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Does stress induce (para)sex? Implications for *Candida albicans* evolution

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Abstract

Theory predicts that stress is a key factor in explaining the evolutionary role of sex in facultatively sexual organisms, including microorganisms. Organisms capable of reproducing both sexually and asexually are expected to mate more frequently when stressed, and such stress-induced mating is predicted to facilitate adaptation. Here, we propose that stress has an analogous effect on the parasexual cycle in *Candida albicans*, which involves alternation of generations between diploid and tetraploid cells. The parasexual cycle can generate high levels of diversity, including aneuploidy, yet it apparently occurs only rarely in nature. We review the evidence that stress facilitates four major steps in the parasexual cycle, and suggest that parasex ensues much more frequently under stress conditions. This may explain both the evolutionary significance of parasex and its apparent rarity.

The parasexual cycle in *Candida albicans*

C. albicans, the most prevalent fungal pathogen of humans, is constantly challenged by interactions with the immune system as well as by the need to survive in a broad range of ecological niches within its human host. When exposed to severe stress, such as an antifungal drug, it is capable of evolving resistance, in some cases very rapidly [1-3]. This rapid evolution arises despite the primarily clonal nature of the population structure [4-6] and the apparent absence of a meiotic cycle. For a long time, *C. albicans* was thought to be asexual, but exciting work over the last decade revealed that it can be induced to undergo parasex (reviewed in [7-12]). This parasexual cycle involves mating between diploids to form tetraploids that eventually undergo a non-meiotic process termed ‘concerted chromosome loss’, resulting in approximately diploid cells with high levels of aneuploidy and homozygosity [13]. While this type of diversity has the potential to benefit cells exposed to antifungal drugs (reviewed in [14]), the degree to which these progeny are beneficial or whether they incur a high fitness cost is not known. A central issue is to what extent the

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parasexual cycle occurs in nature and, when it does occur, how important it is as a mechanism for adaptation (reviewed in [12]).

The parasexual cycle requires multiple steps that must occur sequentially (Fig. 1) and each step occurs relatively infrequently under laboratory conditions (reviewed in [8, 9, 11, 12]). First, the mating type-like locus (*MTL*) must become homozygous (*MTLhom*) in diploid cells. In the laboratory, this is usually achieved through gene disruption or by selection for rare whole chromosome loss events. Second, these *MTLhom* cells must undergo a phenotypic switch from the normal 'white' state to a new physiological state, termed 'opaque' [15], a process dependent upon accumulation of the white-opaque regulator 1 (*WOR1*) gene product in *MTLhom* cells (reviewed in [16]). Third, two mating-competent cells must be in close enough proximity to send and receive pheromone signals. Given the potentially low frequencies of the first steps, it is not clear how frequently two cells of opposite mating type would be in close enough proximity to mate although if they were present in biofilms, they could establish more effective pheromone gradients [17]. Are the tetraploid progeny stable? Do they reproduce as tetraploids? Indeed, tetraploid recombinant progeny were detected following inoculation of a mouse with high levels of mating competent cells [18, 19], but tetraploids have not been reported in clinical isolates. Finally, the resulting tetraploid strains must undergo a reduction in chromosome number, via concerted chromosome loss, thought to be due to chromosome instability that triggers sequential rounds of aberrant mitoses, rather than by meiosis [13, 20]. How this process occurs is not understood. It has been proposed that parasex occurs very rarely, if at all, in nature because of the multiple steps required, the fact that each step occurs inefficiently in the lab, and the failure, thus far, to detect tetraploid clinical isolates.

A hypothesis: Stress promotes the parasexual cycle, potentially contributing to *C. albicans* adaptation

We hypothesize that exposure to stress could drive parasex, and that it has the potential to generate progeny that are better able to adapt to a given stress condition. This hypothesis is based upon observations, detailed below, indicating that the major steps of the parasexual cycle occur more frequently upon exposure to stress.

Mating type homozygosity

We propose that the first step of parasex, homozygosity of *MTL*, occurs via recombination (Fig. 2), a process that occurs more frequently following exposure to several types of stress [21]. Laboratory strains may also become *MTLhom* by chromosome loss and reduplication events [22] or via a transcriptional mechanism that does not require alteration at the DNA level [23]. Nonetheless, *MTL* homozygosity due to recombination events was the most frequent event detected in mating competent clinical isolates [24].

