Competition Between Species can Drive Public-goods Cooperation within a Species

by

Hasan Celiker

B.S. Electrical and Electronics Engineering
Bilkent University, 2009

Submitted to the Department of Electrical Engineering and Computer Science
in Partial Fulfillment of the Requirements for the Degree of
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ABSTRACT

Costly cooperative strategies are vulnerable to exploitation by cheats. Microbial studies have suggested that cooperation can be maintained in nature by mechanisms such as reciprocity, spatial structure and multi-level selection. So far, however, almost all laboratory experiments aimed at understanding cooperation have relied on studying a single species in isolation. In contrast, species in the wild live within complex communities where they interact with other species. Little effort has focused on understanding the effect of interspecies competition on the evolution of cooperation within a species. We test this relationship by using sucrose metabolism of budding yeast as a model cooperative system. We find that when co-cultured with a bacterial competitor, yeast populations become more cooperative compared to isolated populations. We show that this increase in cooperation within yeast is mainly driven by resource competition imposed by the bacterial competitor. A similar increase in cooperation is observed in a pure yeast culture when essential nutrients in the media are limited experimentally.

Thesis supervisor: Jeff Gore
Title: Assistant Professor of Physics

CONTENTS

I - Introduction

Costly cooperative strategies are vulnerable to exploitation by cheats[1,2]. Microbial studies have suggested that cooperation can be maintained in nature by mechanisms such as reciprocity[3,4], spatial or temporal heterogeneity[5-7] and multi-level selection[8]. So far, however, almost all laboratory experiments aimed at understanding cooperation have relied on studying a single species in isolation. In contrast, species in the wild live and evolve within complex communities where they interact with other species[9]. Interspecific competition – that is competition between species – has been shown to play a key role in shaping species distributions[10,11] and adaptation[12,13]. Nevertheless, little effort has focused on establishing a link between this ecological pressure and the evolution of cooperation within a species[14-17]. We test this relationship by using sucrose metabolism of budding yeast as a model cooperative system that is subject to social parasitism by cheater strategies. We find that when co-cultured with a bacterial competitor, *E. coli*, yeast populations become more cooperative compared to isolated populations. We show that this increase in cooperation within yeast is mainly driven by resource competition imposed by the bacterial competitor. A similar increase in cooperation is observed in a pure yeast culture when essential nutrients in the media are limited experimentally. We develop a simple logistic growth model that suggests that it is environmental adversity that is driving cooperation within yeast. In support of this, we find experimentally that when the species interactions are primarily mutualistic—as occurs with the bacterium *B. subtilis*— cheating is favored within the yeast population. Our results demonstrate that interspecific competition can be a major force in shaping the evolution of cooperation within a species.

II - Results

Wild-type yeast cells break down extracellular sucrose cooperatively by paying a metabolic cost (Supplementary Fig 1) to synthesize the enzyme invertase[18,19]. Invertase is secreted into the periplasmic space between the plasma membrane and the cell wall where it hydrolyzes sucrose to the sugars glucose and fructose. In a well-mixed environment, most of the sugars produced in this manner diffuse away to be consumed by other cells in the population, making the sugars a shared public good. Under these conditions, an invertase knockout strain can act as a cheater that takes advantage of and invades a cooperating population. However, cooperator cells capture ~1% of the sugar they produce directly due to a local glucose gradient, which provides cooperators an advantage when present at low frequency. The cooperator and cheater strategies are therefore mutually invasible, leading to steady-state coexistence between the two strategies in well-mixed batch culture[19]. For example, starting with an initial cooperator fraction of 10 %, we observed little change in cooperator frequency after 10 days of co-culture (Fig 1). In these experiments, every 48 hours we performed serial dilutions into fresh sucrose media and measured the fraction of cooperator cells within the yeast population using flow cytometry (methods and Supplementary Fig. 2).

To test whether interspecific competition can influence cooperation within the yeast population, we performed the same experiment, but this time co-cultured the cooperator and cheater yeast along with a bacterial competitor, *E. coli* (DH5α). This strain of *E. coli* cannot utilize sucrose[20] but could grow on arabinose, another carbon source present in the media. Arabinose could not be utilized by our yeast strains (Supplementary Fig. 3). We

found that the presence of bacteria led to a dramatic increase in the cooperator fraction in the yeast population over the 10 days of growth. Whereas the cooperator fraction in the pure yeast cultures was only ~14% at the end of the experiment, in cultures with the bacterial competitor the cooperator fraction increased up to ~45% (Fig. 1).

One possible explanation for this increase in cooperation within the yeast population is that bacteria behave as a 'superior' cheater strain by assimilating available free glucose, thus depriving cheater yeast cells of any sugar. In such a scenario, cooperator cells would do better than cheaters since they have at least some preferential access to the produced glucose. However, when we competed yeast against a mutant strain of *E. coli* (JM1100) that has much reduced glucose and fructose uptake rates[21] (Supplementary Information 1), we found a similarly dramatic increase in the cooperator fraction within the yeast population. The increase in cooperation is therefore not simply driven by competition for glucose between the two species. We also confirmed that this increase in cooperator frequency is not due to a hidden fitness difference between the two yeast strains uncovered by the presence of bacteria. Addition of excess glucose (0.2%) completely eliminated any increase in cooperation in all of these conditions, even though bacteria were still present (Supplementary Fig. 4). Therefore, we conclude that the increase in cooperator fraction is strongly related to public good production by cooperator cells.

To gain insight into the dynamics of competition between the two species, we monitored the optical absorbance of batch cultures seeded with yeast and bacteria. We found that the overall growth follows reproducible successional stages (Fig. 2a). Bacteria have a higher

growth rate than yeast and rapidly increase in biomass until they stop growing early during culture. In contrast, the yeast population takes relatively longer to establish but is able to continue growth after bacteria have stopped dividing. We reasoned that this succession might be due to acidification caused by fermentation, since *E. coli* growth can be severely limited at acidic conditions[22,23]. Indeed, when we monitored the fluorescence of a pH sensitive dye (fluorescein) in the media, we measured a sharp drop in fluorescence (~pH) coinciding with bacterial growth and saturation (Fig. 2a and Supplementary Fig. 5). This suggests that the limited bacterial growth is caused by low pH brought about by sugar fermentation. Compared to bacteria, yeast cells are better able to tolerate the harsh acidic conditions[22] present in the later stages and can therefore continue to grow, albeit on depleted resources. In microbial assemblages, such ecological succession is a commonly observed phenomenon[24-26].

We reasoned that if acidic conditions restrict bacterial growth then it should be possible to delay the onset of this limitation by adding more pH buffer in the media. Consistent with this expectation, we found that the final biomass achieved by bacteria increased with the concentration of the pH buffer (PIPES) in the culture (Fig. 2a). We also saw that this increased bacterial density restricted the yeast growth due to pronounced competition between the two species. Prompted by these observations, we decided to use the buffering capacity as an environmental variable to tune the intensity of competition between yeast and bacteria.

If cooperation were indeed driven by interspecific competition, we would expect to see a positive correlation between the level of cooperation within the yeast population and the degree of competition imposed by bacteria. To test this, we performed competition experiments with yeast and bacteria as before and varied the buffering capacity of the media. As expected, increasing the buffering further promotes cooperation within the yeast population, but only when competing against bacteria (Fig. 2b).

We next repeated these experiments by starting out with different initial fractions of cooperators (30%, 50%, 90%) and observed the same trend in all the conditions we examined (Supplementary Fig. 6). Even starting with an initial fraction of 90% cooperators, at high buffering we saw an increase of ~6% in the frequency of cooperators after 10 days of growth. This implies that at equilibrium the cheater cells might be completely purged from the yeast population under the pressure of interspecific competition. Using our mutant *E. coli* strain (JM1100) as a competitor instead of DH5α, we verified that we could get similar results across all of these conditions with the same dependence of cooperation on buffering capacity (Supplementary Fig. 7).

To measure the density of the yeast and bacteria in these experiments, we used flow cytometry at the end of each growth cycle (see Methods and Supplementary Fig. 7). We found that by the end of the last cycle, in cultures without any added buffer, bacteria (DH5α) went extinct, whereas at the highest buffer concentration used (20 mM), yeast was outcompeted by bacteria (Fig. 2c). However, at intermediate levels of buffering, yeast and bacteria could stably coexist. This coexistence is primarily a result of the

temporal heterogeneity mediated by acidification and the fact that bacteria and yeast partition into different niches[27] by utilizing different carbon sources in the media (arabinose and sucrose respectively).

