DOI: http://dx.doi.org/10.7551/978-0-262-32621-6-ch088

Clonal development is evolutionarily superior to aggregation in wild-collected *Saccharomyces cerevisiae*

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Abstract

The vast majority of multicellular organisms develop clonally via 'staying together' after mitotic reproduction. Evolutionary theory predicts that cells staying together provides several key advantages over multicellular construction via cells 'coming together', but little empirical work has directly compared these developmental modes. In our previous work evolving multicellularity *de novo* in the yeast *Saccharomyces cerevisiae*, cells evolved to form clonal clusters exclusively through post-division adhesion of mitotically-produced cells, a result that reflects the strong bias towards clonal development in extant multicellular taxa. An equally parsimonious explanation, however, is that cluster development through incomplete cell separation is simply easier to evolve than the production of the adhesive compounds required for aggregation. To disentangle these hypotheses we repeated the experiment of Ratcliff et al (2012), selecting for rapid settling through liquid medium. Instead of using a unicellular ancestor, however, we started our experiment with five wild strains of yeast capable of aggregating into clusters via flocculation. Clonally-developing 'snowflake' yeast evolved and invaded 36/40 experimental populations within 155 transfers, and competition experiments revealed that invading snowflake yeast were substantially more fit than their floc contemporaries. These results support the hypothesis that clonal development is evolutionarily superior to aggregation, and demonstrate that 'snowflake' yeast can readily evolve in diverse, wild-collected yeast strains.

Introduction

The evolution of multicellular organisms from unicellular ancestors is considered a 'major transition' in the history of life on earth. As such, it was one of a few innovations that allowed for the evolution of increased complexity (Smith and Szathmary, 1995). The transition from uni- to multicellularity has occurred at least 25 times in separate lineages (Grosberg and Strathmann, 2007). This transition involved a fundamental shift in biological organization, as individual cells, formally organisms in their own right, evolve to become integral parts of a new, higher-level organism. A key step in the evolution of multicellularity was a transition to larger size, which necessitated the formation of simple cellular clusters (J. T. Bonner, 1998; Boraas, et al., 1998; Kirk, 2005; Pfeiffer and Bonhoeffer, 2003). Selection must then shift from the single cell level to the cluster level, resulting in clusters that are themselves Darwinian individuals (Damuth and Heisler, 1988; Godfrey-Smith, 2013; Michod, 2005; Smith and Szathmary, 1995).

Construction of an organism from lower-level units that are fully capable of Darwinian evolution is potentially problematic, however, as it may result in evolutionary conflict between the lower- and higher-level units (i.e., cells and multicellular organisms). The potential for conflict is especially strong when selection for multicellular-level functionality results in reproductive altruism among cells (e.g., differentiated somatic cells that are at a reproductive dead-end) (Herron and Michod, 2008; Libby and Rainey, 2013; Michod, 2005). This raises a fundamental question in evolutionary biology: How do the fitness interests of lower and higher-level units become aligned, limiting the negative consequences of evolutionary conflict? Similarly, how are lower-level units (cells) de-Darwinized, limiting the potential for among-cell selection to undermine multicellular-level selection?

Not all multicellular organisms are constructed in the same manner. There are two basic modes of body formation: potentially unrelated cells either 'come together' to form a body, or cells 'stay together' after reproduction, which results in clonal development if the life cycle includes a genetic bottleneck (J. T. Bonner, 1998; Tarnita, et al., 2013). Multicellular organisms have evolved via both routes. For example, the myxobacteria are a group of soil-dwelling bacteria that exhibit a social foraging behavior, coming together to form swarms that increase their feeding efficiency (Olive, 1975; Velicer and Vos, 2009). However, the vast majority of independent transitions to multicellularity have occurred via staying together (J. T. Bonner, 1998), suggesting that this is the superior mode of multicellular development.

Evolutionary theory predicts that multicellular development via cells staying together should provide several key advantages over cells coming together (Grosberg and Strathmann, 2007; Tarnita, et al., 2013). This mode of development limits among-cell genetic variation, especially if the life cycle includes a genetic bottleneck (Grosberg and Strathmann, 1998). Limiting genetic variation among lower-level units has several benefits: 1) It eliminates the potential for evolutionary conflict, since there is little standing genetic d iversity within a higher-level unit for selection to act on (Grosberg and Strathmann, 1998; Hamilton, 1964; Michod, 2005; Michod and Roze, 2001). 2) High among-cell genetic relatedness favors the evolution of traits that increase the fitness of the cluster, even if this reduces the fitness of individual cells.