White-opaque switch

Several elegant studies already demonstrated that the second step, the opaque switch, occurs at higher levels when cells are exposed to stresses including, UV, oxidative stress, or a transition through mouse intestines [25-27]. Increased frequencies of opaque cell formation are tightly correlated with a reduction in the growth rate of the cells [25], a condition expected to occur when cells are stressed, or maladapted to their environment. Thus, the stress that promoted the reciprocal recombination to generate newly *MTL* homozygous cells could also lead to slow growth of these cells, and increase the frequency with which they switch to the opaque state. These results bolstered the argument that the parasexual cycle could occur *in vivo* (reviewed in [7-12]). Furthermore, conditions within the host may be stressful for *C. albicans* (reviewed in [14]).

Mating

Stress-induced recombination also provides a solution to the third step of parasex by positioning mating partners in close proximity (Fig. 2). Reciprocal recombination would produce two sister cells of opposite mating type, which would be physically adjacent to one another. If mating did not occur immediately, the two types of cells would then continue to divide near one another, providing a large population of mating partners in close proximity to one another. In the lab, this type of reciprocal recombination would give rise to a colony with two half-sectors, one of each mating type (Fig. 2). Presumably, *in vivo*, a reciprocal recombination involving *MTL* would give rise to a population of cells of opposite mating types that would be in close proximity to one another. To mate, cells produce conjugation tubes that grow towards one another and fuse to form tetraploid zygotes. Interestingly, mating between opaque cells occurs preferentially on nutrient-poor medium [28]. While tetraploid clinical isolates have not been reported, the inability to detect them is a negative result that may simply reflect the few studies that monitored cell ploidy. Another possibility is that tetraploid zygotes may be unstable and thus short-lived, and might undergo concerted chromosome loss rapidly, perhaps driven to do so by the same stress conditions that promoted increased switching to the opaque form. Consistent with this, tetraploids are less virulent in a systemic murine model of candidiasis [29].

Concerted chromosome loss

Finally, in the lab, tetraploid zygotes undergo concerted chromosome loss at high frequency when exposed to stress conditions such as poor carbon source availability [25]. This yields a high proportion of progeny with new combinations of homologs, frequent whole chromosome aneuploidies as well as a subset of strains that undergo short-range recombination events [13]. For example, of 31 ~diploid progeny from a parasexual cross between two related lab strains, all carried at least one completely homozygous chromosome, the majority (~65%) also carried at least one extra chromosome and a few (~23%) underwent multiple recombination events. Furthermore, a significant proportion of these progeny was homozygous for the *MTL* locus (~29%). We suggest that parasexual progeny have the potential to reenter the parasexual cycle by switching to the opaque state and mating with neighboring cells [20]. Thus, while we do not know if the parasexual cycle occurs *in vivo*, it appears that most of the requisite steps would ensue with increased frequency when cells are under stress.

Parasex and adaptation

If parasex does occur, then does it contribute to adaptation by generating more diverse progeny? Theory predicts that meiotic sex and recombination facilitate adaptation by generating new combinations of genotypes [30]. Stress-induced sex and recombination are expected to facilitate adaptation even further [31], especially in cases of complex adaptation in which multiple mutations that are deleterious when present independently yet are advantageous when present in specific combinations [32]. What about parasex?

Parasex has the potential to facilitate outcrossing—the mating of unrelated individuals, which may occur between different strains that infect the same host [33]. However, outcrossing is expected to be quite rare because a single strain is usually dominant in a given individual and the scenario above suggests that most parasex would involve inbreeding between siblings. Clinical isolates of *C. albicans* are highly heterozygous across much of the genome (average 1 single nucleotide polymorphism (SNP) per ~200 bp [34]). Inbreeding between heterozygous individuals can generate diverse progeny that have significant levels of homozygosity at different combinations of loci. This parallels the predicted ability of rare conventional sex to reveal variation in mostly clonal organisms [35].

