# Chapter 4.

# Yeast Use a Tit-For-Tat Strategy in Ammonia Metabolism to Establish Cooperation

Explaining the emergence of cooperation is a major goal of evolutionary biology.[1-3] Most explanations rely on kin selection,[4] spatial isolation,[5] or rational policing.[6] However, recent theoretical work[7] has suggested that cooperation can evolve via the game theoretic tit-for-tat (TFT) strategy,[8-12] which is able to invade a population of cheaters and is itself resistant to invasion. Here, we found that yeast use a strategy resembling tit-for-tat in regulation of ammonia assimilation.[13] We first identified a tradeoff between maximum growth rate and ammonia utilization efficiency, which creates an opportunity for social conflict in microbial populations.[14-18] Efficient use of resources with a correlated tradeoff in growth rate is regarded as cooperation in microbes, while inefficient use of resources with high growth rate is regarded as cheating.[19, 20] We found that yeast use ammonia efficiently when ammonia is abundant (e.g., they cooperate when resources are abundant, which would indicate cooperation from other cells) and switch to inefficient growth in low ammonia (e.g., they defect when resources are limited, which indicates that other cells may be competing for ammonia). Competition experiments in batch culture with a cheater mutant confirmed that no special conditions (such as spatial isolation) is needed for the TFT strain to invade a cheater population. This data shows that the TFT strategy is a viable mechanism for the emergence of cooperation, even in simple organisms. In addition, this is one of the first demonstrations that microbes use genetic regulation of metabolism to play a game theory strategy.

Cooperation is widespread in nature, yet it remains difficult to explain how it might have emerged in populations via natural selection. This is demonstrated in the "tragedy of the commons": efficient use of a common resource at a cost to an individual can benefit selfish individuals (who incur no costs). Game theory has modeled this situation as the Prisoner's Dilemma, where a player is faced with either cooperation with another player or defection. Both obtain a payoff for mutual cooperation and a lower payoff for mututal defection. If one player defects while the other cooperates, the defector (also known as the cheater) receives the highest payoff while the cooperator receives the lowest payoff (the "sucker's payoff"). Thus, the matrix of fitness payoffs in a Prisoner's Dilemma dictates that defection will overcome cooperation in a population with both strategies. Alternative strategies become possible in the repeated Prisoner's Dilemma, yielding a variety of cooperation and defection decisions depending on the strategy encountered.

While cooperation and social interactions are usually considered in rational agents, microbes have proven valuable model systems for understanding how the use of resources may (or may not) lead to cooperation.[15, 16, 18, 21] Thermodynamic first principles dictate that organisms generally face a tradeoff between rate and yield in metabolic pathways.[15, 18, 22] For example, Pfeiffer and Bonhoeffer have described the tradeoff between the rate of adenosine triphosphate (ATP) production and the yield of ATP production in heterotrophic organisms, and how organisms that produce ATP efficiently can be considered altruistic cooperators. The ATP rate/yield tradeoff is apparent in many microorganisms that use both fermentation and respiration to metabolize glucose. Fermentation of glucose proceeds faster than respiration, but yields less

ATP per glucose (2 versus 32 ATP), meaning that inefficient fermenting strains will be able to outcompete slower but altruistic respiring strains. MacLean and Gudelj[15] have used fermentation and respiration mutants in *Saccharomyces cerevisiae* to demonstrate how competition and cooperation between strain can be influenced by the spatial and temporal parameters of the environment. These and other experimental studies have shown that tradeoffs between rate and efficiency of resource metabolism drive the emergence of metabolic strategies such as cooperation or defection. However, more complex strategies have not been seen observed, which is somewhat surprising given the complexity of metabolic regulatory circuits.[23] As noted by MacLean and Gudelj, the ability to regulate metabolic pathways according to environmental conditions could allow for more complex competitive strategies to arise.

We sought to investigate how the ability to switch metabolic strategies depending on the environment could affect fitness in competition with other strategies. We had previously characterized the fitness of *S. cerevisiae GDH1* promoter mutants that showed tradeoffs between fitness in abundant ammonia and fitness in limiting ammonia. GDH1 is a glutamate dehydrogenase that is responsible for the majority of ammonia assimilation in yeast.[13] We hypothesized that the wildtype strain could use genetic regulation to optimize metabolism for specific ammonia environments. We first examined maximum growth rates ($\mu_{max}$) in continuous culture[24, 25] for the wildtype laboratory strain and two mutants: a strain that showed high fitness in abundant ammonia (denoted as A), and one that showed low fitness in abundant ammonia (denoted as B). In abundant ammonia (> 2.5 g/L), the wildtype and B strain showed similar growth rates, while the growth rate of the A strain was several fold higher (**Fig.1a**). Growth rate

decreased with ammonia concentration for all strains, although the wildtype strain switched from a B-like to A-like rate as ammonia decreased.

We next examined the efficiency of ammonia utilization for each strain at different ammonia concentrations. Utilization efficiency is calculated as the one over the amount of ammonia consumed per unit biomass (Methods) such that high efficiency values indicate efficient use of ammonia per organism. We found that the efficiency of all strains increased with decreasing ammonia concentrations, although the A strain was consistently lower efficiency than the B strain (**Fig.1b**). At high ammonia concentrations the wildtype strain showed an efficiency similar to the B strain. At low ammonia concentrations the wildtype strain switched to relatively low efficiency, similar to the A strain. The tradeoff between growth rate and resource utilization efficiency is a clear situation for social conflict.[15, 18] The A strain shows the hallmarks of defector (or cheater) strains in that it has a high growth rate at the expense of efficiency. The B strain displays cooperator characteristic in that it uses ammonia efficiently (to the benefit of other cells) at a cost to itself (lower growth rate). We will thus refer to the A strain as the defector strain and the B strain as the cooperator strain.

