Nonsex Genes in the Mating Type Locus of *Candida albicans* Play Roles in a/α Biofilm Formation, Including Impermeability and Fluconazole Resistance

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Abstract

The mating type locus (*MTL*) of *Candida albicans* contains the mating type genes and has, therefore, been assumed to play an exclusive role in the mating process. In mating-incompetent a/α cells, two of the mating type genes, *MTLa1* and *MTLa2*, encode components of the a1-a2 corepressor that suppresses mating and switching. But the *MTL* locus of *C. albicans* also contains three apparently unrelated “nonsex” genes (NSGs), *PIK*, *PAP* and *OBP*, the first two essential for growth. Since it had been previously demonstrated that deleting either the a/α copy of the entire *MTL* locus, or either *MTLa1* or *MTLa2*, affected virulence, we hypothesized that the NSGs in the *MTL* locus may also play a role in pathogenesis. Here by mutational analysis, it is demonstrated that both the mating type and nonsex genes in the *MTL* locus play roles in a/α biofilm formation, and that *OBP* is essential for impermeability and fluconazole resistance.

Introduction

The a and α copies of the mating type locus (*MTL*) of *Candida albicans* contain several genes that play highly specialized roles in the mating process. *MATα1* and *MATα2* in the a copy, and *MATα1* and *MATα2* in the α copy [1]. This locus also contains three genes apparently unrelated to mating, the nonsex genes (NSGs) *PIK*, *PAP* and *OBP* [1–3]. Other members of the Candida clade of the hemiascomycetes also contain these NSGs in their *MTLs* [2], but members of the Saccharomyces clade, including *S. cerevisiae*, do not [4]. Interestingly, the mating type loci of several other fungi contain NSGs [3–8]. The mating type locus of *Cryptococcus neoformans*, for instance, contains approximately 20 genes, including mating type genes, mating-related genes and NSGs, both essential and nonessential [5,6]. However, there is no evidence in these other fungi that the NSGs serve related functions or play roles in mating. In *C. albicans*, the NSG *PIK* is an essential gene that encodes a phophatidylinositol kinase involved in signal transduction [9–12]. The NSG *PAP* is an essential gene encoding the poly(A) polymerase, which polymerizes adenosine (A) at the 3’ ends of mRNAs [13–16]. The NSG *OBP* is the only nonessential gene, encoding an oxysterol binding protein involved in sterol sensing in the cytoplasm [17–21]. In *C. albicans*, *PIK*, *PAP*, and *OBP* are not only located in the *MTL* locus [3], but the a and α alleles of each have diverged at rates far higher than those of other genes bordering the *MTL* locus, a characteristic of NSGs in the mating type loci of other fungi [6–8].

Until recently it had been assumed that in mating-incompetent a/α cells, the mating-related genes in the *MTL* locus play exclusive roles in repressing white-opaque switching and mating [22]. No attention had been given to the possibility that the three NSGs played a role in mating or in a common function unrelated to mating. Their known functions predicted no apparent direct role in the mating process. Two of the three genes, *PIK* and *PAP*, were demonstrated to be essential for growth in *S. cerevisiae* [9,13] and, therefore, presumably essential in *MTL* heterozygous and homozygous configurations of *C. albicans*. The mating-type genes in *MTL*, on the other hand, were all nonessential for growth, but *MTLa1* and *MTLa2* did function to suppress mating in a/α cells [1,23]. In *S. cerevisiae*, there were indications that the orthologs of *C. albicans* *MTLa1* and *MTLa2* also played non-mating roles in wall maintenance during the stress response [24] and that *MATα2* played a role in invasive growth [25]. In *C. albicans* there were also indications that genes in the *MTL* locus played non-mating roles. The configuration of the *MTL* locus was implicated in virulence in a mouse model for systemic infection. First, it was observed that natural a/α strains were on average more virulent than natural a/a or α/α strains [26]. Second, it was observed that in every case in which an a/a or α/α derivative spontaneously emerged from a natural a/α strain during *in vitro* culturing, the parental a/α strain was more virulent than the *MTL*-homozygous offspring [26]. Finally, it was observed that deleting either *MTLa1* or *MTLa2* in unrelated a/α strains resulted in decreased virulence [26]. These results indicated for the first time that the mating type locus and mating type genes might play nonsex roles in the virulence of mating-incompetent a/α cells. Given that the three NSGs were also located in the *MTL* locus, we entertained the possibility that they may also...
Nonsex Genes and Biofilms in Candida

Author Summary

Most natural strains of the yeast pathogen Candida albicans are diploid and heterozygous (a/α) at the mating type locus (MTL). The MTL locus contains mating type genes and has been assumed to play roles exclusively in the mating process of a/a and a/α cells. In C. albicans, however, the MTL locus also contains three nonsex genes (NSGs), the essential phosphatidyl inositol kinase gene, PIK, the essential poly(A) polymerase gene, PAP, and the nonessential oxyosterol binding protein gene, OBP. We demonstrate for the first time that both the mating type genes MTLa1 and MTLa2, and the three NSGs play non-mating roles in a/α biofilm formation and virulence. In addition, we show that the NSG OBP is necessary for impermeability and fluconazole resistance of a/α biofilms. These results demonstrate that nonsex genes as well as two mating type genes embedded in the mating type locus, play related roles in pathogenic processes unrelated to mating in a/α cells.

Contribute to the increased virulence and pathogenesis of a/α strains.

