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Competition between species can stabilize public-goods cooperation within a species

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Competition between species is a major ecological force that can drive evolution. Here, we test the effect of this force on the evolution of cooperation within a species. We use sucrose metabolism of budding yeast, *Saccharomyces cerevisiae,* as a model cooperative system that is subject to social parasitism by cheater strategies. We find that when cocultured with a bacterial competitor, *Escherichia coli*, the frequency of cooperator phenotypes in yeast populations increases dramatically as compared with isolated yeast populations. Bacterial competition stabilizes cooperation within yeast by limiting the yeast population density and also by depleting the public goods produced by cooperating yeast cells. Both of these changes induced by bacterial competition increase the cooperator frequency because cooperator yeast cells have a small preferential access to the public goods they produce; this preferential access becomes more important when the public good is scarce. Our results indicate that a thorough understanding of species interactions is crucial for explaining the maintenance and evolution of cooperation in nature.
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Introduction

Cooperation is a widespread phenomenon in nature. However, costly cooperative strategies are vulnerable to exploitation by cheats that do not cooperate but freeload on the benefits produced by the cooperating individuals (Axelrod and Hamilton, 1981; Nowak, 2006). Therefore, the persistence of cooperation in nature has been a puzzling question for evolutionary biologists and there has been much theoretical and experimental research trying to elucidate the mechanisms underlying this phenomenon (Frank, 1998; West, 2007; West *et al*, 2007; Nowak *et al*, 2010). Microbial studies have suggested that cooperation can be maintained in nature by mechanisms such as reciprocity (Queller *et al*, 2003; Smukalla *et al*, 2009), spatial or temporal heterogeneity (Rainey and Rainey, 2003; MacLean and Gudelj, 2006; Diggle *et al*, 2007), and multilevel selection (Chuang *et al*, 2009). Recently, it has become increasingly clear that in addition to population dynamics, external ecological factors can also have significant roles in affecting the evolution of cooperation (Brockhurst *et al*, 2007, 2010).

One such important ecological factor is interspecies interactions (Little *et al*, 2008). 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 (Thompson, 1999). Interspecific competition—that is competition between species—has been shown to have a key role in shaping species distributions (Connell, 1961; Schoener, 1983) and evolution of character displacement (Schluter, 1994; Grant and Grant, 2006). Nevertheless, little effort has focused on establishing a link between this ecological pressure and the evolution of cooperation within a species (Harrison *et al*, 2008; Hibbing *et al*, 2010; Korb and Foster, 2010; Mitri *et al*, 2011). As one of the few studies that tried to answer this question, Harrison *et al* found that interspecific competition with *Staphylococcus aureus* can select for cheats within *Pseudomonas aeruginosa* for the production of an iron-scavenging siderophore molecule. The authors speculated that this result was probably owing to increased competition for iron (Harrison *et al*, 2008). In another study, computer simulations of biofilms showed that in spatially structured environments, when competition for essential nutrients is strong, the addition of more species can inhibit cooperation within a focal species because the added species can outcompete the cooperating cells (Mitri *et al*, 2011). On the other hand, when nutrients were abundant, their model predicted that the public-good-producing cells would be surrounded by other species and insulated from cheater cells of the same species, thus cooperators would be favored. In our paper, we aimed to systematically quantify the effect of interspecific competition on the evolution of cooperation using an experimental microbial system, yeast sucrose metabolism. We found that the presence of a bacterial

competitor could dramatically increase the cooperator frequency.

Wild-type yeast cells break down extracellular sucrose cooperatively by paying a metabolic cost (Supplementary Figure S1) to synthesize the enzyme invertase (Greig and Travisano, 2004; Gore *et al*, 2009). 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 owing to a local glucose gradient (Gore *et al*, 2009; Dai *et al*, 2012). This preferential access to the public good provides cooperators an advantage when present at low frequency, as in this case there is little glucose for the cheaters to consume (experiments here are done in media with 4% sucrose and 0.005% glucose). The cooperator and cheater strategies are therefore mutually invasible, leading to steady-state coexistence between the two strategies in well-mixed batch culture (Gore *et al*, 2009).