Although in co-cultures bacteria went extinct without buffering, in pure cultures we found that bacteria could grow robustly under the same conditions. This observation suggests that the presence of yeast has a negative effect on bacteria. Resource competition and perhaps ethanol production combined with further acidification during yeast growth might be playing a role in producing this outcome[22,28]. When we analyzed the overall relationship between yeast density versus bacterial density across all buffer conditions for each cycle and different initial cooperator fractions, we found a consistent negative linear dependence (Fig. 2d). This relationship is the hallmark of interspecific competition whereby the two species reciprocally repress each other's growth[29].

Next, we hypothesized that if public-goods cooperation within the yeast population is driven by resource competition with bacteria, the same process should act to increase cooperation in a pure yeast culture when essential nutrients in the media are limited experimentally. To test this hypothesis, we competed cooperator and cheater yeast cells in uracil limited cultures. Our yeast strains are uracil auxotrophs and require uracil to be supplied in the media to grow (see Methods). As before, we performed serial dilutions every 48 hrs into fresh media and measured final fraction of cooperators and total yeast density. We found that the frequency of cooperators increased with decreasing concentrations of supplemented uracil (Fig. 3a). To make sure that this result is not due to

an anomaly related to the synthetic nature of auxotrophy, we also repeated this experiment by limiting a universal essential nutrient, phosphate. Again, consistent with our hypothesis, we observed that the cooperator fraction increased at low phosphate concentrations (Fig. 3b). In all these conditions, we saw that yeast density decreased with limiting concentrations of nutrients as expected. Once again, we observed a negligible change in cooperator fraction in cultures with abundant glucose (0.2%), confirming that the observed behavior is intimately related to the sucrose metabolism.

These results suggest that limiting the carrying capacity selects for cooperation within the yeast population. If it is indeed the limited carrying capacity that is driving cooperation, then we would expect the increase in cooperation to be strictly dependent on the yeast density rather than the specific type of nutrient limitation. Consistent with this expectation, when we plotted the final cooperator fraction as a function of the final yeast density for both uracil and phosphate limitation conditions, we found that the resulting relationship was nearly indistinguishable for the two treatments. This observation strongly suggests that the underlying force driving cooperation was the same and related to the limited carrying capacity in both cases. Interestingly, we also found that, for both treatments, the final cooperator fraction was approximately linear as a function of logarithm of final yeast density (Fig. 3c).

To explain the increase in cooperation with limited carrying capacity, we developed a simple logistic growth model simulating the cooperative dynamics within the yeast population (see Supplementary Fig. 8). The model assumes that in the beginning of a

culture, yeast density is low and there is little glucose in the media because there are not enough cooperators to supply it. In these low-density conditions, cooperator cells grow faster than cheaters, as they have preferential access to the produced glucose and 'feel' a higher glucose concentration than cheaters do[19]. However, as the density increases above a critical value, cheating starts to be favored -- cheaters have a higher growth rate than cooperators -- because now there is enough glucose in the media that cooperators are at a disadvantage by carrying the burden of public good production while cheaters do not pay any cost. In the end, the culture logistically saturates to a set carrying capacity, K. To model the dynamics over ten days, the saturated culture is let to grow again after dilution into a fresh environment. To impose resource limitation in the model, we varied the parameter K across our experimental range. We found that this two-phase growth model could fit our experimental data reasonably well and explain the apparent negative correlation between the yeast density and the final frequency of cooperators (Fig. 3c). Thus, we conclude that smaller population size mediated by low nutrient availability should increase cooperation within yeast as long as the population is not driven to extinction[30].

Next, we analyzed our two species competition experiments to see if there is a similar relationship between yeast density and cooperator frequency. We found that competition with bacteria also resulted in a log-linear dependence between yeast density and final cooperator fraction (Fig. 3c). We also found that this relationship was reproducible and could be observed at the end of each growth cycle and for different initial cooperator fractions (Supplementary Fig. 9). Controlling for yeast population size, we found that

competition with bacteria is more effective in driving cooperation within yeast than resource limitation alone. Moreover, we observed a marked difference between the effect of our two bacterial strains – DH5α and JM1100 – in selecting for cooperation within yeast. The fact that DH5α has a higher glucose uptake rate than JM1100 suggests that glucose competition between yeast and bacteria might also be important. To account for glucose consumption by bacteria in our nutrient limitation model, we further lowered the growth rate of cheaters at low density for different treatments. We found that this could reliably reproduce the difference between various treatments shown in figure 3c. These results indicate that in addition to resource competition, other species' ability to directly interfere with the public-goods interaction within a species can also help drive cooperation.

Finally, to probe the generality of our results, we competed cooperator and cheater yeast against bacteria on solid agar with sucrose as the carbon source. Consistent with the results in liquid cultures, we observed that the presence of bacteria (JM1100) strongly selected for cooperation within yeast (Fig 4). Next, we asked: how would the cooperative dynamics within yeast be affected if the competing bacteria were also producing glucose just like cooperator yeast? To test this, we inoculated yeast cells on sucrose plates together with the soil bacteria *B. subtilis* instead of *E. coli*. Similar to wild-type yeast, *B. subtilis* breaks down sucrose with a secreted enzyme and generates extracellular glucose[20]. Surprisingly, we found that now cheating is favored within the yeast population (Fig 4). It seems that although *B. subtilis* cells compete for resources with yeast, they can produce enough glucose to reverse selection for cooperation within the

yeast population. We therefore conclude that other competing species do not necessarily promote cooperation within a species. Thus, caution must be taken in assessing the effect of one species on the other, as the nature of the interaction can drastically modulate the outcome.

III - Conclusion

Our findings provide evidence for a potentially general ecological mechanism —resource competition between species— for the evolution of public-goods cooperation within a species. These findings can help explain the apparent ubiquity of cooperative traits found in nature and improve our understanding of social evolution in natural microbial communities[16]. This study shows that a thorough understanding of the dynamics of interspecies interactions is crucial to resolve the origin of social traits in natural populations. Our results also argue that cooperation may be more stable than would be concluded from experiments that study a single species in isolation. Our two species community, which consists of widely used model organisms, is amenable to genetic manipulation and can be reconfigured to explore more complicated interactions between species – such as parasitism and warfare – that may affect within-species cooperation.

IV - Methods

Strains. All yeast (*S. cerevisiae*) strains were derived from haploid cells BY4741 (mating type **a**, EUROSCARF). The 'wild-type' cooperator strain has an intact *SUC2* gene and yellow fluorescent protein (yEYFP, gift from G. Stephanopoulos) expressed constitutively by the *TEF1* promoter inserted into the *HIS3* locus using the backbone

plasmid pRS303. The mutant cheater strain lacks the *SUC2* gene (EUROSCARF, *suc2Δ::kanMX4*) and has the red fluorescent protein tdTomato expressed constitutively by the *PGK1* promoter inserted into the *HIS3* locus using the backbone plasmid pRS303. Both of these strains had the same set of auxotrophic markers: *leu2Δ0, met15Δ0, ura3Δ0.* Both *E.coli* strains were derived from *E.coli* K-12. JM1100 was obtained from The Coli Genetic Stock Center (CGSC#: 5843). JM1100 strain (*ptsG23, fruA10, manXYZ-18, mgl-50, thyA111*) could grow on minimal media without additional thymine probably due to a picked up deoC mutation, therefore no additional thymine was used in the media for experiments with this strain. *B.subtilis* 168 was obtained from ATCC (#23857).

Batch culture media. All experiments were performed in defined media supplemented with the following carbon sources: 4% Sucrose, 0.2% L-Arabinose and 0.005% Glucose. For experiments with excess glucose, extra 0.2% Glucose was added to cultures. Our default defined media consisted of 0.17% yeast nitrogen base (Sunrise Science) plus ammonium sulfate (5 g/L) supplemented with the following amino acid and nucleotide mixture: adenine (10 mg/L), l-arginine (50 mg/L), l-aspartic acid (80 mg/L), l-histidine (20 mg/L), l-isoleucine (50 mg/L), l-leucine (200 mg/L), l-lysine (50 mg/L), l-methinonine (20 mg/L), l-phenylalanine (50 mg/L), l-threonine (100 mg/L), l-tryptophan (50 mg/L), l-tyrosine (50 mg/L), l-uracil (20 mg/L), l-valine (140 mg/L). For uracil limitation, uracil concentration was varied below the amount used in the default media. Uracil concentrations used in figure 3c: 1, 2, 4, 6, 10, 14 mg/L. Phosphate limited media contained 0.071% yeast nitrogen base without KH_2PO_4 (Sunrise Science) supplemented with 80 mM K_2SO_4 and the amino acid mixture used in the default media. To limit phosphate concentration, KH_2PO_4 was added to this media below the concentration (7.3

mM) used in the default nitrogen base. KH_2PO_4 concentrations used in figure 3c: 0.01, 0.03, 0.05, 0.1, 0.2, 0.3 mM. In all the experiments, pH was adjusted to 6.5 with NaOH and PIPES (pKa 6.8 @ 25°C) was used as a buffering agent for different conditions. For nutrient limitation experiments, a set PIPES concentration of 10 mM was used for all the conditions. In competition experiments with DH5α, a buffer range of 0-20 mM was used. We found that JM1100 was more acid tolerant than DH5α, therefore a narrower range of 0-10 mM of buffering was used for this strain.

