Figures and figure supplements

Improved use of a public good selects for the evolution of undifferentiated multicellularity

John H Koschwanez, et al.
Three engineered strategies for growth in low sucrose:

1. Form multicellular clumps.
2. Make more invertase.
3. Import sucrose.

At low cell density in low sucrose concentrations, yeast cells cannot capture enough glucose and fructose to grow.

Figure 1. Three engineered strategies for growth in low sucrose. Strategy 1, form multicellular clumps, was previously verified (Koschwanez et al., 2011). The results of testing strategy 2, make more invertase, and strategy 3, import sucrose, are shown in Figure 2. All three strategies outcompete wild-type strains when the sole carbon source is 1 mM sucrose (Table 1).

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Figure 2. Two strategies for growth from low sucrose concentrations. (A) Strong expression of secreted invertase allows growth from a single cell at low sucrose concentrations. All GAL1 promoter induction data is from the same yeast strain yJHK312 in which transcription of SUC2 is driven by the GAL1 promoter. Galactokinase (GAL1) is deleted from this strain so that galactose acts as an inducer and not as a carbon source, and the Gal regulon has been engineered to produce a graded rather than a bistable response to increased galactose concentrations by overexpressing GAL3 from the ACT1 promoter (Ingolia and Murray, 2007). (B) Sucrose import allows growth from a single cell in low sucrose concentrations. The 'SUC2, import' strain yJHK372 expresses SUC2 from the SUC2 promoter and MAL11 from the ACT1 promoter. The 'SUC2, no import' strain yJHK222 expresses SUC2 from the SUC2 promoter. The 'suc2-1cyt, import' strain yJHK373 expresses cytoplasmic invertase from the SUC2 promoter and MAL11 from the ACT1 promoter. The 'suc2Δ, import' strain yJHK374 has SUC2 deleted and expresses MAL11 from the ACT1 promoter. For both (A) and (B), single cells were inoculated by fluorescence activated cell sorting (FACS) into 150 µl wells at the given sugar and galactose concentration and grown without shaking for 85 hr at 30°C and the results shown are totals of three experiments; each experiment used one plate per sugar concentration, and each plate used 24 wells per strain or galactose concentration. In both figures, 2 mM glucose + 2 mM fructose is used as a positive control, and error bars refer to 95% binomial confidence interval using the adjusted Wald method. FRU is fructose, GLC is glucose, and SUC is sucrose.

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Figure 3. Evolved populations show a clumpy phenotype. (A) An ancestor derivative (yJHK111) after growth in 1 mM sucrose. (B) Schematic of experimental evolution. Cells were inoculated in 1 mM sucrose media, grown to high density, and then $10^5$ cells were reinoculated into fresh media for a total of 25–35 cycles. A sample was frozen down at each serial dilution. (C) Samples taken from the last time point of the evolved populations. Representative DIC images were taken with a 40× objective in a glass-bottomed, 96-well plate. All scale bars are 50 µm. DOI: 10.7554/eLife.00367.006

Figure 3—figure supplement 1. Eleven of twelve clones show a clumpy phenotype. EvoPopulation7 had three morphologically distinct clones, named EvoClone7A, 7B, and 7C. For each of the remaining populations, we selected one clone from a group of eight morphologically indistinguishable clones. Representative DIC images were taken with a 40× objective in a glass-bottomed 96-well plate. Scale bars are 50 µm. DOI: 10.7554/eLife.00367.007
Figure 3—figure supplement 2. Size distribution of evolved clones. Sizes were measured on a Multisizer 3 Coulter counter. The thick line in the center of the box is the median size of the cells. The left and right side of the box correspond to the first and third quartiles. The lines extending from the boxes (the whiskers) correspond to the values within 1.5 times the inter-quartile range (IQR). Clumps or cells with sizes outside the whiskers are shown as dots. Cells were grown in the media shown for at least 12 hr, and sizes were measured during log-phase growth. Note that the x-axis is log-scale.

