprague Jr and Herskowitz 1981), and *ASG7*, which inhibits signaling from Ste3 (Roth *et al.* 2000). However, these cells are still *MAT***a** at the *MAT* locus and, thus, will have *MAT***a**-specific expression patterns for all genes except those we manipulated. *MAT*α-playing-**a** cells are *MAT*α at the *MAT* locus but have been engineered to produce **a**-factor and the α-factor receptor by replacing the open reading frame of *STE3* with *STE2*, *MFα1* with *MFA1*, and *MFα2* with *MFA2* (Figure 1B). We also drove the expression of *BAR1* with an engineered version of the haploid specific promoter, P_{FUS1} (Ingolia and Murray 2007), which is expressed in both *MAT***a** and *MAT*α cells (Trueheart *et al.* 1987), and the expression of the **a**-factor transporter, *STE6* with the *MFα1* promoter. Since we tested mating

efficiency on solid media, we did not manipulate the expression of the mating agglutinins, which function mainly when cells mate in liquid (Lipke *et al.* 1989; Roy *et al.* 1991).

We asked if the manipulated genes are the only mating-type specific proteins required for efficient mating. Crossing these cells with wild-type cells of their original mating type (e.g. *MAT*α-playing-**a** crossed to *MAT*α) has a potential caveat. When two wild-type cells mate, the combination of the transcription factors expressed from *MAT***a** and *MAT*α turns off the pheromone and receptor genes of both mating types (Haber 1998), and the zygotes escape pheromone-induced G1 arrest (Roth *et al.* 2000). But zygotes produced by crossing a transvestite to a wild-type cell of the same mating type will keep expressing pheromones and receptors from both mating types (since both parents have the same *MAT* locus), raising the concern that these zygotes respond to their own pheromones and remain arrested in G1. To measure the mating efficiency of these crosses, we selected for viable diploids by crossing transvestite cells and wild-type cells with complementary nutritional requirements. We obtained both *MAT***a**-playing-α/*MAT***a** and *MAT*α-playing-**a**/*MAT*α diploids. Most of these diploids progress normally through the cell cycle and have normal cell morphology (Figure 1C). We did find an occasional population of *MAT*α-playing-**a**/*MAT*α diploids with abnormal morphology, suggesting delayed progression through G1, but even these are capable of budding (Figure 1C). The ability of these diploids to bud indicates that it is possible to measure the mating efficiency of transvestites crossed with wild-type cells.

We used quantitative mating assays to measure the mating efficiency of the transvestite cells. Cells of the two mating types are incubated together and then plated on media that distinguishes diploid cells from either parental haploid. When wild-type *MAT***a** cells are mated with wild-type *MAT*α cells, 66% of haploids form diploids (Figure 1D). However, the mating

efficiency of the *MAT***a**-playing-α cells crossed with *MAT***a** cells is three-fold lower than that of a wild-type cross, and the mating efficiency of the *MAT*α-playing-**a** cells crossed with *MAT*α cells is about 60-fold lower than the efficiency of a wild-type cross (Student's *t*-test, $p<10^{-6}$) (Figure 1D). These mating defects are synergistic: crossing the transvestite strains to each other decreases mating efficiency about 700-fold, (Student's *t*-test, $p<10^{-6}$) (Figure 1D). Our observation that transvestites can mate with unmanipulated strains with the same *MAT* locus, confirms earlier work showing that pheromones and receptors define a cell's mating type (Bender and Sprague 1989). But the low mating efficiency of the transvestite crosses implies that there are additional requirements for efficient mating.

MAT*a-playing-α cells produce too little α-factor:** We studied the mating defects of transvestite cells. *MATa**-playing-α cells mate three-fold less efficiently than genuine *MAT*α cells (Figure 1D). Because the engineered genes in this strain encode the pheromone and pheromone receptor, the best candidates for this difference were the ability of the *MAT***a**-playing-α cells to respond to **a**-factor and to produce α-factor.

We began by testing the response to **a**-factor. We made mating mixtures of *MAT***a**-playing-α cells expressing YFP under the pheromone-inducible promoter, P_{FUS1}, and *MAT***a** cells expressing mCherry under the *ACT1* promoter and assayed for the expression of YFP in the *MAT***a**-playing-α cells after 2.5 hours. The expression of YFP in the *MAT***a**-playing-α cells indicates that they can successfully detect **a**-factor using the **a**-factor receptor and activate pheromone-induced genes (Figure 2A). The two cell types mated to form zygotes that continue to signal to themselves, thus forming diploid cells, which express both YFP under the *FUS1* promoter and mCherry under the *ACT1* promoter (Figure 2A). Since the G-protein and

downstream components of the pheromone signaling pathway should be the same in *MAT***a** and *MAT*α cells (Bardwell 2005), we asked if reduced pheromone production is the cause of the mating defect.

Pheromone production is important for zygote formation (Kurjan 1985; Michaelis and Herskowitz 1988), and *MAT***a** cells prefer the *MAT*α cell that produces the highest amount of α-factor (Jackson and Hartwell 1990). Since we manipulated pheromone genes to make the transvestites, we measured the pheromone production of *MAT***a**-playing-α cells using a bioassay. We grew cells in rich medium, filtered out the cells, incubated the medium with *MAT***a** cells lacking the α-factor protease, Bar1, measured the fraction of cells that arrest and shmoo (the shmooing index), and compared this data to a standard curve generated with synthetic α-factor. Unstimulated, *MAT***a**-playing-α cells produce about 70 times less α-factor than *MAT*α cells (Student's *t*-test, p=0.01) (Figure 2B). To measure the α-factor production of stimulated cells, we mixed the α-factor producing cells in a 10:1 mixture with *MAT***a** *bar1Δ* cells (which produce **a**-factor and do not destroy α-factor) and measured the α-factor present in the supernatant. Stimulated *MAT***a**-playing-α cells produce 20-fold less α-factor than stimulated wild-type *MAT*α cells (Student's *t*-test, $p=9x10^{-6}$) (Figure 2B).

To test the effect of reduced pheromone production in *MAT*α cells, we knocked out *MFα1*, which is the majority α-factor producer in *MAT*α cells (Kurjan 1985). This reduces α-factor production 12-fold compared to wild-type *MAT*α cells in unstimulated cells (Student's *t*-test, p=0.02) and 9-fold in stimulated cells (Student's *t*-test, $p=10^{-5}$) (Figure 2B). We compared the mating efficiency of *MAT*α *mfα1Δ* cells, which have decreased mating efficiency (Kurjan 1985), to that of *MAT***a**-playing-α cells and determined that *MAT*α *mfα1Δ* cells only mate 1.5-fold more efficiently than *MAT***a**-playing-α cells (Student's *t*-test, p=0.004) (Figure 2C). This

suggests that the reduced mating efficiency of the *MAT***a**-playing-α cells is due, at least in part, to low α-factor production.

To test this hypothesis, we increased α-factor production in the *MAT***a**-playing-α cells by expressing *MFα1* from the *TDH3* promoter. This promoter is not pheromone-regulated, but it is one of the most highly expressed promoters in the yeast genome (Krebs 1953; McAlister and Holland 1985) and should increase α-factor production to at least wild-type *MAT*α levels. Unstimulated *MAT***a**-playing-α P_{TDH3}-*MFα1* cells secrete twice as much α-factor as unstimulated *MAT*α cells (Student's *t*-test, p=0.04), but when stimulated, *MAT***a**-playing-α P_{TDH3}-*MFα1* cells secrete 5-fold more α-factor than stimulated *MAT*α cells (Student's *t*-test, $p=10^{-4}$) (Figure 2B), suggesting that α-factor production is regulated by both post-translational mechanisms, such as pheromone maturation and secretion and transcription of the pheromone genes. If low α-factor production accounts for the weak mating of *MAT***a**-playing-α cells, *MAT***a**-playing-α P_{TDH3}-*MFα1* cells should have a mating efficiency approaching that of wild-type *MAT*α cells, which is indeed what we found (Figure 2C). This confirms previous results, which showed that sufficient α-factor production is important for efficient mating (Kurjan 1985) and shows that the principle defect of *MAT***a**-playing-α cells is insufficient α-factor production. This defect could reflect a difference in the strengths of the *MFA1* versus the *MFα1* promoter or differences in the translation or processing of α-factor between *MAT***a** and *MAT*α cells. Our analysis also shows that there are no additional *MAT***a**-specific genes, beyond those we manipulated (*STE2*, *MFA1*, *MFA2*, *BAR1*, and *ASG7*), that interfere with the ability of *MAT*α cells to mate.