Here we have investigated this hypothesis by analyzing the effects of independently deleting, in two unrelated natural a/α strains, the a or α copy of the MTL locus, MTLa1 or MTLa2, all three a alleles of the NSG simultaneously, all three α alleles of NSGs simultaneously, each a allele of the three NSGs individually, each α allele of the three NSGs individually and both OBPa and OBPα simultaneously. The results reveal that the sex genes MTLa1 and MTLa2, and the NSGs, located in the mating type locus, play roles in biofilm formation and in the virulence of mating-incompetent a/α cells. In the case of the only nonessential NSG, OBP, analysis of the homozygous deletion derivative revealed that it played no discernible role in the pheromone response or fusion in the mating process, but it did play a role in the formation of a complete a/α biofilm that was both impermeable and drug-resistant, two of the most important traits of an a/α biofilm. Together, these results suggest that in mating-incompetent a/α cells all of the tested genes in the MTL locus play roles in common pathogenic processes that are unrelated to the mating process.

Results

Nonsex genes in the MTL locus

An a/α strain of C. albicans harbors an a copy of the mating type locus, MTL, on one homolog of chromosomes 5 and an α copy on the other [1]. The a copy of the locus contains the mating genes MTLa1 and MTLa2, as well as the non-sex genes (NSGαs) PIKa, PAPA, and OBPa, and the α locus contains MTLα1 and MTLα2, as well as the non-sex genes (NSGaαs) PIKα, PAPA and OBPα (Figure 1A) [1,2]. Three of four diploid species of the Candida clade (C. albicans, Candida dubliniensis, Candida tropicalis) similarly possess the three NSGαs in the MTLa locus and the three NSGaαs in the MTLα locus [2,3] (Figure 1B). In C. albicans mating type and non-mating type genes differ both in the direction of transcription and in their relative position in the opposing a and α copies of the MTL locus (Figure 1A). A fourth diploid species, Candida parapsilosis, contains the NSGaαs in MTLa, but the composition of MTLα is unknown [27] (Figure 1A). Candida orthopsilosis, a close relative of C. parapsilosis, has both a and α loci with NSGαs and NSGaαs [27]. Two additional Candida species (Candida guilliermondii, Candida lusitaniae), for which only haploid strains have been identified, contain either the NSGa in an MTLa locus or the NSGaαs in an MTLα locus [3] (Figure 1B).

The DNA homologies of the open reading frames of the a and α copies of PIK, PAPA, and OBP in C. albicans are 58, 66 and 66%, respectively, which is in marked contrast to the mean DNA homology of 99.5% computed for the open reading frames of the alleles of 50 genes neighboring the MTL locus (Figure 1C). The identity of the deduced a and α proteins encoded by PIK, PAPA, and OBP are 81, 89 and 91%, respectively, which again is in marked contrast to the mean identity of 99.6% computed for the deduced proteins encoded by a and α proteins of the same 50 genes neighboring the MTL locus (Figure 1C).

The homology between the allelic promoter regions of nine genes located upstream of MTL and nine genes located downstream were compared with the putative promoter regions of the NSGs. These 18 genes were chosen from the same 50 genes neighboring the MTL locus, because they exhibited the most polymorphic open reading frames of the set. The average DNA homology of the promoter regions (700 ± 28 bp) of the alleles of the 18 genes was 96.3% ± 3.1%. The promoter regions of alleles of all 18 genes revealed unambiguous alignment. However, comparison of the 1 kb or smaller upstream intergenic region of the a and α alleles of the NSGs revealed that sequence divergence was too high to support bona fide sequence alignments, and hence, did not allow a measure of homology. These differences indicate that the upstream regulatory regions as well as the open reading frames of the a and α copies of the three NSGs have diverged at far higher rates than genes immediately outside the MTL locus on chromosome 5.

Growth rates of all mutants are normal

To test whether MTLa1, MTLa2 and the a and α alleles of the NSGs played roles in biofilm formation and virulence, we generated the following deletion mutants in each of two unrelated natural a/α strains, P37039 and P37037 [28]: the entire MTLa locus (Δmtla); the entire MTLα locus (Δmtlα) [26]; MTLa1 (Δmtla1) [26]; MTLa2 (Δmtla2) [26]; all three of the NSGa alleles simultaneously, ΔpikΔpap Δobp (Δsoppa); all three of the NSGaα alleles simultaneously, ΔpikΔpap Δobp (Δsoppa); PIKa (Δpika); PIKα (Δpikα); PAPA (Δpapα); PAPa (Δpapα); OBPa (Δopapa); OBPα (Δopapa); and both the a and α OBP alleles, (Δobpa/Δobpa) (Figure 1D). All of the mutants formed colonies on nutrient agar after five days with average diameters similar to those of their respective parent strains (data not shown). All of the mutants of both strains generated in strain P37039 exhibited growth kinetics in suspension cultures containing modified Lee’s medium [29] that were indistinguishable from those of the parent strains (Figure 1E). All of the mutants generated in strain P37039 also exhibited growth kinetics in suspension cultures containing the richer medium YPD [30] that were indistinguishable from those of the parent strain (Figure 1F). Changes in biofilm formation or virulence by any of the mutants generated for this study could, therefore, not be attributed to a major growth defect.

Deletion of the a or α copy of the MTL locus affects biofilm formation

Deletion of either the entire MTLa locus (Δmtla), which includes the genes MTLa1, MTLa2, PIKa, PAPA and OBPa, or deletion of the entire MTLα locus (Δmtlα), which includes the genes MTLα1, MTLα2, PIKα, PAPα and OBPa, resulted in reductions in the three assessed characteristics of biofilm formation. These characteristics included adhesion to a plastic surface after 16 hours of incubation [31] (Figure 2 A, B), thickness of the biofilm that developed on a silicone elastomer surface after 48 hours [31] (Figure 2 C, D), and...
Figure 1. The *MTL* locus of *C. albicans* contains three nonsex genes (NSGs), the a and a alleles of which have diverged. A. The locus contains NSGs, poly(A) polymerase (PAP), phosphatidyl inositol kinase (PIK) and the oxysterol binding protein (OBP). The arrow heads in the genes indicate direction of transcription. B. Species in the *Candida* clade of the hemiascomycetes with a and a NSGs. C. Comparison of the DNA homology and protein identity of the a and a alleles of the NSGs and 50 genes bordering both sides of the *MTL* locus on chromosome 5. D. Genotypes of the parent strains and basic mutants used in this study. E. Growth kinetics in supplemented Lee’s medium and YPD medium revealed that none of the mutants used in this study had a growth defect. *These strains were previously described [28]; the remaining mutants were generated for this study.