Importantly, parasex is expected to generate more dramatic diversity than meiotic sex (Fig. 3). First, concerted chromosome loss often results in complete homozygosis of one or more chromosomes, which has the potential to reveal large numbers of recessive traits in new combinations [9]. Accordingly, loss of heterozygosity (LOH) of specific transcription factors that regulate drug efflux has given rise to antifungal drug resistance in both *in vivo* and *in vitro* [36-38]. Parasex generates a second type of diversity: in stark contrast to classical meiosis, a large proportion of ~diploid parasexual progeny will be aneuploid, carrying at least one extra chromosome. This has the potential to reveal much higher levels of copy number variation than seen with conventional mating and meiosis [9]. Even the less precise meiosis in *Candida lusitanae* resulted in much less aneuploidy (6%, [39]) than what was observed for parasex (80%, [13]). Thus, when inbreeding is the only option, parasex could offer an advantage in comparison with conventional sex.

Consider, for example, the effects of sex and parasex on the diversity of offspring at a single locus (see Figure 3). Conventional sex with inbreeding would have a small effect if the locus is heterozygous (Bb), by producing 3 possible genotypes: BB, Bb, and bb. Parasex would allow a much wider range of progeny, including these 3 diploids as well as 4 ~diploid progeny that are trisomic for the chromosome that carries the locus and, theoretically, ~diploid progeny monosomic for the locus of interest, or the tetraploid could be stable. Furthermore, if parasexual progeny re-mate with one another, they could generate new ratios of alleles in the second-generation tetraploid zygotes as well.

Of course, as the numbers of loci that are considered increase, the potential variation that parasex can produce increases further, more than the potential variation produced by sex. Of note, variations in chromosome number have been found in clinical isolates as well as in laboratory strains, and they are dramatically overrepresented in drug-resistant isolates [40]. In this case, it is extra copies of two specific genes on a single chromosome (one involved in ergosterol biosynthesis (*ERG11*) and a transcriptional activator of efflux pump genes (*TAC1*) on chromosome 5) that confer increased antifungal drug resistance [3]. Thus, some aneuploidies clearly have the potential to be adaptive under specific stress conditions, as they affect copy number at multiple loci. Accordingly, the parasexual cycle, even if it occurs only between siblings, would be expected to generate genetic diversity that has the potential to be beneficial. Importantly, *C. albicans* appears to be much more tolerant of chromosome imbalances [14] than is *S. cerevisiae* [41, 42]. Indeed, some aneuploidies that confer increased drug resistance do not have obvious fitness costs when cells are grown in the absence of the drug [2]. Furthermore, it has been suggested that parasex avoids the production of antigenic ascospores, thereby possibly avoiding increased stimulation of an immune response [9].

We propose that stress-induced parasex be viewed in the general context of stress-induced variation. Multiple mechanisms increase the generation and/or revelation of genetic variation in times of stress, potentially contributing to evolution [43]. Examples include stress-induced mutagenesis [44], fitness-associated recombination [21, 45, 46], condition-dependent sexual reproduction [47-49] and revelation of phenotypic variation – through capacitors that buffer the effect of genetic variation in times of well being, and possibly reveal it in times of stress [50, 51], such as the heat shock protein HSP90 in the fruit fly *Drosophila melanogaster* [52]. We suggest that stress-induced parasex may function as a capacitor of adaptation, revealing variation that accumulated during many generations of clonal reproduction. Thus, even if it occurs only rarely and only under stress conditions, it would provide new diversity, some of which would be better capable of surviving the stress condition than their parents, thereby providing an adaptive advantage to the organism.

In this context, it is tempting to speculate about the forces driving the evolution of the parasexual cycle: is it maintained because it increases the ability of cells to evolve in response to their environment or, alternatively, is it a meager remnant of a complete sexual cycle that is deteriorating? We propose that parasex contributes to *C. albicans* evolution through the increase in genotype diversity exactly at the times when such diversity is needed. As such, it may be a case of evolution of evolvability [44, 53].

One problem when considering the evolution of parasex is its high cost, even in comparison with conventional sex. That is, in addition to the probability that the shuffling of genotypes will disrupt existing advantageous gene combinations [30], parasex between siblings produces offspring that have high levels of whole chromosome homozygosity and that are frequently aneuploid [13], which are genome changes that usually result in a reduced growth rate (reviewed in [41, 42]). However, in the context of high stress, this cost may not be as significant: if the stress is severe enough to inhibit mitotic growth, then asexual reproduction would be a dead end. Thus, as in the case of condition-dependent sex, a gene affecting the switch to parasex would benefit from paying almost any cost for a chance to change its genetic background when it resides in a maladapted/highly stressed genotype [48].