***AFB1* encodes a novel a-factor barrier protein:** We examined the decreased mating efficiency of the *MAT*α-playing-**a** cells. Because pheromone production is important for

efficient mating (Kurjan 1985; Michaelis and Herskowitz 1988), we investigated **a**-factor production of *MAT*α-playing-**a** cells. Both α-factor and **a**-factor go through several processing steps before secretion (Betz and Duntze 1979; Kurjan and Herskowitz 1982). But while α-factor is secreted as a small, unmodified peptide (Kurjan and Herskowitz 1982), mature **a**-factor is modified with a 15 carbon farnesyl group, causing it to be very hydrophobic (Betz and Duntze 1979; Chen *et al.* 1997) and hard to quantify biochemically. We therefore used a bioassay to measure the relative **a**-factor production of the *MAT*α-playing-**a** cells: we plated patches of **a**-factor producing cells and then sprayed the plates with a suspension of *MAT*α cells that were made supersensitive to pheromone by deleting *SST2*, which encodes a GTPase activating protein that reduces the duration of signaling from the pheromone-activated G protein (Chan and Otte 1982a; Chan and Otte 1982b; Dohlman *et al.* 1996; Apanovitch *et al.* 1998). The **a**-factor secreted by the patch of cells arrests the *MAT*α *sst2Δ* tester cells in G1, producing a halo of growth inhibition (Chan and Otte 1982a; Chan and Otte 1982b); the halo's diameter increases with the amount of **a**-factor produced by the cell patch (Figure 3A). The halo produced by *MAT*α-playing-**a** cells is smaller than that of wild-type *MAT***a** cells, implying that *MAT*α-playing-**a** cells secrete less **a**-factor than wild-type *MAT***a** cells (Figure 3B).

We considered two explanations for the low **a**-factor secretion of *MAT*α-playing-**a** cells: *MAT*α-playing-**a** cells secrete less **a**-factor than *MAT***a** cells, or *MAT*α cells secrete a protein that provides a barrier to **a**-factor that is analogous to the *MAT***a**-specific α-factor protease, Bar1. We tested for the presence of a *MAT*α-specific **a**-factor blocker secreted from *MAT*α-playing-**a** cells by comparing the halo sizes of two mixtures of cells: *MAT***a** cells mixed with *MAT*α-playing-**a** cells and *MAT***a** cells mixed with *MAT***a** cells that lack the genes encoding **a**-factor and, thus, produce no **a**-factor (*MAT***a** *mfa1Δ mfa2Δ*). If the *MAT*α-playing-**a** cells secrete an **a**-factor

blocker, we would expect the halo size of the *MAT***a** cells mixed with *MAT*α-playing-**a** cells to be smaller than that of the *MAT***a** cells mixed with pheromone-less *MAT***a** cells because the **a**-factor blocker would interfere with the **a**-factor from both the *MAT*α-playing-**a** cells and the *MAT***a** cells. However, if there is no **a**-factor blocker, we would expect the halo size of the *MAT***a** cells mixed with *MAT*α-playing-**a** cells to be larger than that of the *MAT***a** cells mixed with pheromone-less *MAT***a** cells because both the *MAT***a** cells and the *MAT*α-playing-**a** cells are capable of secreting **a**-factor. The halo produced by *MAT***a** cells mixed with *MAT*α-playing-**a** cells is smaller than the halo produced by *MAT***a** cells mixed with pheromone-less *MAT***a** cells, indicating that *MAT*α-playing-**a** cells secrete an **a**-factor blocker (Figure 3C).

We searched for the gene responsible for this activity by comparing the transcriptomes of *MAT***a** and *MAT*α-playing-**a** cells. Although the gene expression of pheromone-stimulated *MAT***a** cells has been investigated, the extreme hydrophobicity of **a**-factor has made similar experiments on pheromone-stimulated *MAT*α cells difficult (Roberts *et al.* 2000). The *MAT*α-playing-**a** cells make it possible to study the transcriptome of pheromone-stimulated cells that are *MAT*α at the *MAT* locus but are stimulated by α-factor in a controlled fashion. We chose a concentration of pheromone, 10nM, in a regime in which *MAT***a** *bar1Δ* and *MAT*α-playing-**a** cells with *BAR1* under its endogenous promoter have a similar shmooing index (Figure 4A), to compare the transcriptomes of stimulated and unstimulated *MAT***a** and *MAT*α-playing-**a** cells using RNA sequencing.

Just as *MAT*α cells do not secrete Bar1 to cleave their own α-factor (Sprague Jr and Herskowitz 1981), we would not expect *MAT***a** cells to secrete an **a**-factor blocker to inhibit their own **a**-factor. Thus, we hypothesized that a *MAT*α-specific **a**-factor blocker would be expressed more highly in both pheromone-stimulated and unstimulated *MAT*α-playing-**a** than *MAT***a** cells.

Ten genes fit this criterion and of these, only one, *YLR040C*, is annotated as encoding a secreted protein that is not already known to be important in mating (Yeast Genome Database, http://www.yeastgenome.org) (see Table S2).

YLR040C was previously identified as an α-specific gene by its reduced transcription in a *MAT*α cell that lacked the transcription factor Matα1 (Galgoczy *et al.* 2004), which induces expression of α-specific genes (Strathern *et al.* 1981). It has also been shown to be translated by ribosome profiling (Brar *et al.* 2012) and localized to the cell wall (Hamada *et al.* 1999; Giaever *et al.* 2002). Deletion of *YLR040C* was reported as having no effect on mating (Galgoczy *et al.* 2004). We found that in unstimulated cells the gene is expressed 11-fold more strongly in *MAT*α-playing-**a** than in *MAT***a** cells and that its transcription is not significantly induced when *MAT*α-playing-**a** cells are exposed to pheromone (see Table S2). The protein is conserved in yeasts that experienced the whole genome duplication around 100 million years ago and is also found in some yeasts, such as *Hansenula polymorpha*, that substantially predate this event (Wolfe and Shields 1997; Dietrich *et al.* 2004; Dujon *et al.* 2004; Kellis *et al.* 2004) (Figure 3D). The experiments described below demonstrate that *YLR040C* encodes a protein that provides an **a**-factor barrier function, leading us to name this gene *AFB1* for **a**-factor barrier.

To determine whether *AFB1* is indeed the **a**-factor blocker, we knocked it out in *MAT*α-playing-**a** cells. The halos produced by *MAT*α-playing-**a** *afb1Δ* cells are larger than those of *MAT*α-playing-**a** *AFB1* cells, indicating that deleting *AFB1* increases the amount of pheromone secreted from a patch of *MAT*α-playing-**a** cells (Figure 3B). The halo around the *MAT*α-playing-**a** *afb1Δ* cells, however, is still smaller than the halo produced by wild-type *MAT***a** cells suggesting that *MAT*α-playing-**a** cells secrete less **a**-factor than wild-type *MAT***a** cells (Figure 3B). We also placed *AFB1* under a strong (*ACT1*) promoter in *MAT***a** cells and observed a

decrease in halo size compared to wild-type *MAT***a** cells, indicating that Afb1 is able to block **a**-factor secreted by *MAT***a** cells (Figure 3B).