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the level of β-glucan released from biofilms after 48 hour [31,32] (Figure 2E, F). The decreases in thickness were significant (all p values were <2×10^{-4}) for the Δmtla and Δmtla mutants generated in each of the two strains. The decreases for adhesion were not significant (p values ranged from 0.18 to 0.068), and those for the release of β-glucan close to significant or significant (p values ranged from 0.081 to 0.043). However, the decreases were reproducible within a mutant strain and between mutant strains generated in different natural strains. The differences were consistent with those previously reported between the biofilms formed by parental strains and spontaneous a/a or a/a derivatives [33]. Deletion of either the a or a copy of MTL, however, had no discernible effect on the general architecture of biofilms (data not shown). Laser scanning confocal microscopy (LSCM) and scanning electron microscopy (SEM) revealed that after 48 hours, mutant biofilms, like parental P37039 and P37037 biofilms, possessed a basal layer of yeast cells at the substratum and an upper region of vertically oriented hyphae embedded in an acellular matrix (data not shown).

**Deletion of MTLa1 or MTLα2 affects only biofilm thickness**

We next tested whether deletion of either of the two genes encoding the α1-α2 corepressor of switching and mating, MTLa1 and MTLα2, affects biofilm parameters in a similar manner to Δmtla and Δmtla. Figure 2 shows that deletion of MTLa1 or MTLα2 caused decreases only in biofilm thickness. A, B. Cell adhesion to a plastic surface after 16 hours of incubation. C, D. Biofilm thickness after 48 hours. E, F. The release of β-glucan by biofilms after 48 hours.}

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**Figure 2. Deletion of the a or α copy of the MTL locus caused decreases in three biofilm parameters.** Deletion of MTLa1 or MTLα2 caused decreases only in biofilm thickness. A, B. Cell adhesion to a plastic surface after 16 hours of incubation. C, D. Biofilm thickness after 48 hours. E, F. The release of β-glucan by biofilms after 48 hours. doi:10.1371/journal.ppat.1002476.g002
or MTLe2, affected biofilm formation. Deleting either of these genes in both strains P37039 and P37037 had no apparent effect on either adhesion (Figure 2A, B) or the release of β-glucan (Figure 2E, F). The differences between wild-type and mutants were statistically indistinguishable (p values were close to 1.0). However, deleting either caused decreases in biofilm thickness (Figure 2C, D) that were significant (p values ranged from $6 \times 10^{-3}$ to $10^{-4}$). These differences were obtained in three independent experiments performed for each of the two parental strains and mutant derivatives, measuring three locations in each of three biofilms of each strain. This provided 27 individual measurements per mutant or parental strain. In every experiment, the thickness of the biofilms formed by the Amt1a mutant derivative was 10 to 20% less than that of the parental strain and the biofilm of the AMT22 mutant 20 to 40% less than that of the parental strain (data not shown). LSCM and SEM revealed that the thinner biofilms formed by the Amt1a and AMT22 derivatives still exhibited the basic cell architecture of parental a/a biofilms, including a basal yeast cell layer and a larger upper portion containing vertically oriented hyphae interspersed with an acellular matrix (data not shown).

Simultaneous deletion of all three NSGa or NSGα alleles affects biofilm formation

Deletion of MTLa1 and MTLa2, therefore, did not affect adhesion or release of β-glucan, and the decreases in the thickness of the biofilms formed by Δmtla1 and Δmtla2 cells, although significant, were not as great as those that occurred when the entire a or α locus was deleted in the mutants Δmtla1 or Δmtla2, respectively (Figure 2C, D). These results suggested that it may have been the deletion of the NSGa or NSGα in the full MTL deletion derivatives Δmtla1 and Δmtla2 that was responsible for the decreases in adhesion and β-glucan release, and a portion of the decreases in thickness observed in the full locus deletions. To test this directly, we generated the triple deletion mutant Δnsa1, in which Pika, PαA, and OBPa were deleted (Δpikα Δapα Δobpα) and the triple deletion mutant Δnsa2, in which Pikα, PαA, and OBPα were deleted (Δpikα Δapα Δobpα), in both the natural strain P37039 and P37037 (Figure 1D). The homozygous triple deletion of the NSGas and the NSGαs was not possible because both Pikα and PαA are essential genes [13,19]. The deletion mutants Δnsa1 and Δnsa2 generated from both test strains exhibited reductions in adhesion (Figure 3A, B), biofilm thickness (Figure 3C, D), and the release of β-glucan (Figure 3E, F). The reductions in adhesion and in the release of β-glucan were similar to those observed for the deletion mutants of the entire a or α copy of the MTL locus, Δmtla1 and Δmtla2 (Figure 2A, B and E, F, respectively). As was the case for the mutants Δmtla1 and Δmtla2, the p values for reductions in adhesion and the release of β-glucan for the nsa and nsgα mutants were close to significant or significant (p values ranged from 0.05 to 0.23 for adhesion and 0.04 to 0.13 for the release of β-glucan). However, the decreases were reproducible within a mutant and between mutants generated in different natural strains. The decreases in thickness, however, for the two nsa and nsgα mutants were highly significant (p values ranged from $3 \times 10^{-3}$ to $2 \times 10^{-5}$). The reductions in thickness were, however, highly significant with p values ranging from $10^{-4}$ to $6 \times 10^{-4}$. The decreases in thickness for the nsa and nsgα mutants were less pronounced than those for the AMT1 or AMT2 mutants (Figure 3C, D). That was consistent with the conclusion that MTL1 and MTL2 were each partially responsible for biofilm thickness (Figure 2A and B). LSCM and SEM revealed normal general architecture for both Amtsa andAmtsgα biofilm (data not shown).