Results

Effect of interspecific competition on the evolution of cooperation within yeast

First, we confirmed that there is coexistence between cooperator and cheater strategies in pure yeast cultures. Starting with an initial cooperator fraction of 10%, we observed little change in cooperator frequency after 10 days of coculture (Figure 1). In these experiments, every 48 h we performed serial dilutions into fresh sucrose media and measured the fraction of cooperator cells within the yeast population using flow cytometry (Materials and methods and Supplementary Figure S2).

To test whether interspecific competition can influence cooperation within the yeast population, we performed the same experiment, but this time cocultured the cooperator and cheater yeast along with a bacterial competitor, *E. coli* (DH5α). This strain of *E. coli* cannot utilize sucrose (Reid and Abratt, 2005) but could grow on arabinose (another carbon source present in the media), on the other hand arabinose could not be utilized by our yeast strains (Supplementary Figure S3). We found that the presence of bacteria led to a large and rapid 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 to ~45% (Figure 1). 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 the tested conditions, even though bacteria were still present (Supplementary Figure S4). Therefore, the increase in cooperator fraction upon addition of the bacterial competitor is indeed related to sucrose metabolism.

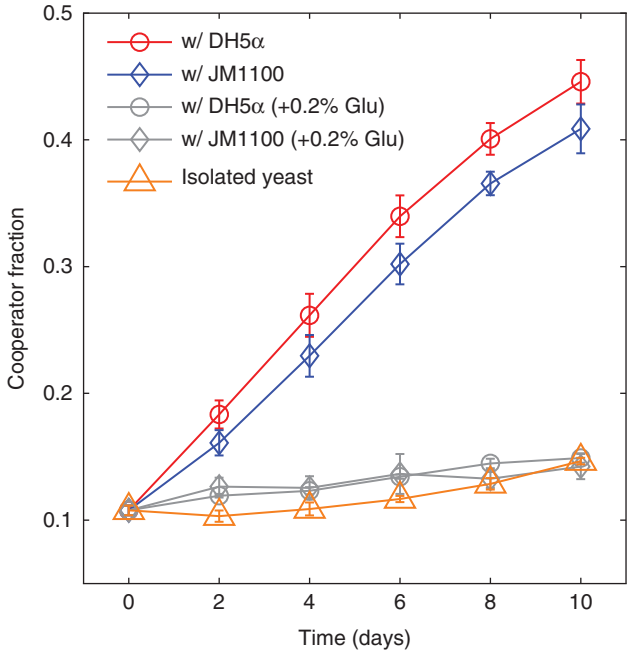

Figure 1 When cocultured with bacteria in sucrose media, cooperator cell fraction increases within yeast populations. Both with *E. coli* strains DH5α or JM1100—a mutant strain that grows poorly on glucose and fructose—a significant increase in cooperator fraction was observed compared with a pure yeast 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 cooperators is linked to sucrose metabolism. In this experiment, culture media contained 4 mM buffer (PIPES). Total final yeast and bacterial densities did not change significantly over the course of five cycles of growth (Supplementary Figure S9). Error bars, ± s.e.m. ($n = 3$). Source data is available for this figure in the Supplementary Information.

A possible explanation for this increase in cooperator fraction 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 as they have at least some preferential access to the produced glucose. To test this, we competed yeast against a mutant strain of *E. coli* (JM1100) that has much reduced glucose and fructose uptake rates (Materials and methods) (Henderson *et al*, 1977). We found a somewhat smaller albeit still significant increase in the cooperator fraction within the yeast population under the same conditions (Figure 1). Bacterial competition for the public good may therefore be a contributing factor toward increasing cooperator frequency in the yeast population, but there is another mechanism at work as well. We will show later that the other mechanism by which bacterial competition is selecting for cooperator cells in yeast is by limiting the yeast population density.