To test whether Afb1 is responsible for blocking **a**-factor produced by other cells, we made cell mixtures of *MAT***a** cells with *MAT*α-playing-**a** *afb1Δ* cells and compared the halo produced by this mixture to the halo produced by the mixtures of *MAT***a** cells with *MAT*α-playing-**a** cells and to the halo produced by *MAT***a** cells with *MAT***a** *mfa1Δ mfa2Δ* cells. As expected from our other results, the mixture of *MAT***a** cells with *MAT*α-playing-**a** *afb1Δ* cells has a slightly larger halo than the *MAT***a** cells mixed with *MAT***a** *mfa1Δ mfa2Δ* cells and a significantly larger halo than the *MAT***a** cells mixed with *MAT*α-playing-**a** cells (Figure 3C). This result indicates that when Afb1 is not present in the cell mixtures, the **a**-factor from the wild-type *MAT***a** cells as well as that from the *MAT*α-playing-**a** cells is free to interact with the supersensitive *MAT*α cells. Taken together, our results provide strong evidence that Afb1 has an **a**-factor barrier function.

We asked whether the expression of *AFB1* affected the mating efficiency of *MAT*α-playing-**a** cells. We crossed *MAT*α-playing-**a** *afb1Δ* cells with wild-type *MAT*α cells and observed a five-fold increase in mating efficiency over a similar cross with *MAT*α-playing-**a** cells (Student's *t*-test, $p<10^{-6}$) (Figure 3E). However, deleting *AFB1* from wild-type *MAT*α cells does not reduce their mating efficiency (Figure 3E), perhaps because small changes in **a**-factor production do not have a large effect on mating efficiency (Michaelis and Herskowitz 1988). We tested this possibility in two ways. The first was to delete *MFA1* from wild-type *MAT***a** cells. We saw a small decrease in the halo size of *MAT***a** *mfa1Δ* compared to that of wild-type *MAT***a** cells, but as previously reported (Michaelis and Herskowitz 1988), the mating efficiency of *MAT***a** *mfa1Δ* cells was statistically indistinguishable from that of *MAT***a** *MFA1* cells (Figure 3B

and 3E). We also tested the mating efficiency of *MAT***a** cells with *AFB1* placed under the *ACT1* promoter. These cells produce a smaller halo than wild-type *MAT***a** cells (Figure 3B) but mate slightly better than wild-type *MAT***a** cells, implying that there is a range of **a**-factor production that results in efficient mating, at least in the absence of additional mutations (Student's *t*-test, p=0.03) (Figure 3E).

MATα*-playing-a cells shmoo but arrest only transiently in the presence of pheromone:** Although the expression of *AFB1* in *MAT*α-playing-**a** cells was responsible for a portion of the reduced mating efficiency of *MAT*α-playing-**a** cells, *MAT*α-playing-**a** *afb1Δ* cells still mate 12-fold worse than wild-type *MATa** cells (Student's *t*-test, $p<10^{-6}$) (Figure 3E). We hypothesized that the response of *MAT*α-playing-**a** cells to pheromone could also reduce their mating efficiency.

There are three cellular responses to pheromone stimulation: altered gene expression, cell polarization, and cell cycle arrest (Bardwell 2005). We compared the transcriptomes of *MAT*α-playing-**a** and *MAT***a** cells both with and without exposure to α-factor (see Table S2), excluding those genes, such as *STE3* and *BAR1*, that had been removed during the construction of the strains. Twenty-one genes showed a more than two-fold variation in both comparisons. Ten genes showed a more than two-fold variation when comparing the two stimulated cell types but were not significantly different when comparing the unstimulated cells, and another 8 genes showed the opposite pattern. As expected, known α-specific genes, such as the *MAT*α-specific agglutinin gene, *SAG1*, were expressed more strongly in *MAT*α-playing-**a** cells than in *MAT***a** cells, and known **a**-specific genes, such as the **a**-specific agglutinin gene, *AGA2*, were expressed less strongly in *MAT*α-playing-**a** cells than in *MAT***a** cells. Despite our attempts to engineer their

expression to match the levels seen in *MAT***a** cells, three important **a**-specific genes, *STE2*, *MFA1*, and *MFA2* are expressed at five-fold, seven-fold, and 120-fold lower levels, respectively, in *MAT*α-playing-**a** cells compared to *MAT***a** cells (see Table S2).

We assayed cell polarization (Segall 1993; Butty *et al.* 1998) by measuring the shmooing index of *MAT*α-playing-**a** cells stimulated with known quantities of synthetic α-factor. We found that *MAT*α-playing-**a** P_{BAR1}-*BAR1* cells have a similar shmooing index to *MAT***a** *bar1Δ* cells at low concentrations of α-factor, indicating that the *MAT*α-playing-**a** cells are as sensitive to low concentrations of α-factor as *MAT***a** cells (Figure 4A) and suggesting that *BAR1* is not expressed in *MAT*α cells. Because efficient mating in *MAT***a** x *MAT*α crosses depends on the secretion of Bar1 by the *MAT***a** cells, we investigated the pheromone response of *MAT*α-playing-**a** cells that express *BAR1* from a mutant version of the pheromone-induced *FUS1* promoter (Trueheart *et al.* 1987): the mutant promoter, P_{FUS1*}, was selected to have a low basal and a high pheromone-stimulated level of expression (Ingolia and Murray 2007). These cells make fewer shmoos at 50nM α-factor than *MAT*α-playing-**a** P_{BAR1}-*BAR1* cells (which lack detectable Bar1 activity) make at 2nM α-factor, indicating that *MAT*α-playing-**a** P_{FUS1*}-*BAR1* cells are able to secrete Bar1 (Student's *t*-test, p=0.02) (Figure 4A and 4B). In contrast, the *MAT*α-playing-**a** P_{FUS1*}-*BAR1* cells make significantly more shmoos than wild-type *MAT***a** cells at each concentration of α-factor tested (Student's *t*-test, $p<0.005$) (Figure 4B), suggesting that *MAT*α-playing-**a** P_{FUS1*}-*BAR1* cells secrete less Bar1 than wild-type *MAT***a** cells. This difference is unlikely to be the sole remaining cause of the mating defect of *MAT*α-playing-**a** P_{FUS1*}-*BAR1* cells: reducing the expression of Bar1 in *MAT***a** cells, by expressing it from the *FUS1** promoter, does not reduce their mating efficiency (Figure 4C).

We observed α-factor-induced cell cycle arrest (Chang and Herskowitz 1990) in a microfluidic device. Pheromone stimulation arrests *MAT***a** cells in G1 through phosphorylation of Far1, a protein that binds to cyclin-dependent kinase/cyclin complexes (Chang and Herskowitz 1990; Tyers and Futcher 1993; Peter and Herskowitz 1994). When *MAT***a** *bar1Δ* cells are exposed to 10nM α-factor, their cell cycle remains arrested for many hours while they form multiple successive shmoos (Figure 4D and see File S3). However, even at this high α-factor concentration, *MAT*α-playing-**a** *bar1Δ* cells form shmoos but arrest only transiently (Figure 4D and see File S4).

The transient cell cycle arrest in *MAT*α-playing-**a** cells could be due to a difference in the response of *MAT*α and *MAT***a** cells to pheromone stimulation or the inhibition of Ste2 by *MAT*α-specific proteins in the *MAT*α-playing-**a** cells. We tried to find the responsible genes by looking for differential expression of genes that might have an effect on cell cycle arrest between pheromone-stimulated *MAT***a** and *MAT*α-playing-**a** cells. We manipulated the expression of three candidates, *PCL1*, *GYP8*, and *TOS4*, which had at least a two-fold difference in expression between stimulated *MAT***a** and *MAT*α-playing-**a** cells and a plausible connection to cell cycle control (see Table S2). None of these manipulations altered the pheromone-induced cell cycle arrest of either *MAT***a** or *MAT*α-playing-**a** cells (Data not shown).