Growth conditions. Before each experiment, yeast strains were grown in minimal media (2% glucose) for 20h at 30°C and bacterial strains were grown in LB at 37°C for 20h. These initial cultures were diluted in fresh media to start the experiments. In all the experiments described, initial inoculation densities were 10^6 cells/mL for bacteria and $7.5x10^4$ cells/mL for yeast. All experiments were performed in 96-well microplates containing 150 μL media per well. To enable gas exchange, microplates were sealed with two layers of a gas permeable tape (AeraSeal) and incubated at 30°C, 70% relative humidity, shaken at 825 r.p.m. Evaporation per well was measured to be 20% over 48h. For multi-day experiments, cultures were serially diluted 1:1,000 into fresh media every 48 hrs, taking evaporation into account.

Flow cytometry. Grown cultures were diluted 1:100 in PBS (phosphate buffered saline) and cells were counted on BD LSR II equipped with an HTS unit. For each well, two separate measurements using different settings were taken for yeast and bacteria. For measuring cooperator fraction and yeast density, a high SSC threshold (300) with SSC voltage 200 V was used to exclude bacterial counts (FSC voltage, 270 V). Cooperator and cheater yeast strains were gated on fluorescence (YFP and RFP respectively). For

each well, 20 μL of sample was measured with flow rate 1.5 μL/sec. Yeast was assumed to be extinct in wells with less than 400 counts and cooperator fraction was not calculated for these cases. To estimate the yeast population density, a calibration was used with measurements of yeast cultures with known densities. To measure bacterial density, SSC voltage was set to be 300 V with threshold 1000 to capture all the bacterial population. For each well, 5 μL sample was analyzed with flow rate 0.5 μL/sec. Bacterial counts overlapped with noise in FSC and SSC plots. To distinguish bacteria from noise, in every cycle, pure yeast culture controls was measured with the same settings used for bacteria (Supplementary Fig. 8). From these control measurements, noise was calculated and found to have a maximum coefficient of variation less than 0.03. To calculate actual bacterial counts, mean noise of 8 control wells of pure yeast cultures was subtracted from bacterial counts in each competition experiment. In conditions where bacterial population was not extinct, the bacterial counts with noise subtracted were always larger than the noise counts; therefore the variation in noise had little effect on bacterial density measurements. Bacterial density was estimated based on a calibration obtained by measurements of bacterial cultures with known densities.

Successional growth assay. Yeast and bacteria were grown and diluted in fresh media with initial densities same as described in 'growth conditions' section. Initial cooperator fraction was 50%. Culture media was the default media used in all two species competition experiments and cells were grown in microplates. Cultures were incubated using an automated shaker Varioskan Flash (Thermo Scientific) at 30°C, 800 r.p.m. To monitor pH, 0.6 μM fluorescein sodium salt (Sigma) was added to cultures. Every 15

minutes, absorbance (600 nm) and fluorescence (excitation: 488 nm, emission: 521 nm) measurements were taken for 40h.

Competition on agar plates. Solid agar media was prepared using 1.6% agar, 1% yeast extract, 2% peptone supplemented with either 2% glucose or 2% sucrose. Cells were spread on plates (100 mm diameter) containing 20 mL solid media using glass beads. In all the conditions, initial cooperator yeast to cheater yeast ratio was 1:5 (~17% cooperators). Plating density for yeast was aimed to be ~900 cells/plate (15 cells/cm^2), for JM1100 it was ~12 cells/plate (0.2 cells/cm^2) and again for *B. subtilis* ~12 cells/plate (0.2 cells/cm^2). Inoculated cultures were incubated for 4 days at 30°C until no further growth could be observed. Then, plates were illuminated under a blue light (~470 nm) transilluminator (Invitrogen) and imaged through an orange filter. Later, plates were destructively sampled by washing off colonies in PBS. Fractions were measured on BD LSR II flow cytometer using the yeast settings (see flow cytometry section). We also tried competing yeast against *B. subtilis* in liquid well-mixed culture, however we could not get coexistence of the two species, and *B. subtilis* was outcompeted by yeast, presumably due to the less acid tolerant nature of this bacterium compared to *E. coli*.

Glucose and fructose uptake measurements for *E.coli* strains. DH5α and JM1100 strains were grown overnight at 37°C in LB and then diluted into media containing 0.2% arabinose plus either 0.05% glucose or 0.05% fructose. Initial cell density for each strain was 5x10^6 cells/mL. For DH5α and JM1100, media contained 8 mM and 4 mM buffer respectively. After inoculation, 5 mL cultures were incubated at 30°C in 50 mL falcon tubes shaking at 300 r.p.m. Sugar uptake rates were determined by measuring the depletion of sugars during exponential growth according to the following equation[31]:

$$r = \mu \frac{S_0 - S(t^*)}{N(t^*) - N_0}$$

where r is the uptake rate of sugar and μ is the growth rate measured during exponential phase. N is the cell density inferred from optical density measurements. S represents the measured sugar concentration in the media. Measurements taken at two time points separated by t^* were used to calculate the uptake rates. The timing of the two measurements was chosen so that there was substantial depletion in sugar concentration during that period. Glucose concentration was determined by using a commercial glucose (hexokinase) assay reagent (Sigma). Fructose concentration was measured by using the same assay reagent in conjunction with the enzyme phosphoglucose isomerase (PGI), which converts fructose 6-phosphate to glucose 6-phosphate. Glucose uptake rates for DH5α and JM1100 were found to be 4.14×10^4 molecules s^{-1} $cell^{-1}$ and 0.72×10^4 molecules s^{-1} $cell^{-1}$ respectively. Fructose uptake rates for DH5α and JM1100 were found to be 0.47×10^4 molecules s^{-1} $cell^{-1}$ and 0.08×10^4 molecules s^{-1} $cell^{-1}$ respectively.

V - Figures

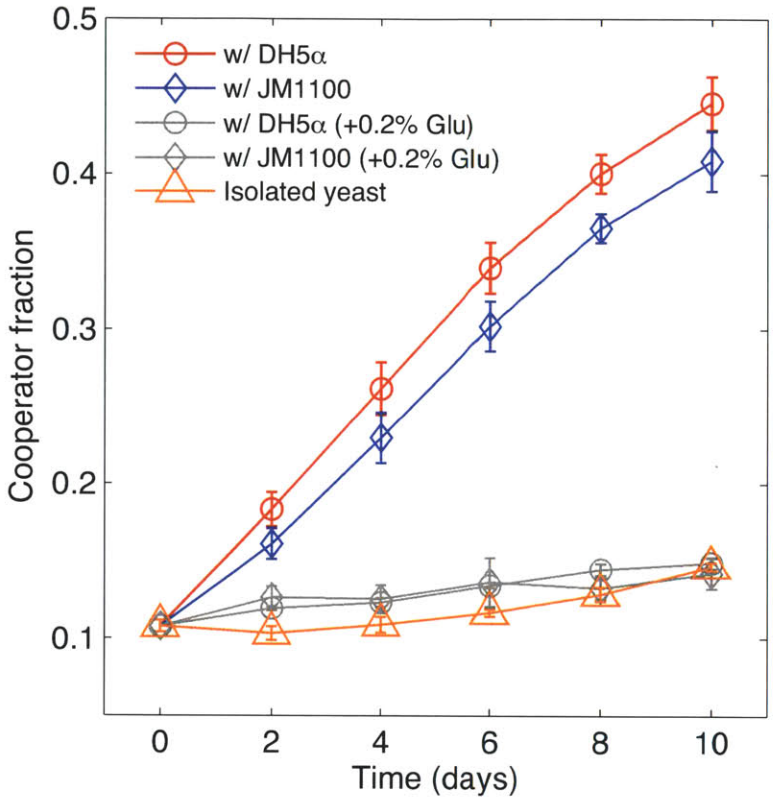


Figure 1: Presence of bacteria drives cooperation within yeast. When co-cultured with bacteria in sucrose media, yeast population becomes more cooperative. Both with DH5α or JM1100 – a mutant strain that grows poorly on glucose and fructose – a significant increase in cooperator fraction was observed compared to a pure yeast culture (isolated yeast) over 10 days of growth. Addition of excess glucose (+0.2%) to these cultures eliminated this increase in cooperator fraction, indicating that selection for cooperation is linked to the sucrose metabolism. In this experiment, culture media contained 4 mM buffer (PIPES). Error bars, ± s.e.m. (n = 3).