Two-species growth dynamics

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 (Figure 2A). Bacteria have a higher growth rate than yeast

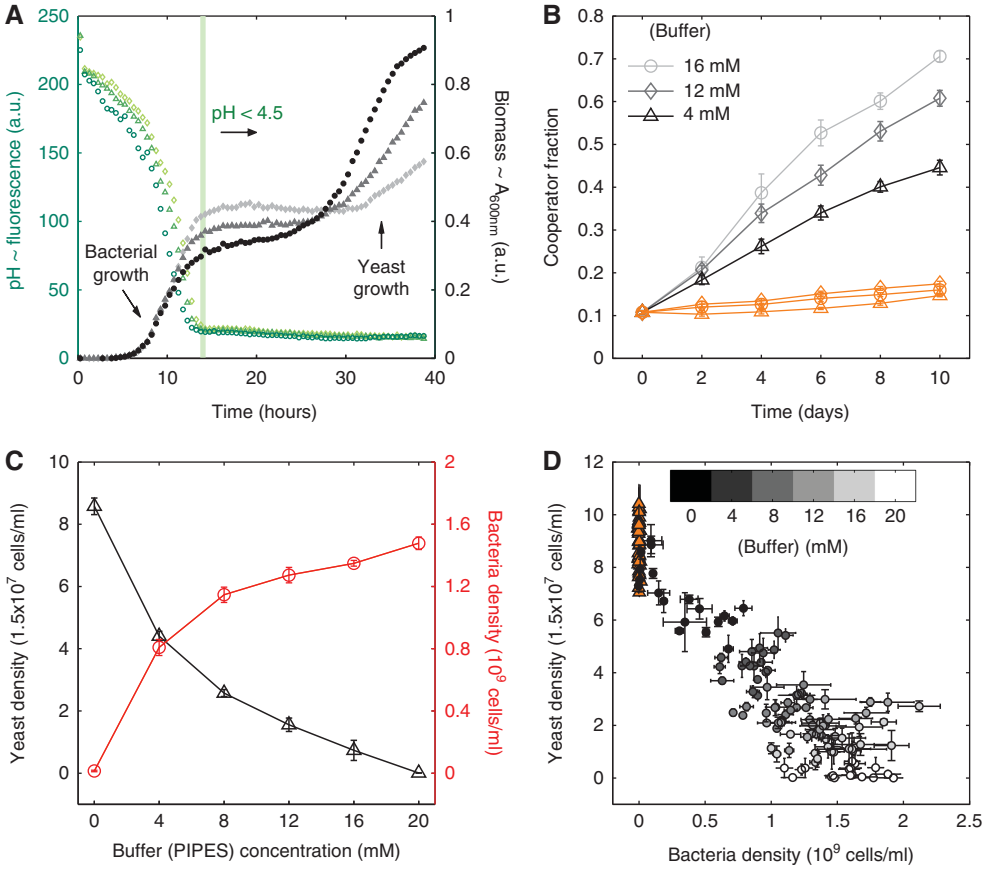

Figure 2 Correlation between the intensity of interspecific competition and cooperator cell frequency within yeast. (**A**) Successional growth dynamics in mixed cultures of yeast and bacteria. Absorbance (600 nm) was measured for different buffer (PIPES) concentrations: 4 mM (circles), 8 mM (triangles), and 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. Initial pH was 6.5 (~ 220 fluorescence a.u.) in all the cultures used in our experiments (see Materials and methods and Supplementary Figure S5B). (**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 (initial fractions: 0.1, 0.3, 0.5, and 0.9). Control cultures (isolated yeast) for the same conditions are shown in triangles. Error bars, ± s.e.m. ($n = 3$). Source data is available for this figure in the Supplementary Information.