How robust is mating?: Mating would be robust to variation in pheromone levels if substantial increases or decreases in pheromone expression had no effect on mating efficiency. The mating of the transvestite strains to each other suggests that mating efficiency is not robust to variation in pheromone production. Mating *MAT***a**-playing-α cells to wild-type *MAT***a** cells reduces mating frequency three-fold, and mating *MAT*α-playing-**a** cells to wild type *MAT*α cells

reduces mating 60-fold, relative to a wild type *MAT***a** x *MAT*α cross, but the mating frequency of the cross between the two transvestites is reduced 700-fold, suggesting that mating defects are synergistic (Student's *t*-test, $p<10^{-6}$) (Figure 1D). If this synergism is largely due to reduced pheromone production by the transvestite strains, increasing pheromone production should increase the efficiency of the inter-transvestite cross. We increased α-factor production from *MAT***a**-playing-α cells by placing *MFα1* under the control of the *TDH3* promoter and **a**-factor production from *MAT*α-playing-**a** cells by deleting *AFB1*. When crossed to each other, these strains mate 90 times better than the cross between the original *MAT***a**-playing-α and *MAT*α-playing-**a** cells. Thus, after improving pheromone production, the inter-transvestite cross is only eight-fold less efficient than a standard *MAT***a** x *MAT*α cross (Student's *t*-test, $p<10^{-6}$) (Figure 5). If reduced pheromone production is the primary cause of the weak mating of the inter-transvestite cross, the cross between a *MAT*α strain making less α-factor and a *MAT*α-playing-**a** cell should mimic the inter-transvestite cross. The mating efficiency of the cross between *MAT*α *mfα1Δ* cells, which produce less α-factor than wild-type *MAT*α cells, and *MAT*α-playing-**a** cells is statistically indistinguishable from that of the double transvestite cross (Figure 2B and 5).

DISCUSSION

Our experiments show that genetic engineering can be used to investigate the factors that control the efficiency of mating in budding yeast. We tested the idea that previous research had found all the genes that control mating efficiency by engineering transvestite strains that switch the mating genes of one mating type for those that are normally expressed in its partner. The behavior of these strains led to two conclusions: there are still more genes that control mating,

such as the **a**-factor barrier protein, Afb1, and mating is not robust to reductions in pheromone production.

Engineering efficient maters: Investigating the pheromone production of the transvestite strains allowed us to account for a significant portion of their mating defects. Unstimulated *MAT***a**-playing-α cells secrete 70-fold less α-factor than wild-type *MAT*α cells, and stimulated *MAT***a**-playing-α cells secrete 20-fold less α-factor than stimulated *MAT*α cells. Increasing the α-factor production of the *MAT***a**-playing-α cells increased their mating efficiency to nearly that of wildtype, showing that the main defect of the *MAT***a**-playing-α cells is low α-factor production and that the level of α-factor secretion is important for efficient mating. The observation that α-factor secretion is still pheromone-inducible, even when α-factor expression is driven by a strong, constitutive promoter, demonstrates that pheromone processing and export respond to pheromone stimulation. Indeed, Ste13, a protein required for the maturation of α-factor (Julius *et al.* 1983), is pheromone-induced (Achstetter 1989).

The mating defects of *MAT*α-playing-**a** cells are more complex. We determined that these cells do not make as much **a**-factor as wild-type *MAT***a** cells and that at least part of this is due to the expression of the novel **a**-factor blocker, Afb1. Increasing the **a**-factor production of *MAT*α-playing-**a** cells by deleting *AFB1* causes a five-fold increase in their mating efficiency, indicating that sufficient **a**-factor expression is important for efficient mating as a *MAT***a** cell. We were unable to engineer *MAT*α cells to mate efficiently as *MAT***a** cells. There are two possible explanations for the remaining defect: even after the removal of Afb1, the *MAT*α-playing-**a** cells make less **a**-factor than *MAT***a** cells, and *MAT*α-playing-**a** cells only arrest

transiently in response to α-factor. We suspect both contribute to the reduced mating of *MAT*α-playing-**a** cells.

Similar pheromone and receptor swaps have been done on other fungi, including *Cryptococcus neoformans* (Stanton *et al.* 2010) and *Ustilago maydis* (Bölker *et al.* 1992). Like the strains we constructed, the engineered versions of these organisms could mate to cells that bore the same genes at the mating type locus. In *Candida albicans*, **a**-**a** or α-α matings can be induced by enhancing autocrine signaling (Alby *et al.* 2009). Studying the mating defects of engineered transvestites in other fungi, should identify additional genes involved in their mating pathways.

AFB1* encodes a novel *MAT*α-specific a-factor barrier protein:** Studies on pheromone-induced genes in *MAT*α cells were hampered by the difficulties in working with **a**-factor. We avoided these by looking at the pattern of gene expression in *MAT*α-playing-**a** cells, which would still express α-specific genes but would increase their expression in response to α- rather than **a**-factor. We argued that novel α-specific genes would be identified by higher expression in *MAT*α-playing-**a** than in *MATa** cells. Mixing experiments suggested that *MAT*α-playing-**a** cells produced an extracellular factor that interfered with the action of **a**-factor, prompting us to look for the secreted product of a *MAT*α-specific gene. This computational sieve produced a single gene, *YLR040C*, which had previously been identified as a gene regulated by the *MAT*α-specific transcription factor, Matα1 (Galgoczy *et al.* 2004). Removing *YLR040C* increased **a**-factor production from *MAT*α-playing-**a** cells and the mating efficiency of *MAT*α-playing-**a** cells, leading us to rename *YLR040C AFB1* for **a**-factor barrier. There have been previous searches for a protein with **a**-factor barrier function. The first reported a

supersensitive *MAT*α mutant, which mapped to a location on Chromosome XII over 600 kilobases away from *AFB1* (Steden *et al.* 1989). The second reported the detection of *MAT*α-specific **a**-factor endopeptidase activity, but the gene responsible for this was not identified, and the protein was not purified (Marcus *et al.* 1991). Without being able to manipulate the genes involved in these studies, it is impossible to assess their effect on **a**-factor activity or stability or their relationship to *AFB1*.

Deleting *AFB1* increased the mating efficiency of *MAT*α-playing-**a** cells. Sequence analysis shows Afb1 is conserved as far as *Hansenula polymorpha* and contains an N-terminal signal sequence and C-terminal motif that suggests it is a GPI-anchored protein (Hamada *et al.* 1999) but lacks other detectable motifs. In particular, Afb1 shows no sequence homology with any other protease but contains a number of conserved aromatic residues (Figure 3D). Our inability to find Afb1 throughout the ascomycete fungi has two possible interpretations: either the protein evolves too rapidly to be detected by standard tools that use sequence homology to identify orthologs, or the protein evolved within in one branch of the ascomycete lineage, rather than in its last common ancestor. Unusually rapid evolution of a single protein or independent evolution of the same function in different lineages may also explain why the α-factor degrading protease, Bar1, in *S. cerevisiae* is not the closest homolog of the same protein in *C. albicans* (Schaefer *et al.* 2007).

We speculate that Afb1 acts to bind and sequester **a**-factor rather than to degrade it. The biological function of Afb1 may mirror that of Bar1, which promotes the efficient mating of *MAT***a** cells by keeping the α-factor concentration at the plasma membrane within the narrow range needed for accurate pheromone gradient detection (Barkai *et al.* 1998). Since Afb1 is predicted to be GPI-anchored, it is possible that the function of Afb1 closely mimics that of Bar1

trapped in the cell wall of *MAT***a** cells: creating a pheromone sink that makes it both more likely that two cells of the same mating type will avoid each other (Jin *et al.* 2011) and easier to distinguish between two, close, potential partners (Rappaport and Barkai 2012). It is also possible that Afb1 in *S. cerevisiae* acts like Bar1 in *C. albicans* (Alby *et al.* 2009): decreasing the threat of autocrine signaling caused by leaky repression of **a**-factor in *MAT*α cells.

MAT*α-playing-a cells only arrest transiently in response to pheromone:** In *MATa** cells, exposure to α-factor leads to a prolonged cell cycle arrest. In contrast, *MAT*α-playing-**a** cells show only a transient arrest, even though their ability to shmoo is statistically indistinguishable from wild-type *MAT***a** cells. This result surprised us because *MAT***a** and *MAT*α cells arrest the cell cycle in the same fashion: by signaling through Far1 (Peter and Herskowitz 1994; Bardwell 2005).