This facilitates the evolution of cellular division of labor (J. Bonner, 2003; Hamilton, 1964; Queller, 2000). 3) Any genetic variation that arises due to mutation gets partitioned among multicellular offspring, allowing selection to act on the multicellular-level phenotypic effects of *de novo* mutation. This also allows selection to act against mildly deleterious alleles (Grosberg and Strathmann, 2007) that would be hard to select against in chimeric organisms.

Despite the clear predictions of evolutionary theory, it has been difficult to test the hypothesis that multicellular development via staying together should be superior to coming together. This is largely due to a lack of model systems in which both modes of development can be induced. The yeast *Saccharomyces cerevisiae* can form clusters either by incomplete cell separation after mitosis, producing 'snowflake yeast' , or by coming together through adhesive glycoprotein production, a process known as flocculation (Smukalla, et al., 2008). In prior experiments, Ratcliff et al (2012) found that snowflake yeast evolved in 10/10 replicate populations selected for faster settling. This raises the possibility that staying together is superior to coming together in this yeast model system, but it is also possible that this trait simply evolves more readily than flocculation. Here we repeat the experiment of Ratcliff et al., 2012, but rather than starting with a unicellular yeast, we start with wild-collected highly flocculent strains. Our experiment thus 'stacks the deck' in favor of floc, as all yeast start out with the ability to form a cluster via aggregation. If staying together is adaptive, it will need to evolve *de novo* and invade a population of aggregative yeast.

Methods

Strains, culture conditions, and selection regime

We used five field-isolated flocculating unicellular *S. cerevisiae:* strains YJM450, YJM454, YPS1000-1, YPS1009-2, and M5-2. Diploids were generated by streaking a and α mating type haploids on YPD agar plates (per liter: 20 g dextrose, 20 g peptone, 10 g yeast extract, 15 g agar). Single strains were isolated through three rounds of single-colony bottlenecking, and diploidy confirmed by tetrad formation after 4 d of shaking incubation at 30°C in sporulation media (per liter: 20 g potassium acetate, 2.2 g yeast extract, 870 mg synthetic amino acid mix, 0.5 g glucose). A single clone of each strain was then used to start eight replicate populations (40 populations total). Yeast were grown in 10 mL liquid YPD in 25 × 150 mm tubes for 24 h at 30°C, with 250 rpm shaking. Every 24 h, the populations were subjected to settling selection by centrifuging at 100 g for 10 seconds (selection protocol described fully in Ratcliff et al, 2012). Whole populations were cryogenically preserved every 7 d at -80°C.

Constructing fluorescently labeled yeast

Single-strain isolates were obtained from a single population of strain M5-2 (replicate 2) after 60 and 120 days. Each strain was transformed to express either the green fluorescent protein yeGFP or red fluorescent protein dTomato constitutively under the TEF2 promoter, using the LiAc/SS-DNA/PEG method of transformation (Gietz, et al., 1995). Transformed strains were then imaged with a SPOT Flex 64 MP camera on an Olympus IX 70 microscope at 10 and 20 X magnification.

Measuring yeast phenotypes

The predominant phenotype (e.g., snowflake or floc) was determined microscopically after 7, 28, 60, 91, 120, and 155 days of evolution. After 24 h of growth in liquid YPD (30°C, 250 rpm shaking), 10 µL of culture was placed on a slide under a 25 × 25 mm cover slip and imaged at 10x and 40x magnification. Snowflake yeast develop by post-division adhesion of cells, as opposed to floc's adhesive aggregation, allowing us to differentiate phenotype via cluster morphology. The numerically dominant phenotype was scored at each time point.