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 owing to acidification caused by fermentation, as *E. coli* growth can be severely limited at acidic conditions (Davison and Stephanopoulos, 1986; Foster, 2004). 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 (Figure 2A and Supplementary Figure S5). This suggests that the limited bacterial growth may be caused by low pH brought about by sugar fermentation. Compared with bacteria, yeast cells are better able to tolerate the harsh acidic conditions (Davison and Stephanopoulos, 1986) 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 (Okafor, 1975; Kuramae *et al*, 2010; Koenig *et al*, 2011).

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 (Figure 2A). We also saw that this increased bacterial density restricted the yeast growth owing 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 niche overlap and thus the intensity of competition between yeast and bacteria.

Cooperator yeast cells do better under interspecific competition

If cooperator cells were indeed selected as a result of interspecific competition, we would expect to see a positive correlation between the level of final cooperator fractions 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 caused the cooperator fractions to increase within the yeast population, but only when competing against bacteria (Figure 2B).

We next repeated these experiments by starting out with different initial fractions of cooperators (30%, 50%, and 90%) and observed the same trend in all the conditions we examined (Supplementary Figure S6). 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 result suggests that at equilibrium the cheater cells might even be completely purged from the yeast population under the pressure of interspecific competition. Finally, to probe the generality of our results, we competed cooperator and cheater yeast against bacteria on solid agar with sucrose as the sole carbon source. Consistent with the results in liquid cultures, we observed that the presence of bacteria (JM1100) strongly selected for cooperator cells within yeast (Supplementary Figure S10).

Although in our experiments the cooperator fractions after 10 days are not necessarily the values at equilibrium, the rapid increase of cooperator fraction in the presence of bacteria is a striking effect of interspecific competition on the evolution of cooperation. The fact that the change in cooperator frequency is extremely slow in isolated cultures as compared with the change in our two-species competition experiments suggests that even transient bacterial competition can have a lasting impact on the fraction of cooperator cells within yeast populations. In fact, in some of the low-buffering conditions the bacterial species went extinct but the cooperator fraction within the yeast population was nevertheless significantly enhanced as compared with isolated yeast populations (Supplementary Figure S6). Given the importance of non-equilibrium dynamics in nature (Murdoch, 1991), we believe that these findings may aid in understanding the evolution of cooperation in wild populations.

To measure the density of the yeast and bacteria in these experiments, we used flow cytometry at the end of each growth cycle (see Materials and methods and Supplementary Figure S7). We found that by the end of the last cycle, in cultures without any added buffer, bacteria went extinct, whereas at the highest buffer concentration used (20 mM), yeast was outcompeted by bacteria (Figure 2C). However, at intermediate levels of buffering, yeast and bacteria could stably coexist. This coexistence is a result of the temporal heterogeneity mediated by acidification and the fact that bacteria and yeast partition into different niches (Tilman, 1982) by utilizing different carbon sources in the media (arabinose and sucrose, respectively). At high buffer concentrations, cooperator yeast are favored relative to cheater yeast. However, as the total yeast population density decreases with increased buffering (eventually going to zero), the absolute number of cooperator yeast decreases as well. It is therefore important to distinguish between selection for cooperator cells in a population (fraction of cooperators in the yeast population) versus the absolute number of cooperator cells.

Although in cocultures 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. We speculate that faster acidification owing to increased glucose concentrations with higher yeast population density combined with ethanol production during the later stages of yeast growth (after bacterial growth stops, i.e., second phase of succession) might be causing bacterial death (Davison and Stephanopoulos, 1986; Thomson *et al*, 2005). 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 (Figure 2D). This relationship is the hallmark of interspecific competition whereby the two species reciprocally repress each other's growth (Molles, 2010).

Two-phase logistic yeast growth model

It has been shown that owing to the cooperative nature of growth on sucrose, the *per capita* growth rate is lower at low cell density and becomes higher as the cell density increases because more of the sucrose has been converted to glucose (Gore *et al*, 2009; Dai *et al*, 2012). Moreover, 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 (Supplementary Figure S8). At high cell density, we found that cheaters have a growth advantage over cooperators, as enough glucose can accumulate in the media to support cheater growth.