There are two possible explanations for the transient cell cycle arrest of *MAT*α-playing-**a** cells. The first is that *MAT***a** and *MAT*α cells have evolved to respond to pheromone stimulation in subtly different ways and that *MAT*α cells shmoo but do not experience enduring arrest. Although it is important for cells to be in the same phase of the cell cycle during nuclear fusion, it is possible that transient arrest of *MAT*α cells is sufficient to allow for the formation of zygotes, while a lasting arrest is required for *MAT***a** cells. Because α-factor is more diffusible, we suspect that initial signaling is usually from *MAT*α to *MAT***a** cells, meaning that it is the *MAT***a** cells that arrest first and, thus, need to wait until the *MAT*α cells receive a strong enough signal to arrest, implying that fusion would usually occur shortly after the arrest of the *MAT*α cell but at a longer and more variable time after the arrest of the *MAT***a** cell.

The second possibility is that interactions between Ste2 and proteins present in the *MAT*α-playing-**a** cells make the cells keep cycling, like cells that express both Ste3 and Asg7 (Bender and Sprague 1989; Roth *et al.* 2000). We looked for genes that might be responsible for the lack of enduring arrest in *MAT*α-playing-**a** cells by focusing on genes that are differentially expressed in pheromone-stimulated *MAT***a** and *MAT*α-playing-**a** cells and might not have been identified in earlier work. Although we tested the effect of deleting or overexpressing several candidate genes individually, we did not find an individual gene responsible for the transient cell cycle arrest in *MAT*α-playing-**a** cells.

Robustness of mating: Characterizing the mating defects of the transvestite strains allowed us to improve our understanding of the pheromone response of *MAT*α cells and study the robustness of mating efficiency to changes in gene expression. We investigated changing the expression levels of three proteins: Bar1, α-factor, and **a**-factor.

The α-factor protease, Bar1, helps *MAT***a** cells to detect an α-factor gradient and choose a mating partner (Sprague Jr and Herskowitz 1981; Jackson and Hartwell 1990; Barkai *et al.* 1998). Reducing Bar1 expression by using an engineered *FUS1* promoter (Ingolia and Murray 2007) reduces the concentration of α-factor required to get 50% of the cells to shmoo four-fold. This change appears to have little effect on mating: expressing *BAR1* under P_{FUS1*} in *MAT***a** cells leaves mating unimpaired, suggesting that mating efficiency is robust to substantial changes in Bar1 expression.

Mating efficiency is not robust to reductions in α-factor secretion, a result that might have been predicted from work that showed that cells make graded responses to increasing levels of pheromone stimulation (Moore 1983; Takahashi and Pryciak 2008). Previous studies have

shown that agglutination, shmoo formation, and pheromone-induced transcription increase with increasing α-factor concentration (Moore 1983; Takahashi and Pryciak 2008). Decreased α-factor production leads to a fusion defect and, thus, a decrease in mating efficiency (Brizzio *et al.* 1996). We show that an approximately 10-fold reduction in α-factor production in otherwise wild-type cells, such as *MAT*α *mfα1Δ* cells, results in a two-fold reduction in mating efficiency when mated to a wild-type partner. Mating *MAT*α *mfα1Δ* cells to a compromised partner, such as the *MAT*α-playing-**a** cells, results in a synergistic reduction in mating efficiency. Although reduced levels of **a**-factor production have also been shown to cause a cell fusion defect and a decrease in mating efficiency (Brizzio *et al.* 1996), the precise regulation of **a**-factor production does not appear to be as important to mating efficiency as precise regulation of α-factor production. *MAT***a** *mfa1Δ* cells have a mating efficiency that is indistinguishable from wildtype (Michaelis and Herskowitz 1988), and reducing the **a**-factor production of *MAT***a** cells by overexpressing *AFB1* actually causes a slight increase in mating efficiency, indicating that the ideal quantity of **a**-factor production may be less than the amount of **a**-factor produced by wild-type *MAT***a** cells but greater than the amount of **a**-factor produced by *MAT*α-playing-**a** cells.

Taken together, these results argue for a molecular arms race in pheromone production. Cells prefer the partner that makes the most pheromone (Jackson and Hartwell 1990), possibly because this is the only indicator of fitness available to a potential mating partner. We speculate that both *MAT***a** and *MAT*α cells have evolved to produce higher and higher concentrations of pheromone, resulting in the need for proteins such as Bar1 and Afb1 to improve gradient detection in dense mating mixtures. Once such functions have been evolved, they imply that mutations that reduce pheromone production back to ancestral levels will decrease mating efficiency because the pheromone antagonists overwhelm the lower pheromone levels.

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Figure 1. **Yeast cells expressing the pheromone and receptor of the opposite mating type are capable of forming zygotes with cells of their original mating type. A.** *MAT***a** cells express the pheromone **a**-factor, the α-factor receptor, Ste2, and the α-factor protease, Bar1. *MAT*α cells express the pheromone α-factor and the **a**-factor receptor, Ste3. **B.** *MAT*α-playing-**a** cells are *MAT*α cells that express **a**-factor instead of α-factor, Ste2 instead of Ste3, and the α-factor protease, Bar1. *MAT***a**-playing-α cells are *MAT***a** cells that express α-factor instead of **a**-factor, Ste3 instead of Ste2, and are *bar1Δ*. **C.** The indicated diploid strains were grown in YPD, and pictures were taken using DIC with 20x magnification. The right panel shows abnormal morphologies indicated of cells secreting pheromones that they can respond to. **D.** Mating efficiency of the indicated crosses. Mating efficiencies are the percentage of diploids that form colonies on double dropout plates relative to the number of colonies formed on single dropout plates. Error bars are standard deviations. Matings were performed as described in Materials and Methods.

Figure 2. **Low mating efficiency of *MAT*a-playing-α cells is due to low α-factor production. A.** *MAT***a**-playing-α P_{FUS1}-*YFP* cells in a mating mixture with *MAT***a** P_{ACT1}-*mCherry* cells. Yellow indicates YFP expression. Red indicates mCherry expression. The orange cell is a diploid expressing both YFP and mCherry. The picture was taken 2.5 hours after mixing the cells using DIC and fluorescence at 20x magnification. **B.** α-factor production is measured by growing cells in YPD, harvesting the supernatant, and exposing *MAT***a** *bar1Δ* cells to the supernatant. The shmooing index of the *MAT***a** *bar1Δ* cells is measured and then compared to a standard curve, produced with synthetic α-factor, to determine the amount of α-factor present in the media. Error bars are standard deviations. **C.** Mating efficiency relative to a wild-type cross between *MAT***a** and *MAT*α cells. Matings were performed as described in Materials and Methods. Error bars are standard deviations.

Figure 3. ***AFB1* encodes a novel a-factor blocker. A.** Halo assays are done by letting cell patches grow on YPD overnight and then spraying super-sensitive *MAT*α *sst2Δ* cells over the cell patches. Where **a**-factor produced by the cell patches has diffused into the YPD, the *MAT*α *sst2Δ* cells cannot grow, forming a halo around the cell patch with a size that corresponds to the amount of **a**-factor secretion. **B.** Halo assays done on various cell patches containing a single cell type. *MAT***a** *mfa1Δ mfa2Δ* is a negative control, and *MAT***a** is a positive control. White bars

indicate the width of the halo. The halo assays were repeated multiple times and the rank order of the halo sizes is consistent. **C.** Halo assays done on cell patches containing two cell types at a 1:8 ratio of *MAT***a** cells to the experimental cell of interest. White bars indicate the width of the halo. The halo assays were repeated multiple times and the rank order of the halo sizes is consistent. **D.** Sequences were obtained from the Yeast Genome Order Browser (http://ygob.ucd.ie) and Blast searches of fungal genomes at NCBI. Sequences were aligned in Jalview using the MAFFT-L-INS-I option, and the aligned core that follows the signal sequence and precedes the GPI anchorage sequence is shown. Amino acids are colored with a scheme that represents chemically similar amino acids in similar colors, and universally conserved amino acids are highlighted in red in the consensus sequence. Full species names: *Saccharomyces cerevisiae, Saccharomyces mikatae, Saccharomyces kudriavzevii, Saccharomyces bayanus, Candida glabrata, Kazachstania africanus, Kazachstania naganishii, Naumovzyma castelli, Naumovzyma dairenensis, Tetraspora blattae, Tetraspora phaffi, Vanderwaltomyzoa polyspora, Zygosaccharomyces rouxii, Torulaspora delbruckii, Kluveromyces lactis, Saccharomyces kluyveri, Eremothecium cymbalariae,* and *Hansenula/Ogataea polymorpha.* Of these, *Z. rouxii, T. delbrueckii, K. lactis, S. kluyveri, E. cymbalariae,* and *H. polymorpha* did not undergo a whole genome duplication. **E.** Mating efficiency of the indicated crosses relative to a wild-type cross between *MAT***a** and *MAT*α cells. Matings were performed as described in Materials and Methods. Error bars are standard deviations.