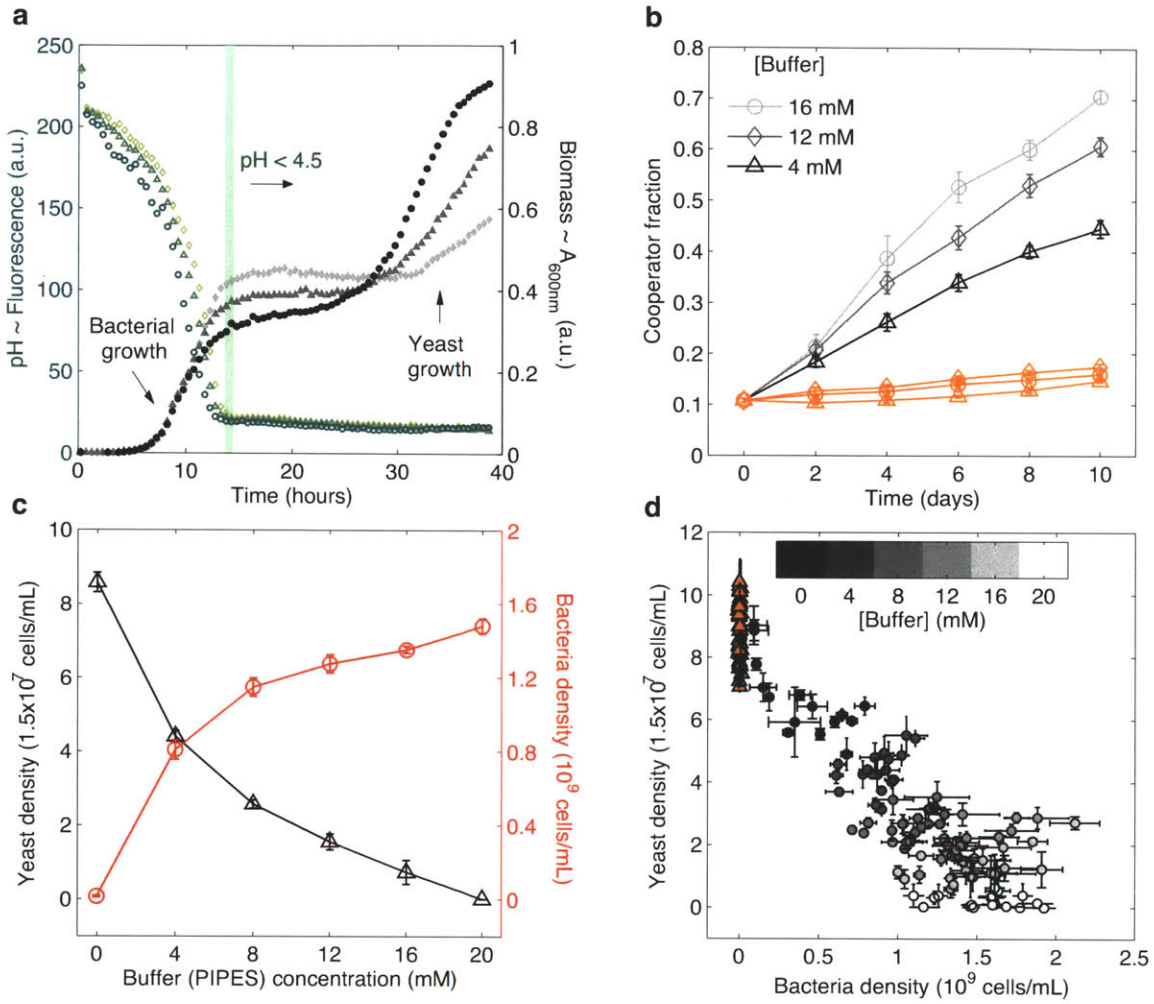


Figure 2: Correlation between the intensity of interspecific competition and cooperation within yeast. a, Successional growth dynamics in mixed cultures of yeast and bacteria. Absorbance (600 nm) was measured for different buffer (PIPES) concentrations: 4 mM (circles), 8 mM (triangles), 12 mM (diamonds). Simultaneously, fluorescence of a pH sensitive dye (fluorescein) was measured and a sharp pH drop was observed coinciding with bacterial growth. Note that as the buffering increases, the pH drop is slower and the final bacterial biomass is higher. **b,** Frequency of cooperators within yeast increases faster with increasing buffer concentration when competing against

bacteria. Isolated control populations under the same conditions displayed little change in cooperator fraction (orange symbols). **c,** Yeast (triangles) and bacterial (circles) density at the end of the last growth cycle as a function of buffering capacity. **d,** Yeast density versus bacterial density across all buffer concentrations and different initial cooperator fractions for each cycle. Control cultures (isolated yeast) for the same conditions are shown in triangles. In all the data presented, DH5α is used as the bacterial competitor. Error bars, ± s.e.m. (n = 3).

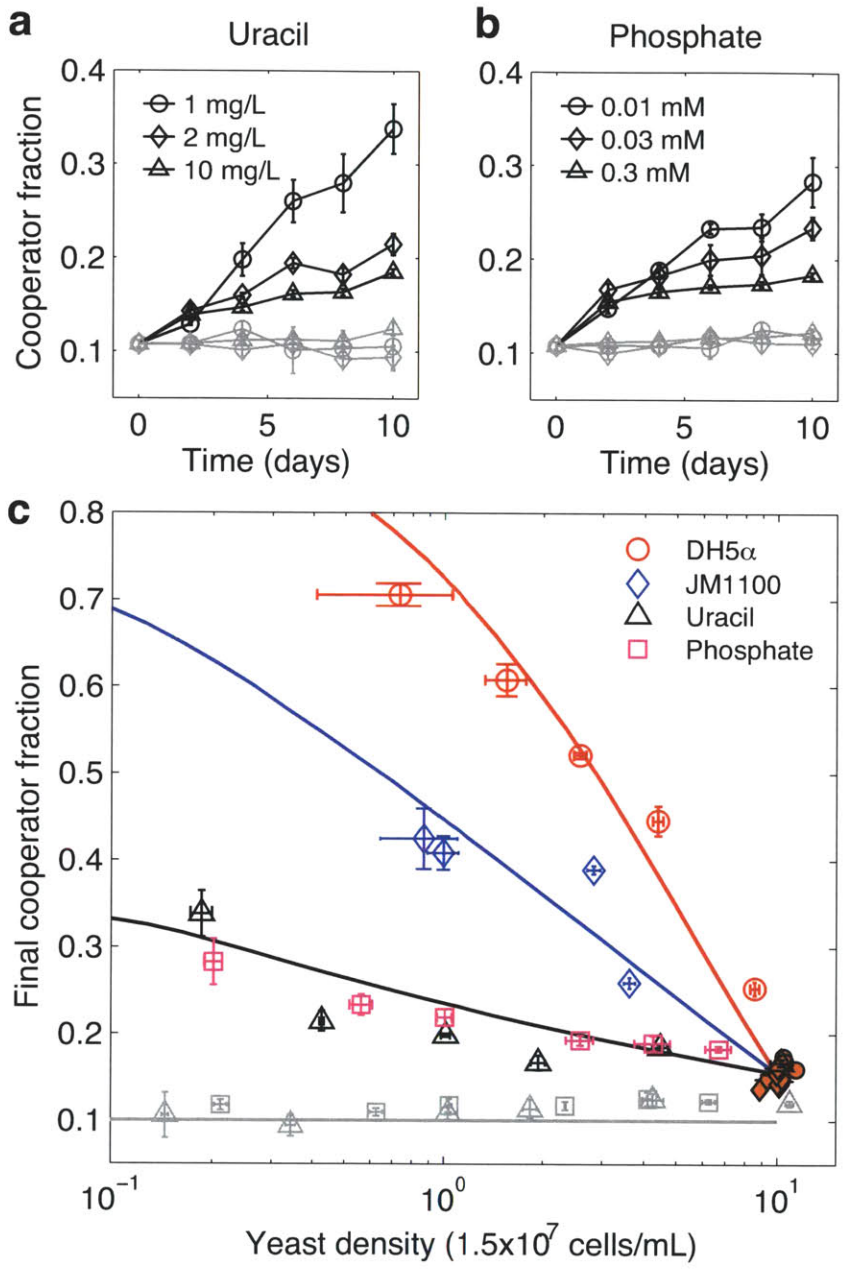


Figure 3: Nutrient limitation can drive cooperation within the yeast population even in the absence of bacteria. Limiting either uracil (a) or phosphate (b) drives cooperation within isolated yeast populations. Control cultures (gray symbols) with excess glucose (0.2%) displayed negligible change in cooperation. **c,** Final cooperator fraction versus final yeast density in bacterial competition and nutrient limitation experiments: DH5α,