Using these results, we developed a simple two-phase logistic growth model describing the cooperative dynamics within a pure yeast population in batch culture (see Supplementary Figure S8). The model incorporates the fact that in the beginning of a culture, the yeast density is low and there is little glucose in the media because there are not enough cooperators to supply it. Therefore, at the beginning of each growth cycle the cooperators have an advantage. However, as the yeast population grows eventually the density of cooperators increases above a critical value, at which point cheating starts to be favored because now there is enough glucose in the media that cooperators are at a disadvantage by carrying the burden of public good (Gore *et al*, 2009). In the end, the culture logistically saturates to a set carrying capacity, K. This phenomenological model has been previously used to yield accurate quantitative agreement to experimental data for yeast growth in sucrose, including the presence of a fold bifurcation that leads to catastrophic collapse of the population in deteriorating environments (Dai *et al*, 2012). Moreover, this simple model is quantitatively identical to a more mechanistic model that incorporates changes in glucose concentration over the course of each growth cycle (Supplementary Figure S11).

Prompted by our experiments with two-species competition, we reasoned that the first-order effect of bacterial competition might be to decrease the carrying capacity of the yeast population by depleting essential nutrients in the media (Figure 2D). Indeed, our model predicts that the cooperator frequency should increase as the carrying capacity decreases (Figure 3 and Supplementary Figures S8 and S11). This increase in cooperation results from the fact that a decrease in the carrying capacity makes the yeast populations spend

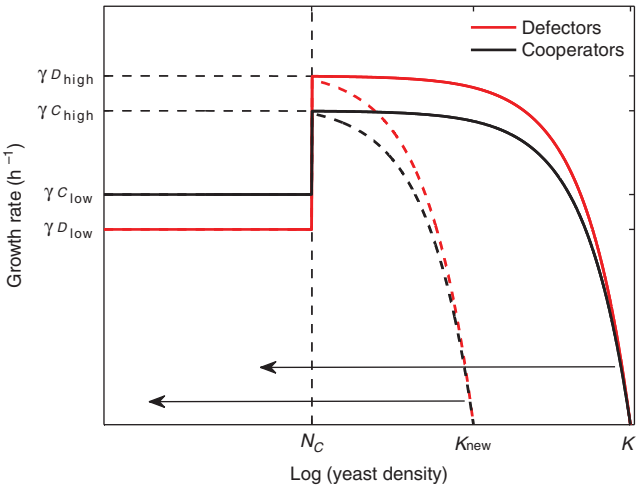

Figure 3 A phenomenological model describes the growth dynamics of cooperator and cheater yeast during each cycle of batch culture. This sketch of our yeast growth model describes how the *per capita* growth rate changes as a function of yeast density. At low density, cooperators have a higher growth rate than defectors. Above a yeast density N_C where cooperator density is at a critical value, it is assumed that the growth rate is higher for both cooperators and cheaters as glucose has accumulated in the media (Gore *et al*, 2009). Then, the growth rate decreases logistically to zero as the yeast density reaches its carrying capacity, *K*. If the yeast-carrying capacity was limited (K_{new}), starting yeast density would be lower after dilution into fresh media.

more time in the low cell density regime (where cooperators have an advantage) and less time in the high cell density regime (where cheater cells have an advantage). Thus, smaller yeast population density mediated by low nutrient availability should increase the frequency of cooperator phenotypes within yeast.

Nutrient limitation causes cooperator frequency to increase within a pure yeast population

If bacterial competition is selecting for cooperator cells within the yeast population via reduced yeast population density, then it should be possible to experimentally induce the same effect even in the absence of bacteria. To test this prediction of our model, we limited essential nutrients in pure yeast cultures experimentally. 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 Materials and methods). As before, we performed serial dilutions every 48 h into fresh media and measured the final fraction of cooperators and total yeast density. Consistent with the predictions of our model, we found that the frequency of cooperators increased with decreasing concentrations of supplemented uracil (Figure 4A). 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 predictions, we observed that the cooperator fraction increased at low phosphate concentrations (Figure 4B). 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 show that limiting the carrying capacity can increase the cooperator frequency within the yeast population. If it is indeed the limited carrying capacity that is causing this effect, then we would expect that the increase in cooperator fractions 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 argues that the underlying force selecting for cooperators was 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 the logarithm of the final yeast density (Figure 4C). Our model could explain this feature of the experimental data and followed a similar log-linear relationship. All the relevant parameters in the model were consistent with independent experimental measurements (Supplementary Figure S8).