Figure 4. ***MAT*α-playing-a cells shmoo but arrest transiently in the presence of pheromone. A.** Shmooing indices of *MAT***a** *bar1Δ* cells and *MAT*α-playing-**a** P_{BAR1}-*BAR1* cells exposed to known concentrations of α-factor. Error bars are standard deviations. **B.** Shmooing indices of *MAT***a** cells and *MAT*α-playing-**a** P_{FUS1*}-*BAR1* cells exposed to known concentrations of α-factor. Error bars are standard deviations. **C.** Mating efficiency relative to a wild-type cross between *MAT***a** and *MAT*α cells. Matings were performed as described in Materials and Methods. Error bars are standard deviations. **D.** *MAT***a** *bar1Δ* cells shmooing and *MAT*α-playing-**a** *bar1Δ* cells shmooing and budding when incubated with SC plus 10nM α-factor in a microfluidic chamber. Pictures were taken using DIC with 60x magnification 8 hours after the addition of α-factor. White arrows point to buds.

Figure 5. **Mating is not robust to changes in pheromone production.** Mating efficiency relative to a wild-type cross between *MAT***a** and *MAT*α cells. Matings were performed as described in Materials and Methods. Note the logarithmic scale for mating efficiency. Error bars are standard deviations.

Figure 1

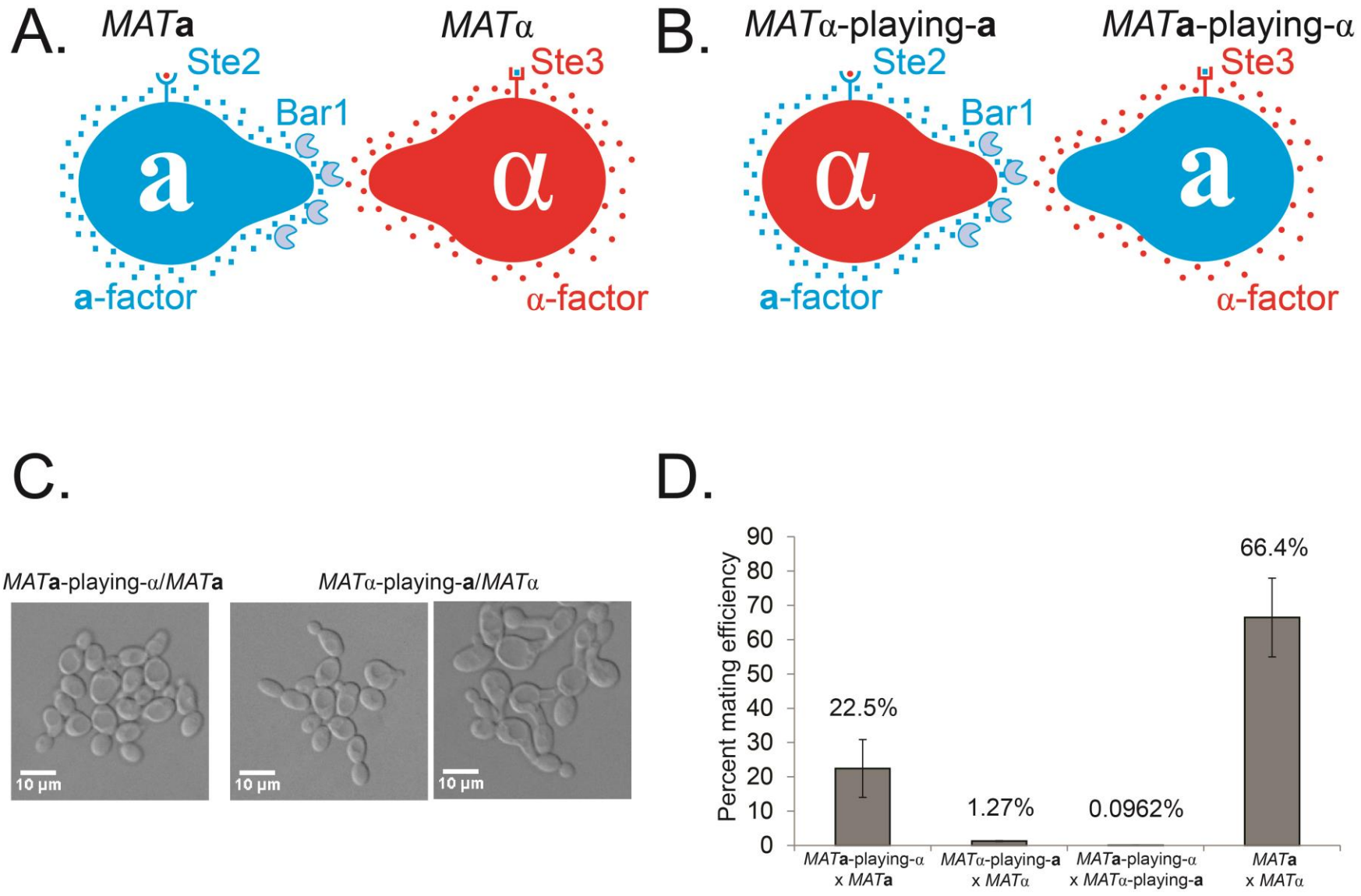

A.
MATa
Ste2
Bar1
a
a-factor
MATα
Ste3
α
α-factor
B.
MATα-playing-a
Ste2
Bar1
α
a-factor
MATa-playing-α
Ste3
a
α-factor
C.
MATa-playing-α/MATa
MATα-playing-a/MATα
10 μm
10 μm
10 μm
D.
Percent mating efficiency
90
80
70
60
50
40
30
20
10
0
22.5%
1.27%
0.0962%
66.4%
MATa-playing-α x MATa
MATα-playing-a x MATα
MATa-playing-α x MATα-playing-a
MATa x MATα