JM1100, uracil, phosphate. With controls: uracil + 0.2% Glucose (gray triangles), phosphate + 0.2% Glucose (gray squares). Controls (isolated yeast) for competition with bacteria are shown in orange circles and diamonds for DH5α and JM1100 conditions respectively. Solid lines are model simulations for each condition. Error bars, ± s.e.m. (n = 3).

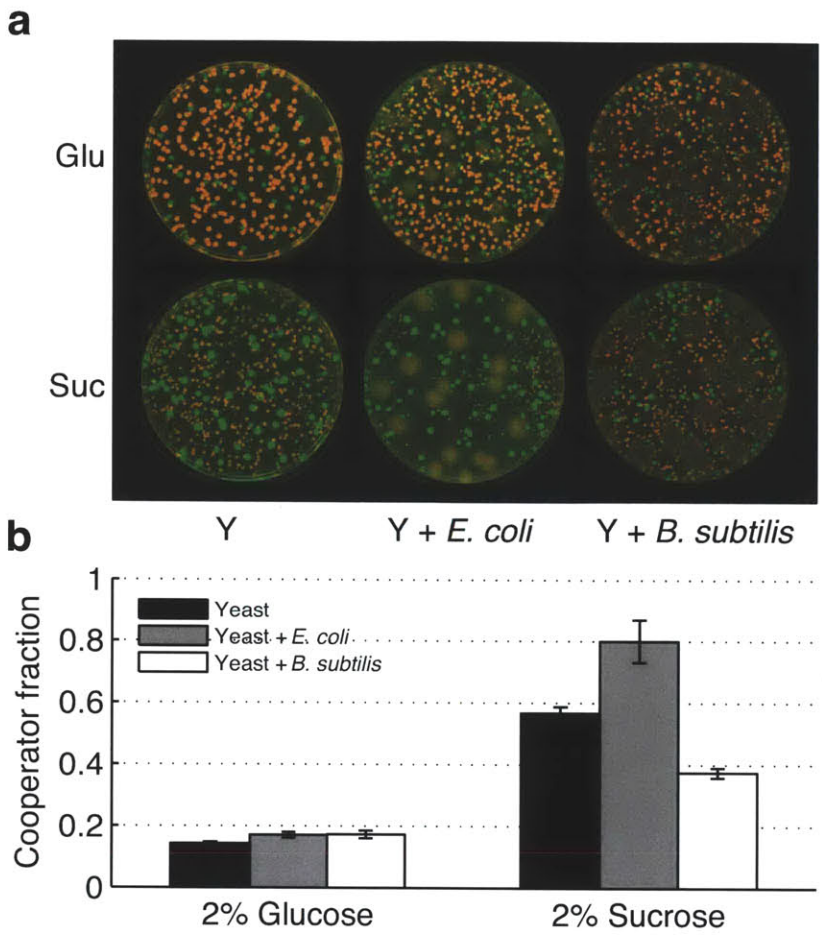
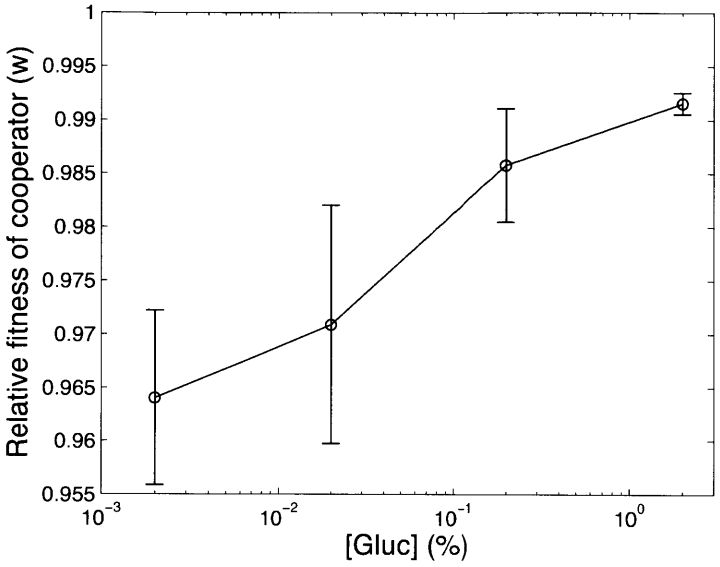


Figure 4: Competition against *E. coli* or *B. subtilis* on agar plates. Growth on agar plates of yeast only, yeast with *E. coli*, and yeast with *B. subtilis* **a,** Images were taken after 4 days of growth at 30°C. Yeast (Y) was competed against either *E. coli* (JM1100) or *B. subtilis* on rich media plates (100mm diameter) supplemented with either 2% Glucose or 2% Sucrose. Cooperator yeast colonies appear yellow/green, cheater yeast colonies appear red and bacterial colonies appear dull colored and bigger compared to yeast colonies. **b,** Colonies were washed off of imaged plates and yeast cooperator fractions were measured by flow cytometry. As expected, competition with *E. coli*

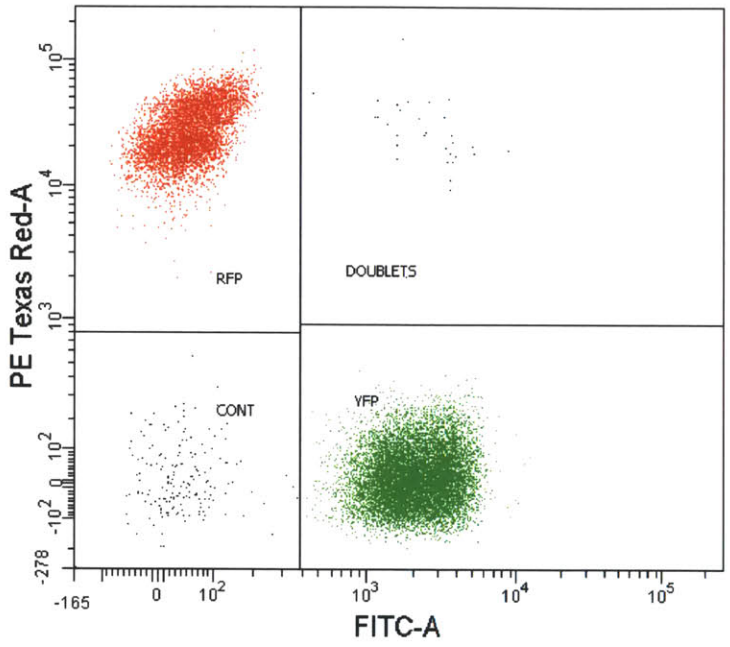
selected for cooperation within yeast. In contrast, *B. subtilis* favored cheating. Error bars, ± s.e.m. (n = 3).