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Figure 3—figure supplement 3. EvoClone9 morphology changes in different media. Representative 40× DIC images of EvoClone9 cells that were grown to log phase in (A) 1 mM sucrose, (B) 80 mM glucose and (C) 1 mM glucose + 1 mM fructose. Scale bars are 50 µm.
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Figure 4. Clumpiness is due to failure to separate and not flocculation. Each image shows two genetically identical strains that are labeled with different fluorescent proteins, shown as magenta and green in the image. The strains were grown together from low density in 1 mM sucrose. (A) Lab strain with constitutively expressed FLO1. Flocculation is evident from the mix of colors in a single clump. (B) Lab strain with the RM11 allele of AMN1. (C) EvoClone2. (D) EvoClone9. The clumps in the AMN1-RM11 strain and the evolved clones are uniform in color, showing that clumpiness is due to failure to separate after cell division. Representative fluorescent images were taken with a 20× objective in a glass-bottomed, 96-well plate. All scale bars are 50 µm.
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Figure 4—figure supplement 1. Clumpiness is due to failure to separate and not flocculation. See Figure 4 for a description of the experiment. Images of EvoClone2 and EvoClone9 in Figure 4 are cropped versions of the images shown here for EvoClone2 and EvoClone9 (cropped area outlined in yellow). Scale bars are 50 µm. DOI: 10.7554/eLife.00367.011
Figure 5. Schematic of bulk segregant analysis and evolved clone reconstruction. (A) A clone is selected from the population and then backcrossed to a derivative of its ancestor. The resulting diploid is sporulated, allowing the mutant alleles to randomly segregate among the haploid progeny. When the haploid progeny are selected for growth in low sucrose, only those cells with causal alleles (red triangles) remain; non-causal alleles (blue diamond, square, and circle) segregate randomly and are present in about half of the spores. (B) The ancestor, evolved clone, and pool of selected progeny are sequenced. Comparing the genome sequences of the ancestor and evolved clone reveals mutations. The allele frequency in the selected spores can then be estimated from the frequency of the reads in the pool of selected progeny. We classified any mutant allele present in >90% of the reads as a putative causal mutation (Table 5). (C) The wild-type alleles in the ancestor were replaced with the putative causal mutations to recreate the evolved clone (Figure 7). (D) Growth of the recreated strain was tested in low sucrose (Table 1).
Figure 5—figure supplement 1. Protocol for replacing alleles in yeast. (A) Design the region of the gene to be included in the plasmid as follows: (1) determine the ‘usable’ gene, or the promoter plus the open reading frame (ORF) plus the terminator. (2) Figure out which end of the usable gene is closer to the mutation. This is the side that will not be truncated. (3) Find a cut site >300 bp away from the mutation. If the new mutation is needed after transformation and before loopout, place the cut site toward far end of gene. This is the end of the gene that will be truncated. If old function is needed, place the cut site toward near end of gene. The region between the mutation and the cut site is now the ‘plasmid’ region. The distance between the cut site and the mutation is necessary because of recision. (D) Extend the plasmid region at least 300 bp onto the opposite side of the cut site. This distance ensures efficient transformation. (E) Extend the plasmid region to the nearest end of the usable gene.

Figure 5—figure supplement 1. Continued on next page
This will ensure the gene is intact after transformation. (F) Find the distance from the mutation to each end of the plasmid region. Extend the plasmid region so that the length that does not include the cut site is larger than the length that does include the cut site. This ensures that loopout is more likely to result in the new mutation. (2) PCR amplify the plasmid region of the gene, treat it with polynucleotide kinase, and blunt-end ligate it into a digested URA3 plasmid backbone that has been treated with Antarctic phosphatase. Only pertinent regions of the plasmid are shown in the diagram. An additional yeast drug marker is useful in the plasmid backbone to verify that the insert has looped out. (3) Transform the yeast with the cut plasmid and select on—URA. This will integrate the linearized plasmid into the chromosome. PCR amplify and sequence the new allele to verify insertion; one primer should be outside the included region. (4) Grow the transformed yeast strain overnight in YPD to allow the insert to loop out through homologous recombination and plate on 5FOA. Cells will only grow on 5FOA if the URA3 marker has looped out. PCR amplify and sequence the new allele with both primers inside the included region to verify that the new allele is the only copy of the gene remaining. Replica plate to YP 2% acetate to eliminate petite mutants. DOI: 10.7554/eLife.00367.016