Figure 2

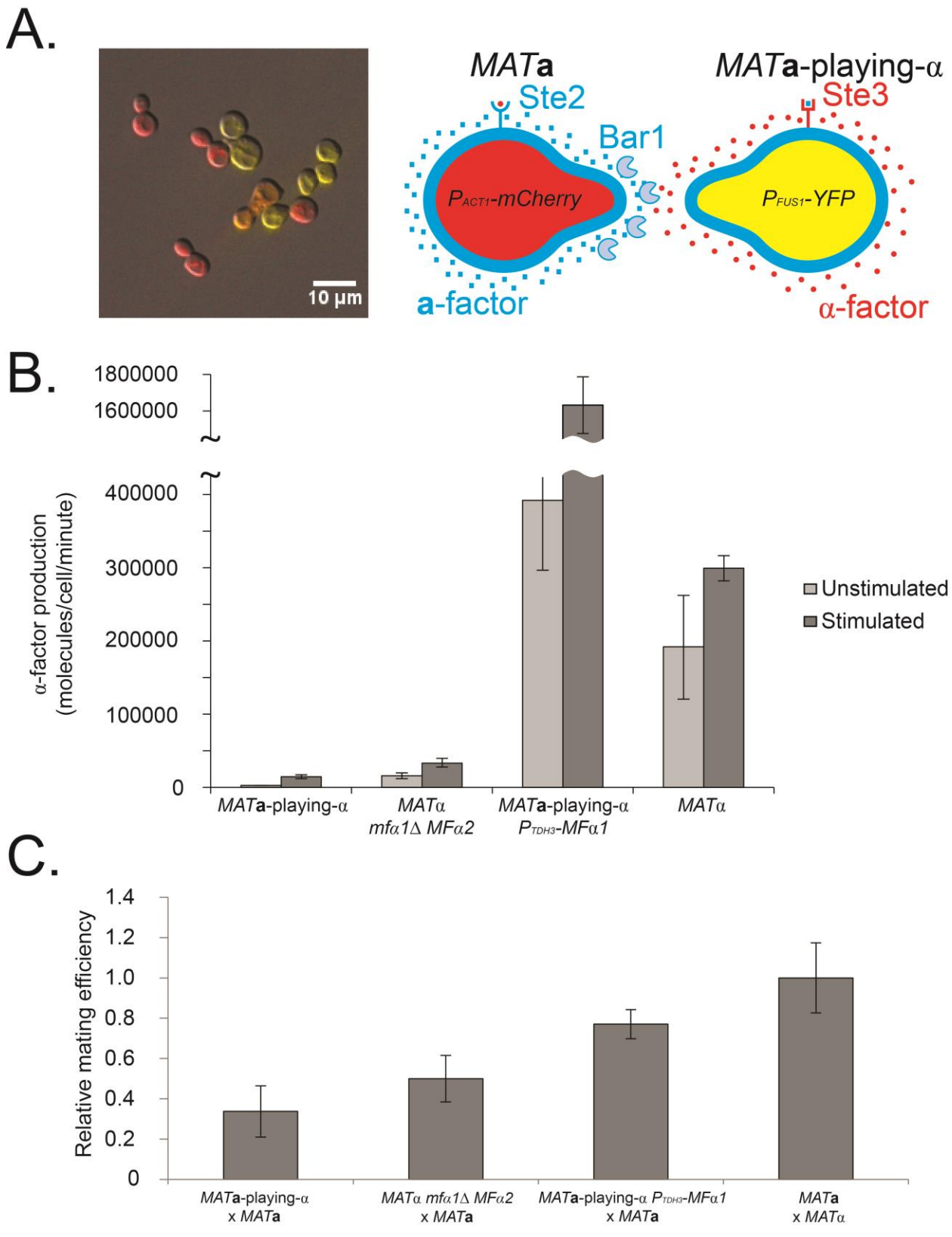

A.
10 µm
MATa
Ste2
Bar1
PACT1-mCherry
a-factor
MATa-playing-α
Ste3
PFUS1-YFP
α-factor
B.
1800000
1600000
400000
300000
200000
100000
0
α-factor production (molecules/cell/minute)
Unstimulated
Stimulated
MATa-playing-α
MATα mfα1Δ MFα2
MATa-playing-α PTDH3-MFα1
MATα
C.
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Relative mating efficiency
MATa-playing-α x MATa
MATα mfα1Δ MFα2 x MATa
MATa-playing-α PTDH3-MFα1 x MATa
MATa x MATα

Figure 3

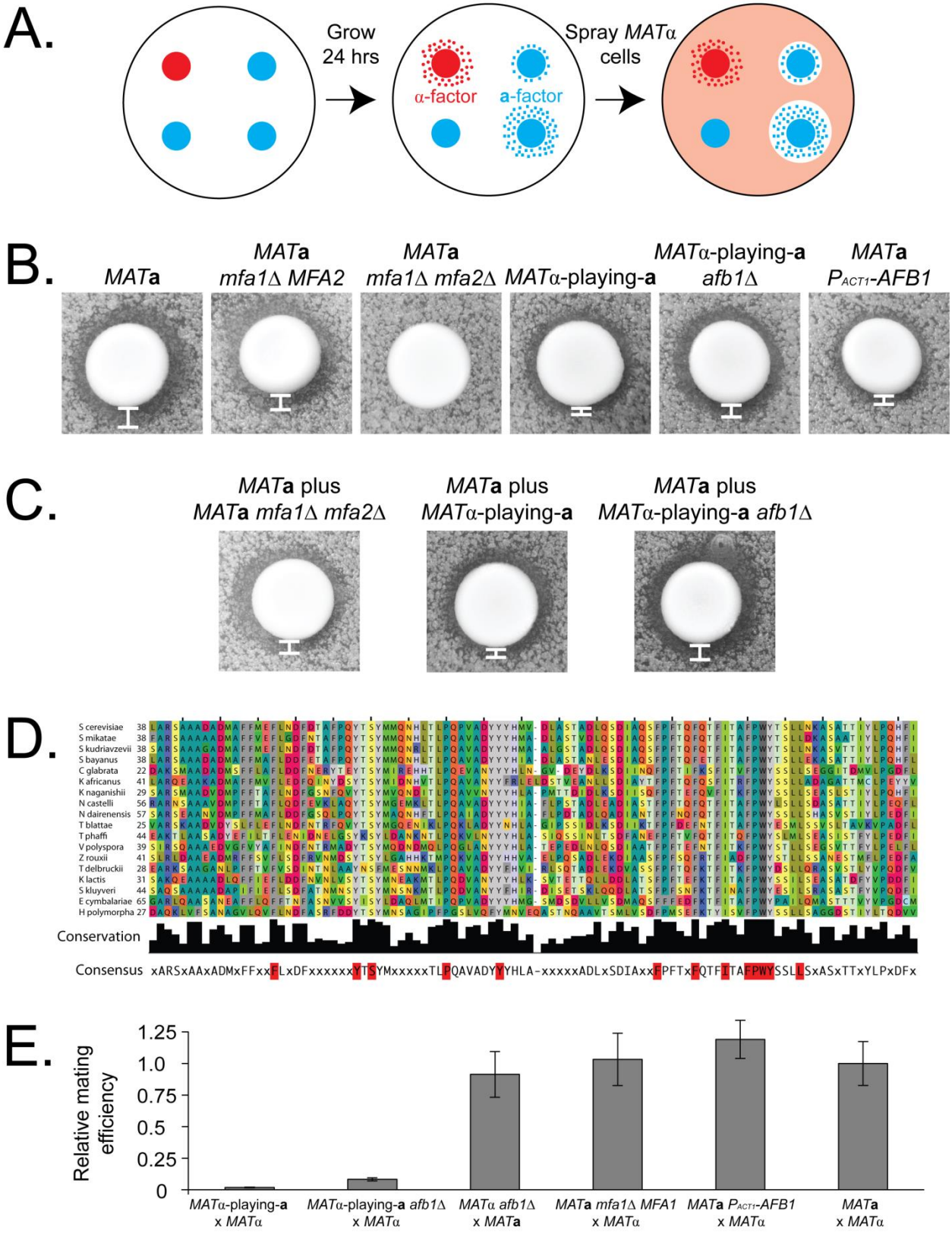

A.
Grow
24 hrs
α-factor
a-factor
Spray MATα
cells
B.
MATa
MATa
mfa1Δ MFA2
MATa
mfa1Δ mfa2Δ
MATα-playing-a
MATα-playing-a
afb1Δ
MATa
PACT1-AFB1
C.
MATa plus
MATa mfa1Δ mfa2Δ
MATa plus
MATα-playing-a
MATa plus
MATα-playing-a afb1Δ
D.
S cerevisiae
S mikatae
S kudriavzevii
S bayanus
C glabrata
K africanus
K naganishii
N castelli
N dairenensis
T blattae
T phaffi
V polyspora
Z rouxii
T delbruckii
K lactis
S kluyveri
E cymbalariae
H polymorpha
Conservation
Consensus xARSxAAxADMxFFxxFLxDFxxxxxxYTSYMxxxxxTLPQAVADYYYHLA-xxxxxADLxSDIAxxFPFTxFQTFITAFPWYSSLLSxASxTTxYLPxDFx
E.
Relative mating efficiency
1.25
1.0
0.75
0.5
0.25
0
MATα-playing-a x MATα
MATα-playing-a afb1Δ x MATα
MATα afb1Δ x MATa
MATa mfa1Δ MFA1 x MATα
MATa PACT1-AFB1 x MATα
MATa x MATα