Deletions of individual NSGs affect biofilm formation

To assess which of the three NSGs played a role in biofilm formation, we deleted either the a or α copy of each NSG individually in the two strains P37039 and P37037. In both strains, the deletion mutant of either the a or α allele of each of the three NSGs resulted in a small or negligible decrease in adhesion (Figure 3A, B) and a small or negligible decrease in the release of β-glucan (Figure 3E, F). Although the consistency of the decreases in both strains suggested a trend, all of the decreases in adhesion and release of β-glucan for the individual NSG allelic mutants were insignificant (p value > 0.05). All of the mutants, however, exhibited decreases in thickness (Figure 3C, D) that were significant (p values ranged between $10^{-3}$ and $10^{-4}$). Together, these results demonstrate that each of the a and α alleles of the NSGs play roles in a/α biofilm formation, most notably in biofilm thickness. Because the two mutants for each NSG allele were generated independently in two unrelated strains and exhibited similar defects, we considered complementation tests on all 26 mutants to be unnecessary. We therefore performed complementation tests on only two, Δapikα and Δapikα of strain P37039. This was accomplished by adding back wild-type Pikα or PαA, respectively, under the regulation of the TET promoter, to generate strains Δpikα-TET-Pikα and Δpikα-TET-PαA. In both cases, addition, of 50 μg per ml of doxycycline, an inducer of the tetracycline promoter, completely rescued the thickness defect (supplemental Figure S1).

The Aobpα/Obpα mutant exhibits severe defects in biofilm formation

Both Pikα and PαA are essential genes [13,19], so simultaneously deleting both the a and α copy of each to produce a homozygous deletion mutant was unattainable. Because OBP is not an essential gene [17,21], we were able to generate the homozygous deletion mutant Δobpα/Obpα in both strain P37039 and P37037. The homozygous deletion mutant Δobpα/Obpα of both strains exhibited far more dramatic decreases in adhesion (Figure 3A, B), biofilm thickness (Figure 3C, D) and release of β-glucan (Figure 3E, F) than either of the heterozygous OBp deletion mutants, Δobpα or Δobpα, the heterozygous deletion mutants of the other two NSGs (Δapikα, Δpikα, Δapikα and Δapikα), the deletion mutants for all three a or α copies of the NSGs (Δnsgα and Δnsgα) and the deletion mutants of the a or α copy of the MTL locus, (Δmtla1 and Δmtla2). The decreases in adhesion were close to significant or significant (p values were 0.06 and 0.01, respectively) and decreases in the release of β-glucan significant (p value were 0.01 and 0.002). The decreases in biofilm thickness were highly significant (both p values were $< 10^{-4}$). The biofilms that were formed by the mutant Δobpα/Obpα, however, still retained the general architecture of parental wild type biofilms. LSCM of Δobpα/Obpα biofilms revealed that they possessed a basal layer of yeast cells just above the substrate (Figure 3H) and vertically oriented hyphae 20 μm above the substrate (Figure 3H), similar to that of the parental strains (Figure 3G). The cell density of the mutants at the substratum and 20 μm above it, however, was lower than that of wild-type biofilms (compare Figure 3G and H).

SEM revealed no differences between biofilms formed by the two parental strains and the deletion mutants Δmtla1, Δmtla2, Δnsgα, Δnsa1, Δnsa2, Δapikα, Δapikα, Δapikα, Δapikα, Δobpα and Δobpα (data not shown). There was, however, a clear difference between the biofilms of the parental strain and that of the mutant Δobpα/Obpα. Δobpα/Obpα mutant biofilms had far less densely packed hyphae resulting in noticeable gaps in the upper hyphal region (Figure 3J). Biofilms of the parental strains lacked these large gaps.
approximately 5% of cells in parental (P37037) biofilms were (Figure 4A, B). We then tested resistance to fluconazole. While

Biofilm permeability and fluconazole susceptibility

Recently we demonstrated that biofilms formed by a/α strains of C. albicans were relatively impermeable to low and high molecular weight molecules, impenetrable by polymorphonuclear leukocytes and resistant to fluconazole [33]. In marked contrast, a/a and α/α biofilms were permeable to low and high molecular weight molecules, readily penetrated by polymorphonuclear leukocytes and susceptible to fluconazole [33]. We therefore first tested whether the biofilms formed by any of the NSG mutants generated in the α/α strain P37037 became more permeable to Sypro Ruby, which has a molecular weight of 1.6 kDa [34,35]. The biofilms formed by the mutant derivatives Δpika, Δpapa, Δpobp and Δpobpa were all slightly more permeable to Sypro Ruby than biofilms of the parental strains (Figure 4A, B). Percent penetration into the biofilm of the parental wild type strain P37037 was approximately 12%, while that of Δpika, Δpapa and were approximately 28% (Figure 4A, B). The percent penetration of Δpobp/Δpobpa biofilms, however, averaged approximately 80% (Figure 4A, B). We then tested resistance to fluconazole. While approximately 5% of cells in parental (P37037) biofilms were susceptible to fluconazole, approximately 20% of cells in biofilms of the mutants Δpika, Δpapa and Δpobp were susceptible (Figure 4C). In marked contrast approximately 40% of cells in biofilms of Δpobp/Δpobpa were susceptible (Figure 4C). These results indicate that PIK, PAP and OBP play roles in a/α biofilm impermeability and fluconazole resistance.