The potential for parasex to reveal variation at high levels, possibly even higher than conventional sex, suggests that parasex may be an effective strategy for the rapid generation of phenotypically diverse progeny, some of which confer better adaptation to the stress. This could be particularly important when considering a commensal/opportunistic pathogen life cycle that undergoes intense inbreeding. Pathogens in general have relatively low chances of meeting unrelated individuals as potential mates, as they are restricted to individuals co-infecting the same host, and the host is usually colonized by a single strain [54]. Nonetheless, as a commensal/pathogen associated with mammals, *C. albicans* experiences constantly changing environmental conditions generated by the host immune system, as well as by the challenge of growing in different host niches. Thus, parasex may provide a better solution than conventional sex to the challenge of generating the variation necessary for survival when the available mating partners are limited to siblings.

Concluding remarks

Most organisms must have mechanisms to survive stress and for many eukaryotes meiosis generates diversity under stress conditions [47, 49]. In *C. albicans*, parasex is the only known form of sexual exchange and there is currently no evidence for conventional meiosis, even if some genes required for meiosis are clearly necessary for recombination during parasex [13]. We propose that parasex in *C. albicans* is an example of a rare phenomenon that is likely to occur much more frequently under stress conditions, such as those found during interaction with the immune system of an animal host or upon exposure to antifungal drugs. Under such conditions, the genetic diversity produced by parasex might be crucial for survival. This advantage may apply to other organisms as well. For example, a parasexual cycle in *Candida tropicalis* shares some regulatory features, but differs in phenotypic details from the *C. albicans* parasexual cycle [55]. In addition, other fungi (e.g., *Aspergillus nidulans*) that have meiotic sexual cycles also undergo a non-meiotic chromosome loss process that provides adaptive advantages and has some similarity to the concerted chromosome loss process in *C. albicans* [56]. Finally, other fungal commensals and pathogens that are assumed to be asexual may undergo parasex as a mechanism to generate diversity in response to stress. We suggest that the use of different stress conditions may facilitate the detection of rare mating events in these organisms, as well as in *C. albicans*.

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Glossary

Aneuploidy	an imbalance in chromosome number. Diploids (organisms with two copies of each chromosome) carrying one chromosome in three copies (trisomics) or one chromosome in only one copy (monosomics) are often less fit under standard lab conditions but can have a selective advantage under some stress conditions.
Biofilms	groups of cells that adhere to a surface and to each other to form a layered structure that often includes an extracellular matrix produced by the cells that promotes adherence.
Loss of heterozygosity (LOH)	homozygosis of alleles due to either recombination or whole chromosome loss. LOH can affect short tracts of the genome, but more frequently occurs via single crossovers that homozygose large portions of a chromosome arm (e.g., Fig. 2). Whole chromosome LOH arises through chromosome loss followed by reduplication of the remaining chromosome.
Parasex	a non-conventional life cycle involving alternation of generations. In <i>C. albicans</i> , diploids mate and the tetraploid products undergo a reduction to diploidy, often with 1 or more trisomic chromosomes, via the poorly understood process of concerted chromosome loss.
Polyploidy	an increase in the number of complete sets of chromosomes. Most organisms alternate between diploidy (two copies of each chromosome) and haploidy (one copy of each chromosome). In <i>C. albicans</i> , the parasexual cycle alternates between diploidy and tetraploidy (four copies of each chromosome).
White/opaque switching	a phenotypic switch from ‘white’ colonies that are shiny, white and domed, composed of cells that are ellipsoid, have smooth cell walls and not mating-competent; to ‘opaque’ colonies that are matte, grey and flat, composed of cells that are oblong, have pimpled cell walls and are mating competent.