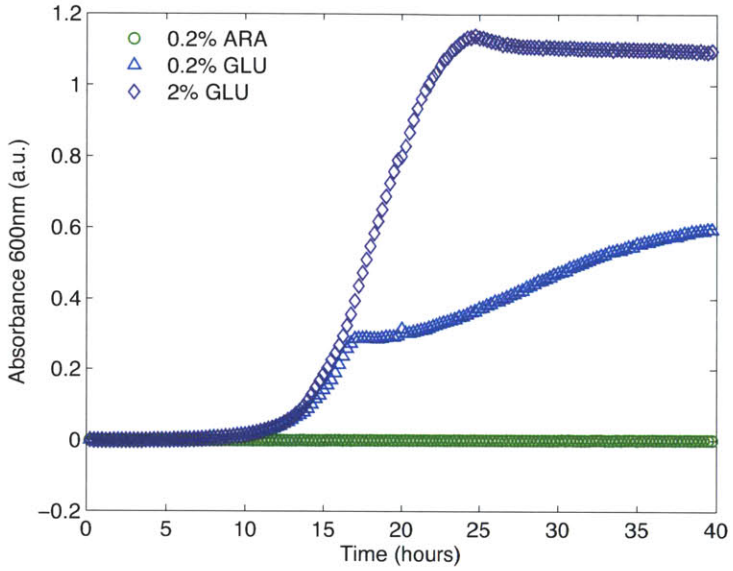
Supplementary Figure 1: Metabolic cost of invertase production. Invertase expression is maximal at low glucose concentrations but repressed when glucose is abundant[32,33]. We measured the metabolic cost of invertase production by co-culturing cooperator and mutant cheater yeast strains in glucose media by daily serial dilution (1:1,000) for three days. Starting cooperator fraction was 50% and initial cell density was $1.5x10^5$ cells/mL. At high concentrations of glucose, invertase expression is repressed and as expected, there was little fitness difference between the two strains. On the other hand, at low concentrations of glucose where invertase expression reached to its maximum, the cooperator strain had a fitness deficit of ~3-4% consistent with a metabolic cost associated with production and secretion of invertase. Plotted relative fitness (w) values are calculated using the following expression[18]:

$$w = \ln\left[\frac{D_f f_f}{D_i f_i}\right] \Big/ \ln\left[\frac{D_f(1-f_f)}{D_i(1-f_i)}\right]$$

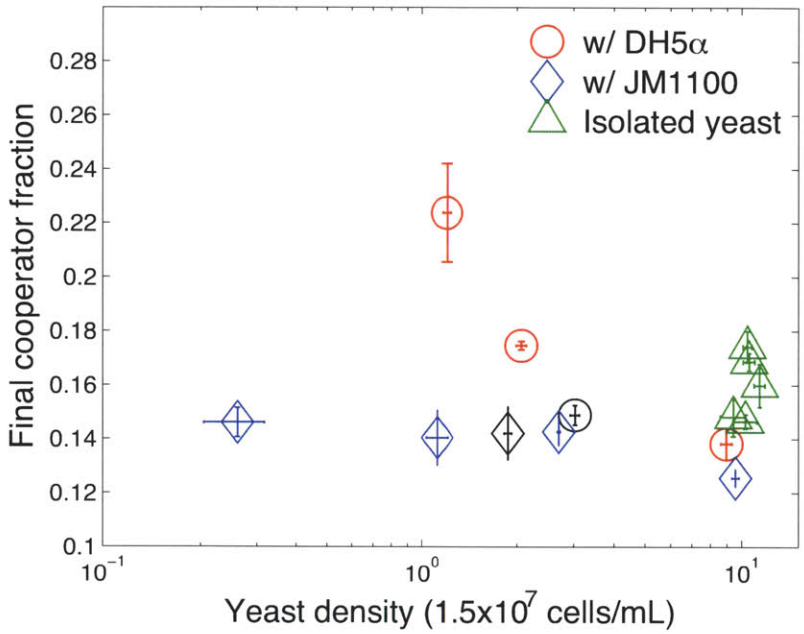
where f_i and f_f are the initial and final cooperator fraction and D_f and D_i are the final and initial total cell densities for each day. Data points represent mean of 3 measurements over 3 days with error bars ± s.e.m.



Supplementary Figure 2: Measurement of cooperator fraction with flow cytometry. Our yeast strains were tagged with constitutively expressed YFP and RFP proteins (cooperator and cheater respectively). We could distinguish between the two strains on BD LSR II flow cytometer. YFP was excited with a blue laser (488 nm) and emission was collected through a 530/30 nm filter (FITC-A channel). RFP was excited with a yellow/green laser (561 nm) and emission was collected through a 610/20 nm filter (PE Texas Red-A channel). The dot plot in the figure is a sample from a competition experiment between yeast and bacteria after 10 days of co-culture. The two strains were well separated on the different fluorescence channels. Cooperator fraction and final yeast density in each well were measured using yeast settings on the flow cytometer (see Methods).



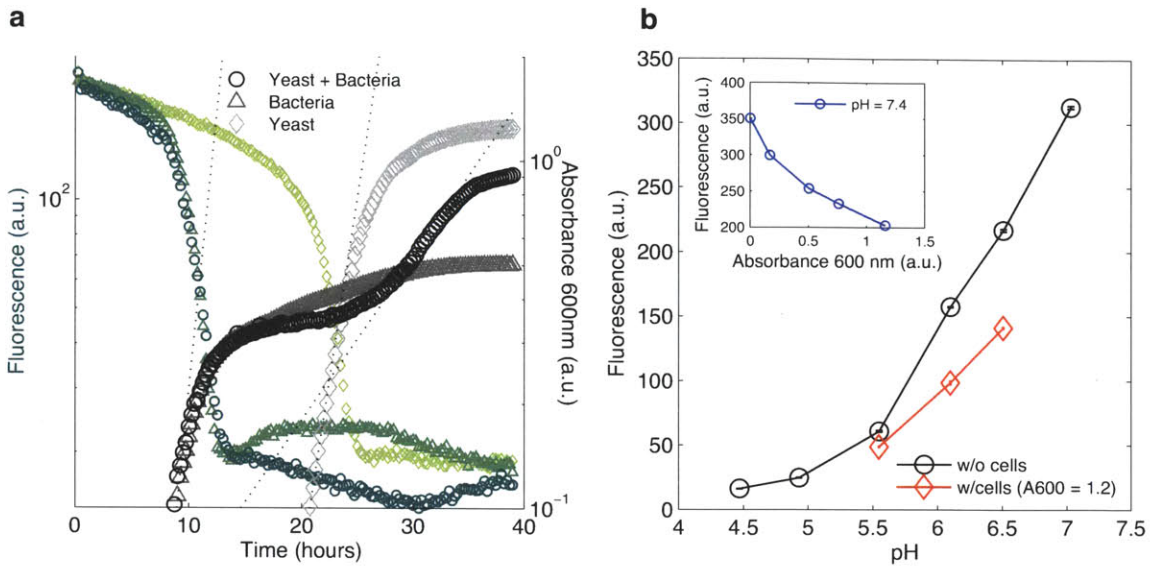
Supplementary Figure 3: Yeast growth on arabinose. We grew yeast (50% cooperator) on 0.2% arabinose, 0.2% glucose or 2% glucose. Initial cell density was the same as in the competition experiments (7.5×10^4 cells/mL). Absorbance at 600 nm was measured for 40 hrs. The results are plotted in the above figure. Our yeast strains were not able to grow on 0.2% arabinose.



Supplementary Figure 4: Excess glucose eliminates selection for cooperation in the presence of bacteria. Cooperator fraction after 5 cycles of dilution (10 days of growth) and corresponding final yeast density in competition against bacteria on default media (see Methods). Starting cooperator fraction was 10% for all the data presented. For the conditions with a bacterial competitor (w/ DH5α and w/ JM1100) media contained additional 0.2% glucose. Each individual data point represents the result for a different buffer concentration used. We see that although the yeast density is limited by the presence of bacteria, there is little increase in cooperator fractions when there is excess glucose in the media. Isolated yeast data (triangles) show the highest density yeast population can reach without the presence of bacteria. Black data points are the results for the condition used in figure 1 (4 mM buffering). The reason that the number of data points differ between DH5α and JM1100 treatments is that with DH5α yeast went extinct

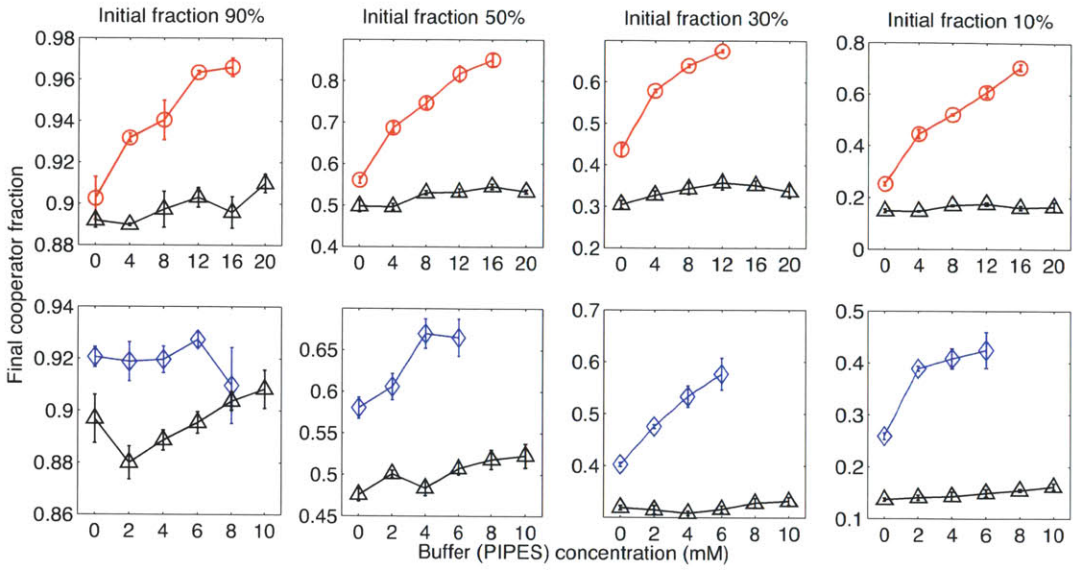
at some of the highest buffer conditions used and fractions were not calculated for those cases (see Methods).