Bacteria both limit yeast-carrying capacity and act as cheaters

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 (Figure 4C and Supplementary Figure S9). However, controlling for yeast population density, we found that competition with bacteria was more effective in increasing cooperator fractions within yeast than resource limitation alone. A possible explanation for this difference is that bacteria behave as a 'superior' cheater strain by assimilating available free glucose as we have indicated earlier.

To test this hypothesis, we again competed yeast against our mutant strain of *E. coli* (JM1100) that has much reduced glucose and fructose uptake rates (Henderson *et al*, 1977)

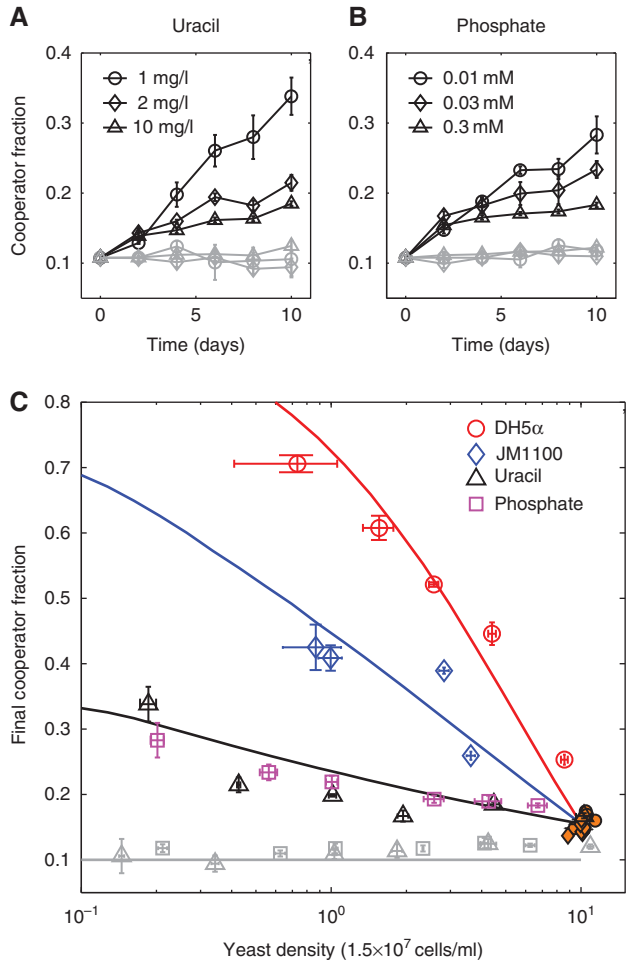

Figure 4 Nutrient limitation can select for cooperator cells within the yeast population even in the absence of bacteria. Limiting either uracil (**A**) or phosphate (**B**) increases frequency of cooperators within isolated yeast populations. Control cultures (gray symbols) with excess glucose (0.2%) displayed negligible change in cooperator frequency. (**C**) Final cooperator fraction versus final yeast density in bacterial competition and nutrient-limitation experiments: DH5α, JM1100, uracil, and phosphate. Note that for both of the limiting nutrients (uracil and phosphate), yeast density versus cooperator fraction relationships are extremely similar, indicating that the underlying force for increase in cooperator fraction is the limited carrying capacity. With controls: uracil + 0.2% Glucose (gray triangles) and 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. ($n = 3$). Source data is available for this figure in the Supplementary Information

compared with DH5α. In this case, across the same yeast densities, the effectiveness of the bacteria in selecting for cooperators within yeast decreased significantly, although the final increase in cooperator fractions was still higher than the resource limitation treatment. This result suggests that competition for glucose and fructose is the reason why bacterial competition favors cooperator cells more than resource limitation alone (controlling for yeast population density).