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Box 1. Outstanding questions

- How frequently does parasex occur *in vivo*? Does it occur at different rates in different host niches? While this might be measured using strains with marked chromosomes, it may also require the presence of different stresses to promote parasexual cycle progression.
- How do cells become *MTLhom in vivo*? Do recombination events predominate or is whole chromosome loss more frequent—either as an initial event or as a feature of parasexual progeny? Surveys of clinical isolates will require analysis of multiple single nucleotide polymorphisms along chromosome 5 in order to distinguish different mechanisms of LOH.
- What is the frequency of reciprocal vs non-reciprocal homozygosis at the *MTL* locus? This type of analysis can be performed by screening half-sectorized colonies that arise from LOH events.
- What is the maximal distance between mating competent cells that can be successfully traversed by conjugation tubes to yield tetraploid progeny? This will require analysis both in the presence and absence of biofilms and, if an appropriate niche is found, within host tissue.
- What triggers the concerted chromosome loss process? Are tetraploid progeny always stable or does the tetraploid zygote often undergo concerted chromosome loss soon after conjugation? How many divisions are required to go from tetraploid to ~diploid genome content? Time-lapse microscopy of the mating and concerted chromosome loss process using fluorescent markers to follow individual chromosomes and/or nuclei has the potential to reveal the types of mitotic divisions that occur after mating.
- Does re-mating of parasexual progeny occur *in vivo*?
- Does stress induce parasex or non-meiotic chromosome loss in other organisms as well?

Figure 1. Stress and the para-sexual cycle

Shown are the different steps of the parasexual cycle. Each oval represents a cell, and the letters represent the genotype at the mating type locus: *a/a*, *a/α*, or *α/α*. Parasex in a wild type heterozygous strain requires four consecutive events all of which occur with increased frequency under stress conditions. (i) the mating type-like (*MTL*) locus must become homozygous; (ii) cells must switch to the opaque state; (iii) cells of opposite mating type (*a/a* and *α/α*) must grow towards each other and mate to form tetraploids; (iv) tetraploids must undergo concerted chromosome loss to return to a ~diploid state. Progeny from parasexual crosses have high levels of homozygosity and are often trisomic (genome cartoons, lower right) [13]. Some progeny are homozygous for *MTL*, and thus can re-enter the parasexual cycle at step (ii). LOH - loss of heterozygosity.

Figure 2. Reciprocal recombination yields adjacent cells with opposite homozygous mating types
 Shown are chromosomes undergoing inter-homolog recombination during G2 of the cell cycle. Upon chromosome segregation, this event results in potential mating partners located adjacent to one another. In the lab on a petri plate, this type of event within a single cell results in production of a half-sectored colony. Stress dramatically increases the rate of reciprocal recombination [21], thus increasing the likelihood of *MTL* homozygosis (from a/α to a/a or α/α) via reciprocal recombination.

Figure 3. Sex versus parasex: possible progeny at one locus

Shown are the possible genotypes of inbred progeny, where each oval represents a possible cell genotype and letters represent the genotype at a single locus with two alleles, B and b, that is unlinked to the *MTL* locus. Ploidy of the majority of the genome is $2n$ (not indicated) or tetraploid ($[4n]$). Four different types of genetic exchange are considered: **(A)**

Heterozygous parents (genotype Bb) undergoing parasex. Approximately diploid progeny (following concerted chromosome loss) usually carry two alleles at the considered locus (genotypes BB, Bb, and bb) but are sometimes trisomic (genotypes BBb and Bbb), shown in orange, are possible progeny of the first Bb x Bb mating. Re-mating of progeny can generate additional genotypes (e.g., BBB from BB x Bb or bbb from bb x Bb, shown in yellow).

Approximately tetraploid progeny usually carry 4 alleles (genotype BBbb from the first mating, in orange, and BBBB, BBBb, Bbbb, and bbbb from remating, in yellow) or can be trisomic (BBb and Bbb from the first mating, in orange, and BBB and bbb from remating, in yellow). Note that each trisomic genotype can occur in either a $\sim$ diploid or a $\sim$ tetraploid background, possibly with different phenotypes. Rare aneuploidies such as monosomies are not shown. **(B)** Heterozygous parents (Bb) undergoing meiotic sex. 3 different offspring

genotypes are possible (BB, Bb, bb), plus a BBbb zygote. Re-mating would not increase diversity further. **(C)** Homozygous parents (BB) undergoing parasex. Progeny can be

approximately diploid or tetraploid at most loci in the genome. Approximately diploid progeny are usually BB, but can be aneuploid with BBB, and possibly monosomic (genotype B). Approximately tetraploid progeny can include BBBB and BBB. **(D)**

Homozygous parents (BB) undergoing meiotic sex: All offspring would be of genotype BB, plus a BBBB zygote.