The Δpobp/Δpobpa mutant mates normally

When NSGs are embedded in a mating type locus, there is always the question of whether they play a role in the mating process. Because only OBP was nonessential for growth, we were only able to test whether an a/α derivative of the homozygous OBP deletion mutant Δpobp/Δpobpa could mate with an α/α strain, in this case strain WO-1 [36,37]. a/a derivatives were generated by screening colonies of the a/α homozygous mutant Δpobp/Δpobpa grown on sorbose medium for opaque colonies[38,39], which reflect MTL homoygosis, and then by testing for the a/a genotype by polymerase chain reaction[38,39]. Opaque cells of the a/a derivatives of Δpobp/Δpobpa mated with opaque cells of the α/α strain WO-1 (Figure 5). Both the formation of evaginated cells (shmoos) and fusion occurred normally (Figure 5). These results demonstrate that at least one of the NSGs, the nonessential gene OBP, are not required for shmoos formation or fusion in the mating process.

Overexpression of PIK and PAP partially rescues Δpobp/Δpobpa defects

The decreases in adhesion and the release of β-glucan, the dramatic decrease in thickness, the decreases in hyphal density,
and the loss of impermeability and drug resistance by the \( \Delta obp^{\alpha}/\Delta obp^{\alpha} \) mutant, demonstrate that \( OBP \) plays a major role in \( \alpha/\alpha \) biofilm development. But because we could not generate homozygous \( PIK \) and \( PAP \) deletion mutants due to the essential roles they play in growth [9,13], we were unable to distinguish if \( OBP \) alone or if all three of the NSGs were necessary for the above biofilm characteristics. To explore this question, we used an overexpression strategy. If overexpression of \( PIK \) or \( PAP \) rescued the \( \Delta obp^{\alpha}/\Delta obp^{\alpha} \) defects in biofilm formation, it would answer two questions, first whether \( PIK \) or \( PAP \) contributed to \( \alpha/\alpha \) biofilm permeability and fluconazole resistance, and second, whether over-expression of one NSG could rescue the defects of the homozygous deletion of another. We therefore placed wild type \( PIK \) or \( PAP \) under the regulation of the \( TET \) promoter, and inserted the construct into one allele of the gene \( ADH1 \) [40] in the \( P37039 \) mutant \( \Delta obp^{\alpha}/\Delta obp^{\alpha} \). Overexpression of the inserted gene was achieved by adding 50 \( \mu \)g per ml of doxycycline at 0 hr followed after 24 hr by addition of 25 \( \mu \)g per ml during biofilm development. Overexpression wild type \( PIK \) returned biofilm thickness to that of wild type, and overexpression of \( PAP \) to a thickness greater than wild type (supplementary Figure S1A). Overexpression, however, only partially rescued impermeability to Sypro Ruby and fluconazole resistance (supplementary Figure S1B and C, respectively). Sypro Ruby penetrated into 5\% in \( P37039 \) biofilms, but into approximately 80\% of \( \Delta obp^{\alpha}/\Delta obp^{\alpha} \) biofilms (supplementary Figure S1B). It penetrated into approximately 55\% of \( \Delta obp^{\alpha}/\Delta obp^{\alpha}-TETp-PIK^{\alpha} \) biofilms and 50\% of \( \Delta obp^{\alpha}/\Delta obp^{\alpha}-TETp-PAP^{\alpha} \) biofilms (supplementary Figure S1B). Cell death due to fluconazole treatment was approximately 4\% in \( P37039 \) biofilms, but approximately 43\% in \( \Delta obp^{\alpha}/\Delta obp^{\alpha} \) biofilms. It was approximately 25\% in \( \Delta obp^{\alpha}/\Delta obp^{\alpha}-TETp-PIK^{\alpha} \) biofilms and 20\% in \( \Delta obp^{\alpha}/\Delta obp^{\alpha}-TETp-PAP^{\alpha} \) biofilms (supplementary S1C). Therefore, overexpression of either \( PIK^{\alpha} \) or \( PAP^{\alpha} \) in a \( \Delta obp^{\alpha}/\Delta obp^{\alpha} \) background completely rescued the thickness defect, but only partially rescued the permeability and fluconazole susceptibility defects.

**MTL\textsubscript{a1}, MTL\textsubscript{a2}, and the three NSGs are constitutively expressed**

Using RT-PCR, we compared the expression levels in the natural strains \( P37037 \) and \( P37039 \), and the laboratory strain SC5314, of \( MTL^{\alpha}1 \), \( MTL^{\alpha}2 \), and each allele of the three NSGs. Measurements were made after 14 hours of planktonic growth or biofilm formation. \( MTL^{\alpha}1 \) and \( MTL^{\alpha}2 \), each of the two alleles of the three NSGs were expressed both during planktonic growth and biofilm formation (Figure 6). The only difference consistent

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**Figure 5. The homozygous OBP deletion mutant \( \Delta obp^{\alpha}/\Delta obp^{\alpha} \) mates normally.** A. Shmoo formation in a cross between an \( \alpha/\alpha \) derivative of \( P37039 \) and \( \alpha/\alpha \) WO-1 cells. B. Shmoo formation in a cross between \( \alpha/\alpha \) \( \Delta obp^{\alpha}/\Delta obp^{\alpha} \) and \( \alpha/\alpha \) WO-1. C. Fusion between \( \alpha/\alpha \) \( P37039 \) and \( \alpha/\alpha \) WO-1. D. Fusion between \( \alpha/\alpha \) \( \Delta obp^{\alpha}/\Delta obp^{\alpha} \) and \( \alpha/\alpha \) WO-1. All crosses were done between opaque cells.