Figure 4

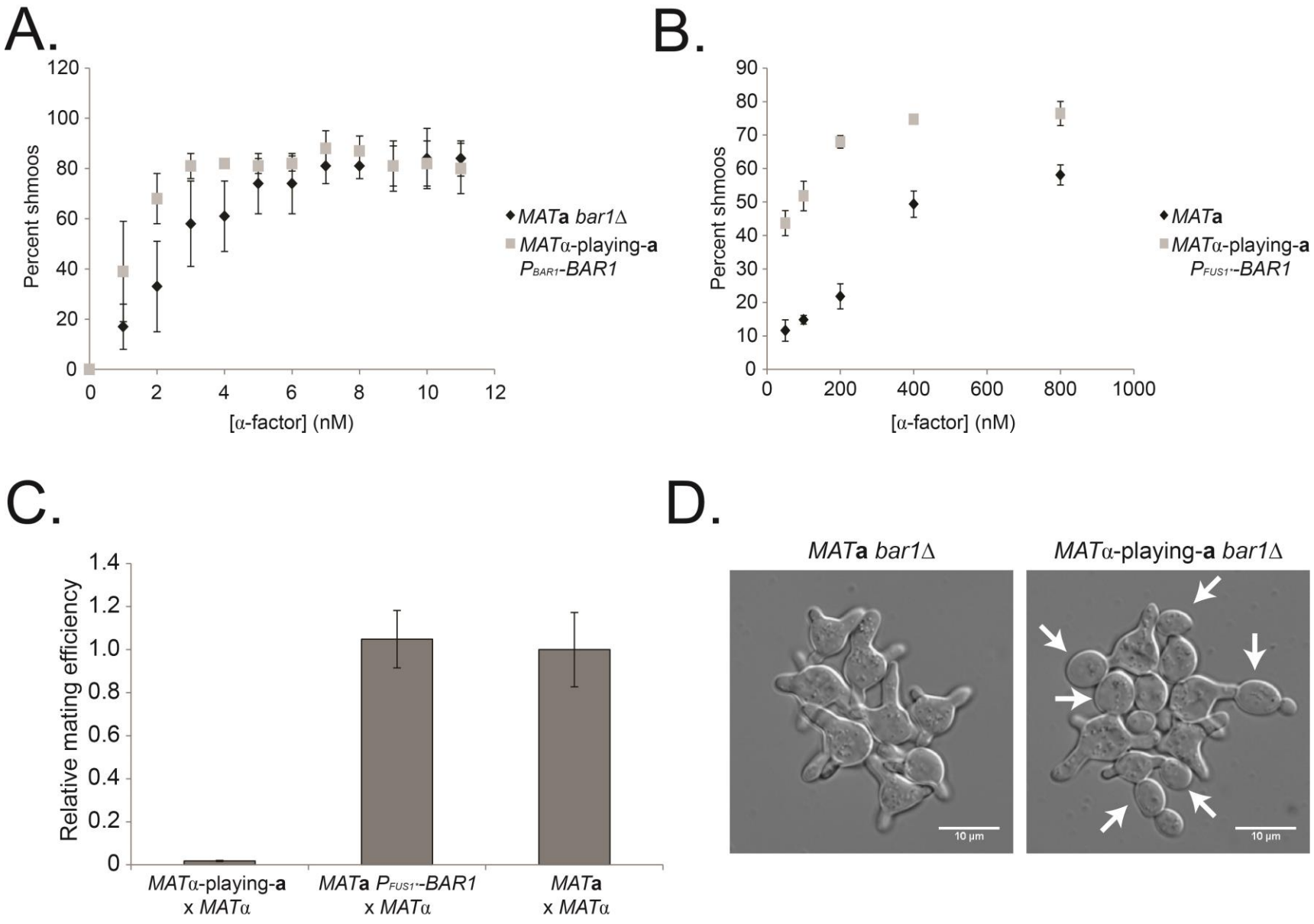
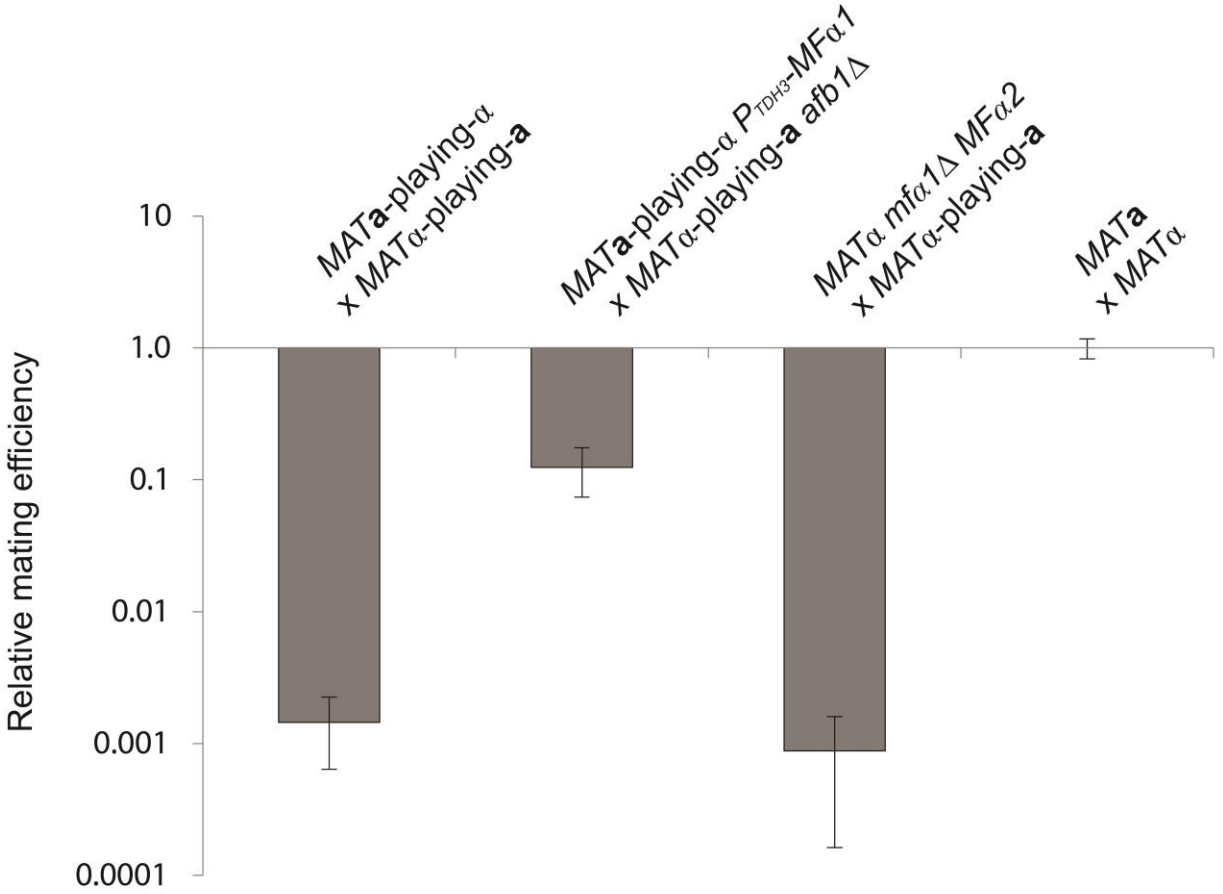

A.
Percent shmoos
[α-factor] (nM)
MATa bar1Δ
MATα-playing-a
PBAR1-BAR1
B.
Percent shmoos
[α-factor] (nM)
MATa
MATα-playing-a
PFUS1-BAR1
C.
Relative mating efficiency
MATα-playing-a x MATα
MATa PFUS1-BAR1 x MATα
MATa x MATα
D.
MATa bar1Δ
MATα-playing-a bar1Δ
10 μm
10 μm

Figure 5


MATa-playing-α
x MATα-playing-a
MATa-playing-α PTDH3-MFα1
x MATα-playing-a afb1Δ
MATα mfα1Δ MFα2
x MATα-playing-a
MATa
x MATα
Relative mating efficiency
10
1.0
0.1
0.01
0.001
0.0001