Relative fitness of early snowflake yeast vs. floc yeast

To measure the relative fitness of early snowflake yeast, we isolated a pair of snowflake and floc yeast strains from the 10 populations that contained both phenotypes at transfer 60 (4 from M5-2, 4 from YPS1009-2, 1 from YJM454, and 1 from YPS1000-1, respectively). All strains were grown in liquid YPD for 24 h, then snowflake/floc pairs were diluted 1:200 into 10 mL liquid YPD. These 10 strain pairs were competed over 5 rounds of selection for both growth and settling (100 x *g* for 10 s). For each pair, three replicate competition tubes were established. Snowflake and floc colonies have a distinct morphology (i.e., smooth colonies for floc and rough colonies for snowflake; see Figure 2a&b). We therefore used plate counts to determine fitness. Each competition tube was plated out at 1:10,000 dilution onto YPD plates after 24 h of growth (pre settling selection), and again after five rounds of growth and settling selection. Colonies were counted on digital images (in ImageJ) made from each plate after two days of growth at 30°C, taken with a Pentax K10D DSLR with a SMC Pentax-D FA 1:2.8 macro lens. Relative fitness was calculated using the ratio of Malthusian growth parameters (Lenski, et al., 1991).

Results and Discussion

Prior experiments selecting for rapid settling of yeast through liquid medium (Koschwanez, et al., 2013; Oud, et al., 2013; Ratcliff, et al., 2012) found that clusters rapidly evolved, but these developed strictly via cells 'staying together' after reproduction, producing the 'snowflake' phenotype. It is unclear, however, if this is because snowflake yeast are actually superior to floc yeast that develop via aggregation, or if snowflake yeast simply arise more readily via mutation. By repeating our prior experiment with five wild-collected highly flocculent *Saccharomyces cerevisiae* strains, we sought to determine if snowflake yeast could invade populations of aggregative yeast. We found that snowflake yeast invaded readily, and in head-to-head competition possessed a substantial fitness advantage over their floc competitors.

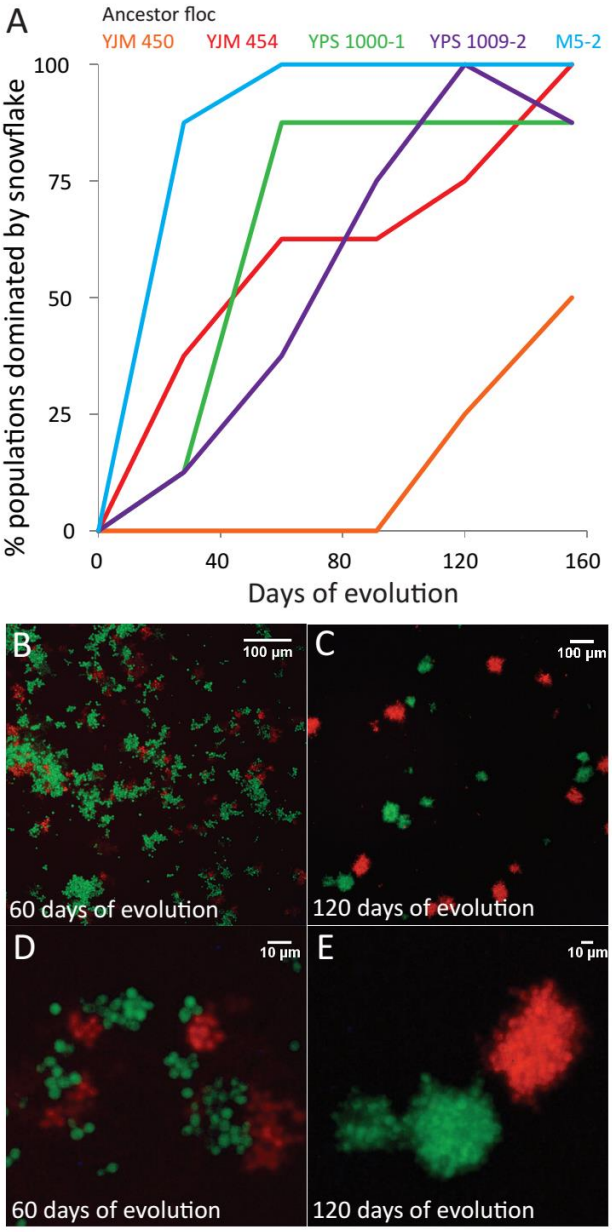

Figure 1. Snowflake yeast evolve and displace floc in all five genetic backgrounds. A) Shown are the percentage of the eight replicate populations of each starting strain that are dominated by snowflake yeast, as determined by cluster morphology. Snowflake yeast develop by cells staying together after reproduction, while floc develop by the coming together of adhesive cells. Morphological differences between clusters are readily apparent in flocculant yeast (B,D) and snowflake yeast (C,E). These yeast were isolated from the same replicate population (M5-2) at either 60 or 120 days of evolution. In each case, a single isolate is labeled with either either the green-fluorescent yeGFP or red-fluorescent dTomato.