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among the three test strains was expression of the PIKa transcript, which showed higher levels in biofilms (Figure 6). Control experiments using RT-PCR to assess expression in mutants verified that the probes used were specific for the mating type genes and NSG alleles.

Virulence in a mouse systemic model

Finally, we compared the virulence of the select mutants Δmtla, Δmtla, Δnsga, Δnsgx and Δobpa/Δobpx with the parental strain P37039, using the mouse tail injection model for systemic infection [26,41]. Host survival increased for every tested deletion mutant (Figure 7A, B, C). The differences in survival, computed by the log rank test for survival, were significant between P37039 and the derivative mutants Δnsga (p value 0.008) and Δnsgx (p value 0.005) (Figure 7A). The differences in survival were significant, for the pooled data of the repeat experiments (Figure 7B and C), between P37039 and Δmtla (p value 0.003), between P37039 and Δmtla (p value 0.007) and between P37039 and Δobpa/Δobpx (p value 0.006). These results indicate that in addition to a/x biofilm formation, the MTLa locus and at least one specific NSG embedded in it, OBP, play roles in virulence in the mouse model for systemic infection.

Discussion

The majority of C. albicans biofilms that form on catheters, prosthetics and tissues are due to strains that are heterozygous (a/x) at the mating type locus MTI [42–44]. Such biofilms are thick, impermeable to molecules ranging in molecular weight from 300 to 140,000 daltons, impenetrable by leukocytes and resistant to fluconazole [33]. The developmental program for the formation of

Figure 6. Both the NSGs and the mating genes MTLa1 and MTLa2 are constitutively and similarly expressed during planktonic growth (P) and biofilm formation (BF). Analyses were performed in three unrelated a/x strains, P37037, P37039 and SC5314. RT-PCR was used to assess expression. The constitutively expressed gene TDH3 was analyzed as a control. doi:10.1371/journal.ppat.1002476.g006

Figure 7. Deletion of either copy of the mating type locus, MTLa or MTLa, of either the NSGa or the NSGx, or the two OBPs, OBPa and OBPx, caused a decrease in virulence of the a/x strain P37039 in the mouse tail injection model for systemic infection. Fifteen mice were injected with each noted strain. A, B and C represent three separate experiments in which injected mice were monitored over a 22 day period. Uninjected control mice exhibited 0 to 7% death after 22 days. doi:10.1371/journal.ppat.1002476.g007
these biofilms is complex, resulting in a multicellular structure containing multiple phenotypes embedded in a multimolecular, extracellular matrix. The film is more than 50 times thicker than a yeast cell monolayer. These a/α biofilms are composed of a thin basal layer of highly adherent and cohesive yeast cells, and a thick upper layer of vertically oriented hyphae, originally formed from the yeast cells in the basal layer. These intertwined hyphae are embedded in a multimolecular matrix produced by the cells in the developing biofilm [45–48]. At the upper edge of a biofilm, yeast cells are formed by the hyphae, which appear to function as a dispersal mechanism [45,49,50]. a/α biofilms are regulated by the Ras1/cAMP pathway, which targets a transcription factor cascade consisting of Efg1→Tec1→Bcr1 [45,49,50]. Because the majority of biofilms formed in nature must be a/α, elucidating the molecular mechanisms that mediate their resistance to antifungals [33,50,51], their impermeability to molecules in the molecular weight range of antibodies, and their capacity to exclude polymorphonuclear leukocytes, will facilitate therapeutic strategies for this pervasive human pathogen.

Here we have investigated whether the nonsex genes embedded in the mating type locus play common or related nonmating roles in pathogenesis, in particular in the formation of a/α biofilms. We had previously presented evidence that natural strains that were homozygous at the MTL locus at the time of isolation, derivatives of natural a/α strains in which the a or α copy of MTL was specifically deleted and derivatives of natural a/α strains that had undergone spontaneous homoygosity in vitro at the MTL locus, were on average less virulent in the mouse tail-injection model for systemic infection than a/α strains [26]. In addition, we had demonstrated that although a/a or α/α cells biofilms were architecturally similar to a/α biofilms, they were thinner, far more permeable to low and high molecular weight molecules, far more susceptible to fluconazole, and readily penetrated by polymorphonuclear leukocytes. Therefore, only a/α biofilms exhibit those traits one would expect of a pathogenic biofilm. Moreover, MTL-homozygous biofilms were found to be regulated by the MAP kinase pathway, in contrast to a/α biofilms, which are regulated by the Ras1/cAMP pathway [33]. Together, these results suggested to us that the genes in the MTL locus, including the NSGs, could play non-mating roles in the formation of pathogenic a/α biofilms and in virulence in the mouse model for systemic infection.

The roles of the MTL genes in adhesion, thickness and β-glucan release

One might assume that one or more of the NSGs may play roles in mating, as was suggested as a possibility by Fraser et al. [8] for nonessential genes embedded in the MAT locus of Cryptococcus. In C. albicans, we have, however, found that this is not the case for the single nonessential NSG, OBP. The homozygous deletion of OBP in an a/a background had no effect on α-pheromone-induced evagination (“shmoo” formation) or on fusion with cells of the opposite mating type.

Deleting simultaneously all of the a or the α alleles of the NSGs did, however, had an effect on six a/α biofilm parameters. Individual allelic deletions of NSGs caused significant decreases in biofilm thickness, but had very limited effects on adhesion and β-glucan synthesis. Decreases in these two parameters were not statistically significant within an experiment, but were reproducible between experiments for a mutant of a single strain and among experiments for the two mutants of the unrelated parental strains.