In addition to these controls, we also tried to grow yeast on media spent by bacteria. To achieve this, we first grew bacteria on default media with varying buffer concentrations. Then, bacteria were spun down and yeast was grown in the supernatant with added glucose (0.2%) for 48 hrs. The results showed no change in the cooperator fraction, again ruling out a fitness difference between our two yeast strains that might be mediated by bacterial resource depletion. However, we could not dilute and propagate these cultures into new spent media, as the final yeast density was much lower than we observed in our competition experiments. Error bars, ± s.e.m. (n = 3).

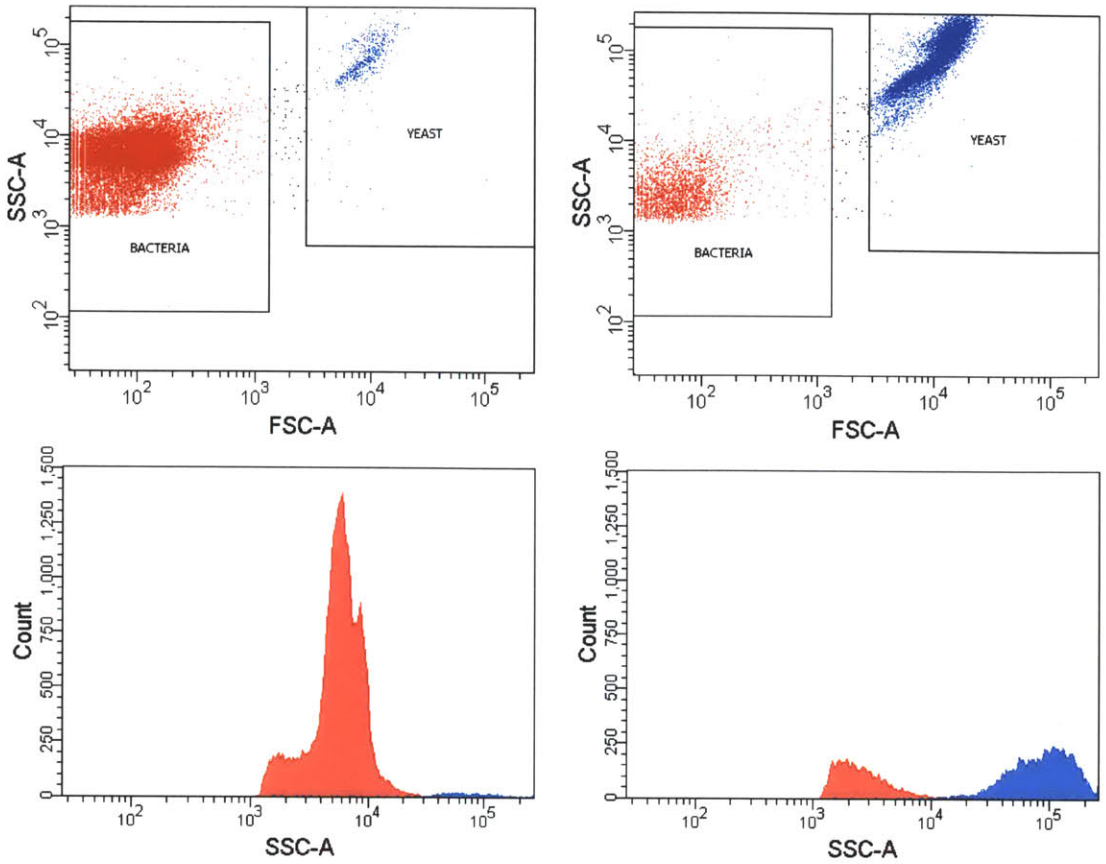


Supplementary Figure 5: Successional growth dynamics in mixed batch culture. a, Absorbance and fluorescence (~pH, see Methods) measurements for a co-culture of bacteria (DH5α) and yeast, an isolated bacterial culture and an isolated yeast culture. Initial cell densities were as described in Methods and were the same for each species in competition with the other species or by itself. All cultures were buffered with 4 mM PIPES. Dotted lines are the tangents to the absorbance traces during exponential growth. We see that the initial drop in pH in the mixed culture coincides with the pH drop in the isolated bacterial culture, which indicates that initial acidification in the mixed culture is strongly mediated by bacterial fermentation. In contrast, pH drop occurs much later in the isolated yeast culture, as the yeast population takes longer to establish. **b,** Fluorescein vs. pH calibration curve with and without cells in the media. pH of our default media was adjusted using NaOH without any added buffer and fluorescence was measured as described in Methods. Fluorescein was fluorescent across the relevant pH range (~4.5 to 6.5) and lost its fluorescence completely around pH 4.5, which is also quite close to the pH value where bacterial growth is limited[23]. The drop in pH shown in (a) and figure 2a

is not due to accumulating cell mass obscuring fluorescence measurement. By suspending yeast cells in the media at a density of $15x10^7$ cells/mL ($A_{600} \sim 1.2$) – which is the maximum density we observed in our experiments – we show that although there is a drop in fluorescence due to the presence of cells, it is not as dramatic as measured during growth. Inset shows the fluorescence versus absorbance (~cell density) relationship measured by suspending yeast cells in PBS (pH = 7.4) at different densities. Error bars, ± s.e.m. (n = 3).

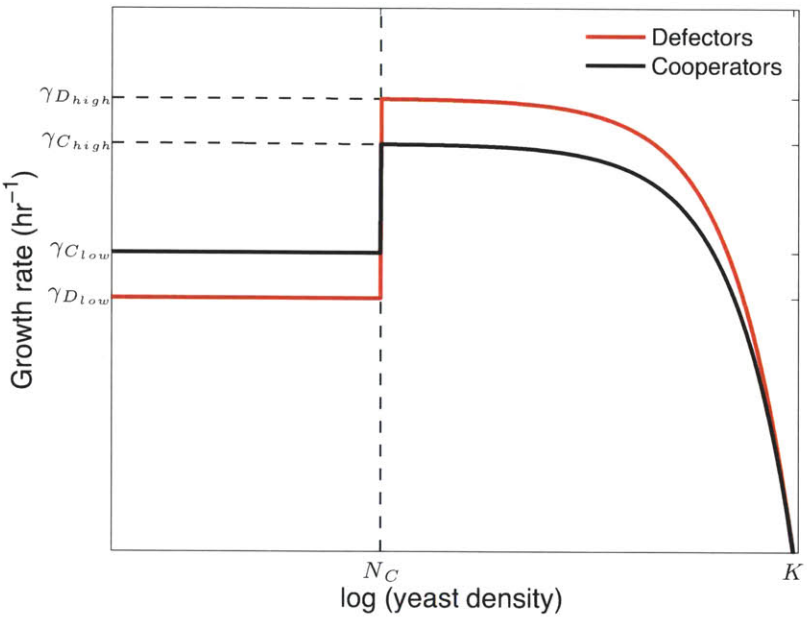


Supplementary Figure 6: Competition between yeast and bacteria with different initial cooperator fractions. Each individual plot shows the final cooperator fraction (after 10 days of growth) as a function of buffer concentration in the media. Top row shows the results for competition between yeast and DH5α (circles) and the bottom row shows the results for competition between yeast and JM1100 (diamonds). In all the plots, pure yeast controls are shown in triangles. Note that when competing against DH5α, even starting with 90% initial cooperator frequency, cooperator fraction increased in most of the buffering conditions, suggesting that at equilibrium yeast population might consist of only cooperators. For experimental details see Methods. Error bars, ± s.e.m. (n = 3).