Snowflake yeast rapidly appeared in our experimental populations. Within just 28 transfers, snowflake yeast had evolved *de novo*, and risen to high frequency (>50%) in 12/40 experimental populations. Over the course of the experiment (155 days), snowflake yeast evolved in 36/40 populations, generally driving their floc ancestors to extinction (Figure 1a). Indeed, floc regained numerical dominance in just one population taken over by snowflake yeast (Figure 1a).

To measure the relative fitness of invading snowflake yeast, we isolated pairs of floc and snowflake yeast from the 10 populations undergoing invasion at 60 transfers. We grew these pairs in isolation, and then inoculated competition populations with a 50:50 ratio (biomass) of each strain and measured relative fitness over 5 transfers. Invading snowflake yeast were more fit than their floc counterparts in all cases, but this was significant for only 6/10 strain pairs (Figure 2c; $F_{9,40} = 14.543$, $P < 0.0001$; ANOVA).

So why are snowflake yeast superior to floc yeast? We propose both proximate and ultimate hypotheses. A key aspect of fitness in our experimental system is the ability to rapidly settle to the bottom of the test tube, and for this task snowflake yeast may possess a direct advantage. While floc yeast are certainly able to settle quickly, they rely on a stochastic process for cluster formation. Specifically, clusters are formed through the collision and adhesion of sticky cells, and fragment when shear forces separate cells. In contrast, snowflake yeast form through a far more deterministic process, with daughter cells adhering to their parents after mitotic reproduction. Clusters only fragment when the among-cell tension exceeds cell-cell adhesive strength. Cluster size, and therefore settling speed (Ratcliff, et al., 2013), of individual clusters is heritable and is consistent for different genotypes of snowflake yeast (Ratcliff, et al., 2012; Rebolleda-Gomez, et al., 2012). Alternatively, floc's stochastic aggregative method of cluster formation may result in clusters that are more variable in size and settling speed. As a result, they may lack the ability to consistently produce large, fast settling clusters, allowing invasion by snowflake yeast.

Snowflake yeast may also possess an ultimate advantage, and be more capable of multicellular adaptation. Snowflake yeast develop clonally, while floc yeast form genetically chimeric clusters (Figure 1b,d). Since snowflake yeast clusters are clonal, there is little potential for the evolution of "cheating" lineages that realize a cell-level fitness benefit at the expense of cluster-level fitness (Grosberg and Strathmann, 1998). The chimeric clusters formed by flocculation may result in the evolution of selfish lineages of cells, interfering with multicellular adaptation (Diggle, et al., 2007; Smith and Szathmary, 1995).

Even if cheating is not an issue, the high relatedness among cells in snowflake clusters should favor the evolution of reproductive altruism and cellular division of labor (Hamilton, 1964; Willensdorfer, 2009). The ability for clusters to partition tasks amongst cells may offer many benefits (Smith and Szathmary, 1995; Szathmáry, et al., 2011). Snowflake yeast evolved a simple among-cell division of labor in response to a growth rate-settling rate trade-off. Large clusters grow less quickly than small clusters, due to limited nutrient availability to internal cells. Large snowflake yeast evolved an elevated rate of apoptosis, resulting in the production of proportionally smaller clusters that grow more rapidly (Ratcliff, et al., 2012).