Deleting either of the two mating type genes, MTL1α or MTL2α, also caused a significant decrease in biofilm thickness, but had no discernible effect on adhesion or the release of β-glucan. The differential effects of the mating type genes and NSGs on biofilm parameters was not surprising given that each of the three parameters represented a very different and inherently complex aspect of biofilm formation. First, the increase in adhesion to a silicon elastomer surface, measured after sixteen hours of biofilm development, is a response by the initial yeast cell inoculum and their immediate yeast cell offspring [31]. No hyphae have formed at the time of that assay. Thickness on the other hand is measured after 48 hours of biofilm development and reflects the extent of vertical hyphal elongation supported by extracellular matrix [52,53]. The release of β-glucan is also measured after 48 hours and has been interpreted as a reflection of matrix deposition [54–56]. Permeability is assessed after 48 hours of biofilm development by adding a solution of Sypro Ruby to the top of a living biofilm and then measuring how deep the dye has penetrated after 30 additional minutes [33]. The cell death assay is a similar measure of fluconazole penetration [33]. And finally, we assessed the presence of a basal yeast cell layer, hyphal orientation and hyphal density in 48 hour biofilms using LSCM and SEM. Our results demonstrate that MTL1α and MTL2α play selective roles in biofilm thickness, but not in adhesion or the release of β-glucan, whereas the NSGs may play roles in all of these biofilm traits. The observation that overexpression of Pik or Pap fully rescued the thickness defect of Δobp/Δobp biofilms, but only partially rescued the permeability and fluconazole susceptibility defects again dissociates biofilm parameters. These results underscore the complexity of biofilm formation and the dissociability of traits, and therefore the need to use multiple and independent measures of the different aspects of biofilm development in analyzing each mutant phenotype.

The Δobp/Δobp mutants were defective in all biofilm parameters, and the decreases in these parameters were approximately double those measured for any of the heterozygous MTL deletion mutants or for the MTL1α and MTL2α deletion mutants. Moreover, the gaps in the hyphal network in the upper regions of Δobp/Δobp biofilms were not observed in biofilms formed by the Δmnt1α, Δmnt2α or individual allelic NSG deletion mutants. Yet the qualitative aspects of biofilm architecture, which included the general organization of cell types and matrix, was maintained in the Δobp/Δobp mutant biofilm. It therefore appears that the mating type gene MTL1α and MTL2α, and the NSGs Pik, Pap and OBP affect the quality of a biofilm rather than the basic structure.

The role of NSGs in biofilm permeability and drug susceptibility

Two of the most important characteristics of a pathogenic biofilm are impermeability to such molecular challenges as antibodies [33,57] and antifungals (i.e., drug resistance) [33,58–62], and resistance to white blood cell penetration [33]. The a/α biofilm exhibits all of these characteristics, and for that reason has been distinguished as a “pathogenic” biofilm [33,34]. Here, we have shown that deleting one of the alleles of any of the three NSGs in an a/α strain has a small but reproducible effect on permeability and fluconazole susceptibility, and that deleting both alleles of OBP results in a major increase in both parameters. These results suggest that OBP, as well as Pik and Pap, play major roles in establishing the pathogenic traits of an a/α biofilm.

Total rescue of thickness and partial rescue of impermeability and fluconazole resistance in Δobp/Δobp a/α biofilms in which
either PiKa or PaPa are overexpressed suggests at least partial additivity or roles in a dependent pathway. In the latter case, PiK and PAP may play roles downstream of OBP. Although the disparate functions of the mating type genes and the three NSGs do not provide any obvious explanation for the related roles the MTL genes play in biofilm formation, it is worth considering the known functions and regulations of NSG orthologs in other systems. OBPα encode a family of proteins involved in the transport and metabolism of sterols [17–21,63]. They have also been shown to serve as regulators of genes involved in lipid metabolism and intracellular signaling [63]. OBP gene products not only bind oxysterols, but also phosphoinositides, presumably an interaction involved in the regulation of sterol binding [21]. PiK encodes a kinase involved in the first step in the production of inositol-1, 4, 5-triphosphate [64]. In S. cerevisiae, fractionation studies suggest that the PiK ortholog is involved in a nuclear phosphoinositide regulatory process [64]. The poly(A)/polymerase, encoded by PAP, catalyzes 3’ polyadenylation of mRNAs, a necessary step in transport and stability [65,66], in the nucleus [66,67]. Hence, there are loose or indirect connections between the three NSGs in other organisms ranging in complexity from yeast to man. There have been, however, no reported experiments performed to date in C. albicans that could be used to explain the mutant and overexpression results functionally linking these three colocalized genes, and the two mating type genes MTLα and MTLβ, to the formation of a/α biofilms. Given the importance of a/α biofilms in commensalism and pathogenesis, elucidating such relationships would be highly relevant in the future.

The role of NSGs in virulence in a mouse model

Finally, we tested whether the NSGs contributed to the role the MTL played in virulence in the mouse model for systemic infection [26]. Our results demonstrate that deleting MTLα or MTLβ locus, the NSGαs or the NSGβs, or both copies of OBP results in similar decreases in virulence. Although it is not at all clear that biofilm formation plays any role in this model for systemic infection, it is clear that the MTL locus and the resident NSGs play a role not only in biofilm formation, but also in the virulence of a/α cells, two pathogenic traits apparently unrelated to mating.

Materials and Methods

Animal care and usage was in accordance with the National Institutes of Health guidelines for the use of laboratory animals in biological research. Experimental protocols were reviewed by the Institutional Animal Care and use Committee (IACUC) at the University of Iowa (ACURF no. 1101003).