To account for glucose consumption by bacteria in our phenomenological model, we made the cheater growth rate at low cell density a linearly decreasing function of the final bacterial density (which is linearly related to the yeast-carrying capacity, Figure 2D). We found that this simple assumption could reliably reproduce the effect of bacterial competition on the evolutionary dynamics within the yeast population (Figure 4C). By fixing the final yeast density in our model (yeast-carrying capacity not limited by bacteria), we found that bacterial competition for glucose alone significantly underestimated the final cooperator fraction. Taken together, these results indicate that bacterial competition for both essential resources and glucose increases the frequency of cooperators within the yeast population.

We note that this selection of cooperator cells by bacteria is occurring in a yeast growth regime where there is little to no transfer of benefits between yeast cells (i.e., low cell density conditions). Therefore, the cooperator cells are favored by bacteria not because they 'cooperate' with other cells, but because they have private access to some of the glucose that they create. The cheater cells are therefore deprived of glucose owing to the presence of bacteria (either by direct glucose consumption or by limiting yeast density, which limits the amount of sucrose broken down). So, bacterial competition actually selects for 'invertase producing cells' rather than 'cooperators' *per se*. However, as the invertase-producing cells are breaking down sucrose outside of the cell, ~99% of the resulting glucose diffuses away before it can be captured (Gore *et al*, 2009). All cells in the population then benefit from this sucrose hydrolysis during the high-density growth phase, where the bulk of yeast growth occurs in our experiments (see Figure 3 and Supplementary Figure S8). Selection for invertase-producing cells during the first phase of growth (when yeast density is low) then indirectly acts as a stabilizing agent for the cooperator genotype in the yeast population.

A glucose-producing bacterial species can select for cheats within the yeast population

Finally, we asked how the cooperative dynamics within yeast would be affected if the competing bacteria were also producing glucose just like cooperator yeast. We found previously that bacterial competition for the public good could select for cooperators within the yeast population beyond that expected based on resource competition alone. If the competing bacteria instead produce the public good then it may even be possible for the bacteria to favor cheating behavior within the yeast population. To test this, we inoculated yeast cells on sucrose plates together with the soil bacteria *Bacillus subtilis* instead of *E. coli*. Similar to wild-type yeast, *B. subtilis* breaks down sucrose with a secreted enzyme and generates extracellular glucose (Reid and Abratt, 2005). Interestingly, we found that now cheating is favored within the yeast population (Supplementary Figure S10). Control competition experiments on glucose-only media resulted in no difference among various treatments, strongly suggesting that glucose production by *B. subtilis* is responsible for the decrease in cooperator phenotypes on sucrose plates. Thus, it seems that although *B. subtilis* cells compete for resources with yeast, they can produce enough glucose to reverse selection for cooperators within the yeast population. We therefore conclude that other competing species do not necessarily select for cooperators 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.

Discussion

Our results indicate that social evolution within a species can be greatly affected by interspecies interactions. Specifically, we found that interspecific competition for essential nutrients can limit the carrying capacity of our focal species, yeast, and therefore increase the frequency of cooperator phenotypes. In nature, such interspecific competition is ubiquitous and one of the major factors limiting species ranges (Sexton *et al*, 2009). This fact suggests that our findings should be relevant where communities of species coexist and occupy partially overlapping niches. Evolution of cooperation is strongly related to population density. In general, cooperators feel the burden of exploitation by cheater phenotypes at high population densities (Ross-Gillespie *et al*, 2009; West *et al*, 2007). Our results show that interspecific competition can limit the overall population density of the focal species, and therefore drastically alter the outcome of competition between cooperators and cheaters. As discussed before, this result is owing to the fact that cooperators have an advantage in low-density conditions, as they have preferential access to the produced public goods.