**Figure 5—figure supplement 1. Continued**

**Figure 6.** Putative causal mutation frequency at time points during the evolution. The alleles at the indicated time points were sequenced using Sanger sequencing, and frequencies were estimated from peaks in the trace plots. See [Figure 6—figure supplements 1 and 2](#) for the other evolved populations. See Table 5 for amino acid and nucleotide changes. DOI: 10.7554/eLife.00367.018
Figure 6—figure supplement 1. Putative causal mutation frequency at time points during the evolution for EvoClone 1, 3, 4, 5, 6, 7A, 7B, and 7C. See Figure 6.
DOI: 10.7554/eLife.00367.019
Figure 6—figure supplement 2. Putative causal mutation frequency at time points during the evolution for EvoClone 8 and 10. See Figure 6.
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Figure 7. Engineering in alleles can recreate the evolved and ancestral morphologies. The ancestral strain was converted to the evolved morphology by converting ancestral alleles to those of the putative causal mutations and the evolved strains were converted to the ancestral morphology by converting the putative causal mutations to their ancestral alleles. The strains were grown separately in 1 mM sucrose and then mixed. The top row shows EvoClone2 strains and the bottom row shows EvoClone9 strains. The ancestor constitutively expresses mCherry and is shown in yellow; the evolved clone constitutively expresses YFP and is shown in green. The recreated strain (left) and the reverted strain (right) constitutively express CFP and are shown in magenta (recreated evolved) and cyan (reverted to ancestral). Representative confocal fluorescent (left) and brightfield (right) images were taken with a 60× objective in a glass-bottomed, 96-well plate. All scale bars are 50 µm.
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Figure 8. Bulk segregant analysis with the recreated strains verifies causal alleles and shows that alleles responsible for clumpiness are selected in low sucrose and not low monosaccharide. The recreated strains were backcrossed, sporulated, and selected in three different media: 1 mM sucrose (low sucrose), 80 mM glucose (high glucose), and 1 mM glucose plus 1 mM fructose (low monosaccharide). The mutant allele fraction was estimated from Sanger sequencing across the allelic variants. The size of the data point (small, medium, or large) for each allele and media combination refers to one of three independently derived diploids. (A) Recreated2 allele segregation. (B) Recreated9 allele segregation. (C) Ancestor strain with ACE2 deleted (ace2Δ) has a clumpy phenotype. The ACE2 mutation in Recreated2, a likely loss of function mutation that caused the clumpiness in EvoClone2, was selected for in low sucrose and was not selected for in low monosaccharide. (D) Ancestor strain that has wild type alleles of IRC8, MCK1, and GIN4 replaced with the EvoClone9 alleles has a clumpy phenotype. All three mutant alleles were selected for in low sucrose and were not selected for in low monosaccharide. Representative DIC images were taken with a 40× objective in a glass-bottomed, 96-well plate. Scale bars are 50 µm.
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Figure 8—figure supplement 1. Change in HXT4 and SUC2 expression in various allelic combinations of Recreated2 compared to the ancestor. Change in RNA expression was measured using RT-qPCR using RNA isolated from a culture grown in 1 mM sucrose minimal media. Each point is the mean change in expression from three independent trials. The total length of the error bar is twice the size of the standard deviation. Ancestor alleles are shown in upper case and mutant alleles are shown in lower case. (A) Change in HXT4 expression. Allelic combinations containing mth1 have higher expression than those combinations with MTH1. (B) Change in SUC2 expression. Note that the axes are different in the two plots.

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