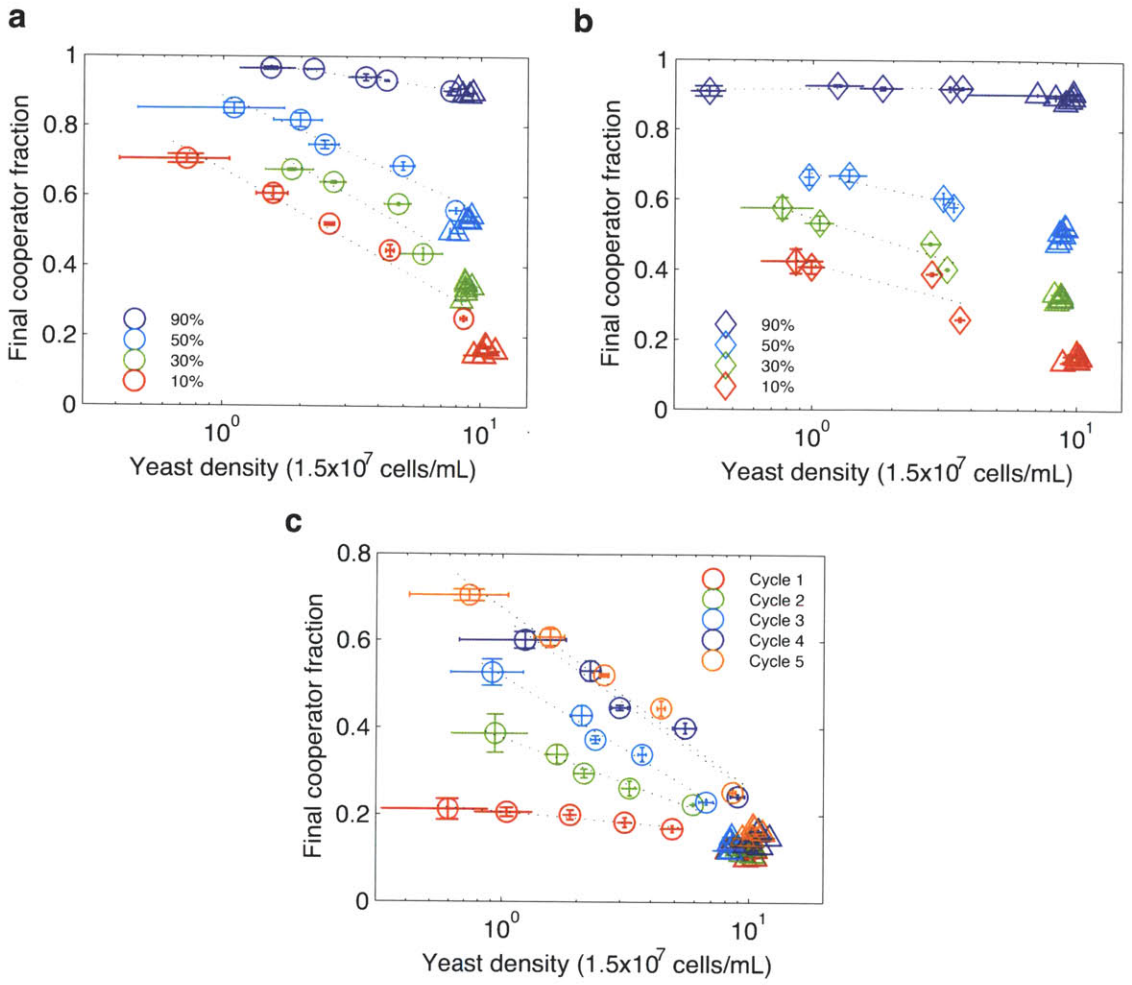
Supplementary Figure 7: Bacterial density measurements using flow cytometry. Left column shows a typical bacterial density measurement from a two species competition culture. This particular sample is competition after 10 days of growth with 30% initial cooperator fraction and 12 mM buffer (PIPES). To collect the data, bacterial settings were used on the flow cytometer (see Methods). As seen in the top SSC/FSC plot, bacteria (red) and yeast (blue) populations were well separated and easily distinguished. Bottom plot shows the histogram SSC counts for the same condition. In this histogram, skewed left tail of the bacterial counts is due to noise overlapping with the bacterial population counts. To quantify the noise and subtract it from bacterial counts, every growth cycle we measured event counts occurring in the 'BACTERIA' gate for 8 pure

yeast cultures (isolated yeast controls) again using the bacterial settings. Right column shows a typical result from such a measurement. This particular sample is from a culture after 10 days of growth with 30% initial cooperator fraction and 12 mM buffer (PIPES) – same as the conditions used in the left column except without bacteria. Top right plot shows SSC/FSC plot with noise appearing in the region where bacteria were before. In the SSC histogram for this sample (bottom right plot), we see that noise counts overlap nicely with the left tail of bacterial counts from the sample with bacteria (bottom left plot). Bacterial counts in mixed culture experiments were corrected by subtracting the mean of such 8 controls from each sample for every different microplate measurement.



Supplementary Figure 8: Two-phase logistic growth model. To model the cooperative dynamics within the yeast population, we developed a simple logistic growth model. A sketch of this model (shown in the above figure, not to scale) describes how the growth rate changes as a function of yeast density. At low density, cooperators have a higher growth rate ($\gamma_{C_{high}}$) than cheaters ($\gamma_{D_{high}}$). Above a critical cooperator density N_C, it is assumed that the growth rate is higher for both cooperators and cheaters since glucose accumulates faster in the media[19]. Then, the growth rate decreases logistically to zero as the yeast density reaches its carrying capacity, K. We measured N_C to be about 3 x 10^5 cells/mL and $\gamma_{C_{low}}$ as 0.33 hr^{-1}. These measurements were taken by observing the time it took yeast cultures to reach a certain density, starting with different initial cell densities. $\gamma_{C_{high}}$ was measured to be 0.45 hr^{-1} on 4% sucrose by measuring growth rate during exponential growth. Taking the cost of cooperation into account, $\gamma_{D_{high}}$ was assigned such

that $\gamma_{C_{high}}$ was 1% lower than $\gamma_{D_{high}}$ (Supplementary Figure 1). Highest K value in a pure yeast culture on our default media was measured to be $15x10^7$ cells/mL (K_{max}). To simulate nutrient limitation or competition with bacteria, K was varied across the experimentally observed range. $\gamma_{D_{low}}$ (cheater growth rate at low density) was varied to fit the data shown in figure 3c. In nutrient limitation conditions, cheaters had a growth deficit of 4.85% at low density compared to cooperators. For JM1100 treatment, this deficit was 4.85% + 5.45% * (1– K/ K_{max}) and for DH5α it was 4.85% + 13% * (1– K/ K_{max}). These values were assigned so as to fit the data. We assumed that cheaters have a lower growth rate than cooperators when competing against bacteria, because bacteria might compete for glucose with yeast and this would further limit the available glucose in the media during low-density conditions (yeast density < N_C). This model enabled us to calculate temporal dynamics and simulate the entire growth process over 5 cycles of growth (10 days) with 1:1,000 serial dilutions in between. Lower carrying capacity due to nutrient limitation or bacterial competition meant that the yeast population would spend more time during the first phase of this growth model where cooperation is favored. According to our model, equilibrium fraction of cooperators without the presence of bacteria is 61%.



Supplementary Figure 9: Log-linear relationship between yeast density and final cooperator fraction shown for different initial cooperator fractions (a,b) and different time points during experiments (c). a, Competition results between yeast and DH5α with different initial cooperator fractions (circles) after 5 cycles. **b,** Competition results between yeast and JM1100 with different initial cooperator fractions (diamonds) after 5 cycles. Triangles represent results for pure yeast cultures both in (a) and (b). **c,** Final cooperator fraction within the yeast population over time while competing against DH5α. Each cycle is 48 hrs. Data points represent different buffering conditions. Yeast density decreases monotonically with buffering in all the plots above. Note the apparent

increase in the final yeast density as the yeast population becomes more cooperative in (c). Dotted lines represent least squares fit for the data. Error bars, ± s.e.m. (n = 3).

VI - APPENDIX

List of Figures

VII - REFERENCES

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