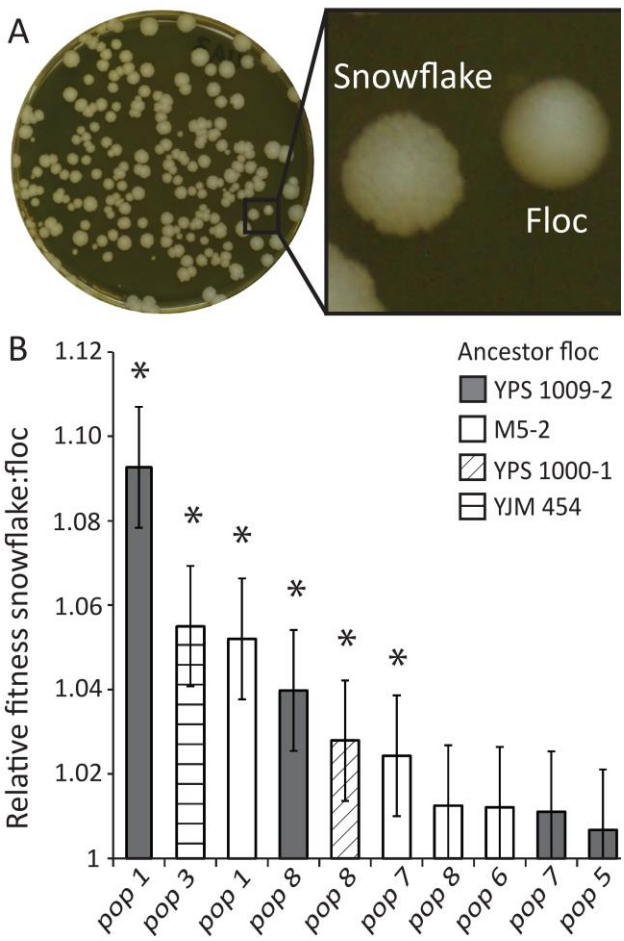

Figure 2. Fitness of early snowflake strains. A) Snowflake and floc colonies have a distinct morphology, allowing us to use plate counts to determine fitness. B) Early snowflake yeast have a significant fitness advantage in 6/10 pairs isolated from the same population at transfer 60. Error bars are the 95% confidence intervals of least-squares means, derived from a 1-way ANOVA. Shown in (A) is a competition plate of a YPS 1009-2 floc-snowflake pair at a 1:10,000 dilution. Asterisks denote significance at the 0.05 level.

Apoptotic cells leave no direct descendants, making this trait costly to individual cells expressing it, but increases the fecundity and fitness of the multicellular cluster (Libby, et al., 2014). This highlights an important shift in the level of selection from the unicellular to multicellular level, which may facilitate the subsequent evolution of multicellular complexity (Damuth and Heisler, 1988; Michod, 2005; Smith and Szathmary, 1995). Genetically chimeric floc clusters are less individuated at the multicellular level, which may limit their capacity for multicellular adaptation (Godfrey-Smith, 2013; Michod, 2005).

Our results raise an obvious question: If snowflake yeast are actually superior to floc yeast, then why are floc more common in nature? We can think of two possible reasons. Flocculation provides protection from environmental stressors (like alcohol (Hu, et al., 2005) and antibiotics (Lachance, 1990). Unlike snowflake yeast, flocculation can provide a fitness advantage even if opportunities for growth are limited. A rare floc strain can still join a group (and obtain a benefit of stress protection) as long as it produces adhesive glycoproteins (Smukalla, et al., 2008). In contrast, snowflake yeast clusters must grow large in order to gain the benefits of size, which requires a relatively resource-rich environment. Floc yeast should be far better at dispersing, as single cells and small clusters readily break away from a larger group. This both increases the number of propagules formed by a single genotype, and may also increase the distance of dispersal of each propagule. If dispersal is important to fitness, floc yeast may possess a substantial advantage.

Conclusion

'Staying together' and 'coming together' describe the two known modes of multicellular development. Evolutionary theory predicts that staying together is superior to coming together, and here we report the first empirical test directly comparing these two modes of development. Consistent with theory, we find that yeast that form clonal multicellular clusters (snowflake yeast) possess a striking fitness advantage over those that form chimeric aggregates (floc yeast). We hypothesize that the superiority of snowflake yeast could be due to either proximate effects of developmental mode (a larger average cluster size with a smaller variance than floc yeast), and/or ultimate evolutionary effects (more capable of multicellular adaptation than floc yeast). Further experiments are under way testing these hypotheses.

Acknowledgments. The authors would like to thank Adaugo Asouzu for laboratory assistance and Barry Williams for providing the ancestral floc yeast isolates. This work was supported by National Science Foundation grant DEB-1051115.

Author contributions. WR and MT conceived of the project. JP and WR conducted the experiments, analyzed the data, and wrote the paper.

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