Strains and media

The names, genotypes and origins of the basic strains and mutant derivatives used in this study are listed in Figure 1D, and the basic strains and mutants used for complementation, mating and overexpression experiments are listed in supplemental Table S1. Growth conditions were previously described [31,36]. Supplemented Lee’s medium [68] was employed for basic growth experiments. MTL genotypes of the parent strains were verified by PCR prior to use in experiments.

Generating hemizygous mutants

All mutants were generated for this study using the pop-out flipper cassette from pSF52A (40) containing the dominant nourseothricin resistance marker CaSAT1. To increase the efficiency of deletion, the Smal sites flanking the SAT-flipper cassette were subcloned into the pGEM-T Easy, to generate pGEM2A. All of the primers used are listed in supplemental Table S1. The genotypes of the mutants employed in this study are presented in Figure 1D. For each hemizygous deletion mutant, the cassettes were constructed in two steps. First, the allele-specific 5’ and 3’ flanking fusion fragment was generated by PCR. Second, the Smal-digested SAT-flipper cassette from pGEM2A was inserted in between the flanking fragment. The deletion of the alleles of all three non-sex genes of MTLα was created in one-step due to their contiguous arrangement in the locus. Deletion of the NSG alleles of MTLα was carried out in two steps. First, two deletion cassettes were created, one for the PaPa allele and second, for the OBPα-PiKa allele pair. Deletion cassettes were isolated from the plasmids by digestion with SacI and SphI, then used for transformation of parental strains. The putative transformants were verified by PCR. These transformants were also verified for a/α heterozygosity using theprimers listed in supplemental Table S2.

Strains for complementation and overexpression

To test for complementation of the mutants Apiα and Apoα of strain P37039, PiKa and PaPa, respectively were reintroduced under regulation of the TET promoter (40), using methods previously described in detail [33]. For overexpression of PiKα and PiKβ in the mutant Obpα/Obpα, the same strategy was employed, using ADH1 (40) as the insertion locus. The primers used to create the constructs are listed in supplemental Table S2.

Generation of mating-competent strains

To test whether the Obpα/Obpα strain could mate, homozygous a/a derivatives of the P37039 mutant were generated using sorbose induction [38,39]. The a/a configuration was verified by PCR. Shmoo formation and fusion in suspension cultures of the a/a derivative of Obpα/Obpα and the a/α strains WO-1 was performed according to methods previously described [37]. The a/a cells were stained with Alexa 488 (Invitrogen) and the a/α cells with TR/TC Con-A (Invitrogen) according to methods previously described [37].

Measuring biofilm parameters

In brief, adhesion was measured by incubating cells in the wells of a 12 well plates for 16 hour, washing gently, then trypsinizing to release cells for counting [31]. Biofilm thickness was measured after 48 hours of biofilm development by Laser scanning confocal microscopy [31]. β-glucan measurements were performed according to the methods of Mitchell and coworkers [32,69] with supernatants of 48 hour biofilms. Measurements of Sypro Ruby permeability and fluconazole susceptibility have recently been described in detail [33]. In brief, 48 hour biofilms were overlaid with Biofilm Tracer Sypro Ruby (Invitrogen) for 30 minutes prior to laser scanning confocal microscopy, as previously described [33], using a Bio-Rad Radiance 2100 MP laser scanning confocal microscope (Bio-Rad, Hercules, Stanford, United Kingdom) equipped with a Mai-Tai IR laser (Spectra-Physics Lasers, Mountain View, CA). Calcofluor was imaged using the Mai-Tai IR titanium-sapphire laser tuned to 818nm. Fluconazole susceptibility was measured as previously described in detail [33]. In brief, 25 μg per ml of fluconazole was added to the top of 48 hr biofilms and incubated an additional 24 hours. Biofilms were dissociated in 20 μM EDTA and cells assessed for viability by Dead Red staining. Scanning electron microscopy was performed by fixing biofilms in 2.5% glutaraldehyde in 0.1M cacodylate buffer. After post-fixation in 1% osmium, the biofilms were dehydrated through a graded ethanol series and further dehydrated by
50% hexamethyldisilazane (HDMS) followed by two rinses in 100% HDMS. The biofilms were allowed to air-dry prior to sputter-coating with a 60:40 mixture of gold and palladium. The biofilms were viewed using a Hitachi S-4800 scanning electron microscope.

RT-PCR
The methods used for RT-PCR have been described previously in detail [33,69].

Virulence in a systemic mouse model
The method used to assess the virulence has been described previously in detail [26]. Mice were examined every 12 hours. Mice exhibiting the first sign of morbidity were euthanized by inhalation of saturated CO₂.

Supporting Information

Figure S1  Complementation and over-expression strategies. A. Thickness of biofilms in P37039, ΔphaΔ, ΔopaΔ, Δpha/Δopa/

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ΔphaΔ and the respective mutants in which Pkha and Paha are expressed under the regulation of the TET promoter, (TETP). B. Sypro Ruby permeability of P37039, ΔphaΔ/ΔphaΔ and the latter mutant in which either Pkha or Paha is overexpressed under the regulation of the TET promoter, TETP. C. Fluconazole susceptibility in the same strains analyzed in panel C.

Table S1 Strains used in complementation, additivity and mating studies.

Table S2 Oligonucleotides used in this study.

Author Contributions
Conceived and designed the experiments: DRS, TS, KJD. Performed the experiments: TS, KJD, CP, NS, SY. Analyzed the data: DRS, KLD, TS, CP. Contributed reagents/materials/analysis tools: TS, KJD, CP, NS, SY. Wrote the paper: DRS.
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