Next, we also showed that competition between species directly for the public goods produced by one of them can select for cooperators within the producing species. In our experiments, bacteria deplete the public good available for both cooperator and cheater yeast, but as cooperators have a 'private' access to some of the glucose produced they grow faster than cheaters during the initial period of low cell density. Such competition between species for a public good is a common phenomenon in nature. Among microbial organisms, there are many cases whereby the diffused products of extracellular enzymes can be assimilated by other species of microbes—examples include the extracellular products of siderophore metabolism (Lesuisse *et al*, 2001; Harrison *et al*, 2008), cellulose degradation(Chen and Weimer, 2001; Flint *et al*, 2008), and starch (Flint *et al*, 2007) and inulin (Belenguer *et al*, 2006) degradation. Therefore, maintenance of the production of these public goods by one species might be mediated by the presence of other species occupying the niche space where cheaters within the same species would have to radiate into. It is often the case that public-good-producing individuals benefit preferentially from being producers, mainly because of spatial heterogeneity (viscous environments in which the produced extracellular products form a diffusion gradient around the producing individuals). This is analogous to our experimental system where we have spatial heterogeneity (despite the fact that we use a well-mixed environment) simply because of the biophysical features of the yeast cell wall. We speculate that such maintenance of cooperation through interspecific competition for public goods might also be present within animal populations, such as primary cavity excavation by woodpeckers (abandoned nests can be utilized by non-excavating bird species instead of next-generation woodpeckers, forcing woodpeckers to excavate new cavities) (Loeb and Hooper, 1997), cooperative hunting by hyenas (exploitative competition from lions and mammalian carnivores for the captured prey) (Caro and Stoner, 2003), and so on.

In conclusion, our findings provide evidence for an important ecological mechanism—competition between species—for the evolution of public-goods cooperation within a species. Our results also suggest that cooperation may be more stable than would be concluded from experiments that study a single species in isolation. 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 (Hibbing *et al*, 2010). Our findings also indicate that depending on the nature of interspecific interaction (e.g., competition versus mutualism), other species may also disfavor cooperation within a species as we have seen in our experiments with *B. subtilis*. 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.

Materials and 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,* and *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,* and *thyA111*) could grow on minimal media without additional thymine probably owing 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-methionine (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), and 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, and 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, and 0.3 mM. In all the experiments, pH was adjusted to 6.5 with NaOH and PIPES (pKa 6.8 at 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 20 h at 30°C and bacterial strains were grown in LB at 37°C for 20 h. 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 7.5×10^4 cells/ml for yeast. These initial inoculation densities were chosen based on preliminary experiments where average densities of two species after stable coexistsence was measured. This ensured that bacteria and yeast would not outcompete each other initially by simple overabundance of one species versus the other. 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, and shaken at 825 r.p.m. Evaporation per well was measured to be 20% over 48 h. For multiday experiments, cultures were serially diluted 1:1000 into fresh media every 48 h, 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/s. Yeast was assumed to be extinct in wells with <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/s. 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 Figure S8). From these control measurements, noise was calculated and found to have a maximum coefficient of variation <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 min, absorbance (600 nm) and fluorescence (excitation: 488 nm, emission: 521 nm) measurements were taken for 40 h.

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 used JM1100 instead of DH5α in these experiments because DH5α formed minute colonies on 2% Glucose agar owing to excessive acidification. 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 owing to the less acid-tolerant nature of this bacterium compared with *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 5×10^6 cells/ml. For DH5α and JM1100, media contained 8 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 (Youk and Van Oudenaarden, 2009):

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

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, 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/cell and 0.72×10^4 molecules/s/cell, respectively. Fructose uptake rates for DH5α and JM1100 were found to be 0.47×10^4 molecules/s/cell and 0.08×10^4 molecules/s/cell, respectively.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Author contributions: HC and JG designed the experiments. HC performed the experiments. HC and JG analyzed the data and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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