As was previously observed, assays of Gdh1p gene expression variability[26-29] (noise) suggest a mechanistic link to growth rate-efficiency phenotypes. The cooperator strain showed low Gdh1p noise across each ammonia concentration, while the defector strain showed high noise (**Fig.1c**). The wildtype strain varied noise in Gdh1p expression according to ammonia concentration – in abundant ammonia it showed low noise, with increasing noise as external ammonia decreased.

This data further suggests that the wildtype strain switches from cooperator-similar growth to defector-similar growth.

We were surprised that the wildtype strain did not show a growth rate and efficiency profile similar to the defector strain. In studies of glucose metabolism, yeast are found to ferment any excess glucose to achieve high rates of ATP production in spite of ATP yield, likely to outcompete neighboring cells.[30-32] In contrast, the wildtype strain in abundant ammonia utilizes ammonia with high efficiency at a cost to growth rate, indicating cooperative behavior. According to evolutionary game theory, the existsence of this cooperation should be overwhelmed by the emergence of defecting mutants (such as the defector strain here). We propose that the wildtype strategy is analogous to the tit-for-tat (TFT) strategy in the Prisoner's Dilemma. TFT players cooperate in the first round of the Prisoner's Dilemma, and for each subsequent round does whatever its opponent did such that cooperation is met with cooperation and defection is met with defection (**Fig.2a**). Although several superior strategies have since been described,[11, 33] TFT remains a primary model for understanding reciprocal altruism. The wildtype strain cooperates when ammonia is abundant (>2.5 g/L), which could be an indicator that either there are no competitors in the environment or that there are other cooperators in the environment using ammonia efficiently (**Fig.2b**). The wildtype strain defects when ammonia is low (< 1.25 g/L) which could indicate that other strains are rapidly consuming ammonia. We note several caveats, such as the fact that yeast in this context are not engaged in a pair-wise contest and that there is a continuum of growth rates and efficiencies instead of a binary division between cooperation and defection. However, because microbes are rarely involved in pair-wise

competitions the wildtype strain and mutants may be a valuable system for understanding how cooperation can persist in populations.

There are several general theoretical predictions for the TFT strategy in competition with alternative strategies: that the strategy is resistant to invasion by a population of defectors, and that TFT is able to invade defectors in finite populations.[7] In particular, the ability of a TFT strategy to invade a population of defectors would be a clear demonstration that TFT is a route for the emergence of cooperation.[7] We were able to experimentally test these predictions by examining frequency dependent selection in batch culture. We chose a batch culture (or "seasonal"[15]) environment so that the dynamic fitness (as ammonia is consumed) could be assessed. We inoculated varying frequencies of wildtype (without the *GDH1:GFP* fusion) and defector strain at low density ($10^3$ cfu/mL) in batch culture and allowed the culture to reach stationary phase (48 hours of growth). We quantitated the frequencies of the wildtype and defector strain by plating the cultures on solid media and assaying for fluorescence (Methods). We found that at high initial wildtype frequencies (> 0.5) the defector strain showed little ability to invade, evidenced by the nearly neutral wildtype fitness (w) observed in these competitions (**Fig.3**). At low initial wildtype frequencies (< 0.3), the wildtype strain showed positive fitness values, indicating that it was able to invade the population of defectors. These conditions are analogous to the immigration or emergence of a small subpopulation of TFT players into a population of defectors, and show that TFT can indeed invade a population of individuals selfishly using resources.

The above data shows that yeast are able to play a TFT-like strategy by adjusting the growth rate and ammonia utilization efficiency according to external ammonia concentrations. This strategy is notable because the wildtype strain does not have the optimal growth rate (compared to the defector mutant) or the optimal efficiency (compared to the cooperator mutant) for a wide range of ammonia environments. Instead, the wildtype strain has a regulatory scheme well suited for competing with alternative metabolic strategies in dynamic fitness landscapes.[34] We believe that this is the first demonstration of a game theoretic strategy being played in a microbial population. In addition, this work suggests that the control of metabolism in response to environmental conditions is a route for the emergence of cooperation.

**Methods Summary**

**Strains and media.** All strains were derivatives of the *GDH1:GFP* fusion strain of the S288C background. Cells were grown in synthetic complete media with 2% glucose and the indicated amount of ammonia by addition of ammonium sulfate. Construction and selection of the low-noise and high-noise *GDH1* mutants were described previously. Briefly, primers flanking 500 nucleotides upstream of the *GDH1* coding region (1043500 - 1043050, chromosome XV) were used to amplify the fragment from yeast genomic DNA. The fragment was diluted into mutagenic PCR buffer[35]. The *GDH1* fragment was assembled with a *LEU2* gene fragment transformed into yeast strains using a standard lithium acetate procedure.[36]

**Continuous growth conditions and growth rate assay.** Cells were inoculated in synthetic complete media with the appropriate ammonia concentration in a well-stirred vessel with a

working volume of 250mL maintained at 30 degrees. Cells were allowed to stabilize for 12 hours at a dilution rate of 0.2 $hr^{-1}$. To measure maximum growth rates ($\mu_{max}$) the washout method[24, 25] was used: when the dilution rate of the chemostat is greater than $\mu_{max}$ the cell number decreases by the expression $\ln X = (\mu_{max} - D)t + \ln X_0$ ; where X is the cell number after time t, $X_0$ is the initial cell number and D is the dilution rate. We increased the dilution rate to 4.0 $hr^{-1}$ and collected samples at regular time points. Cell number was quantified by $OD_{600}$ and by serial dilution and plating on YPD-agar.

**Ammonia utilization assays.** Cells were grown in continuos culture as above at low dilution (0.1 $hr^{-1}$) to standardize growth rates. Cells were collected from the outflow spun down. The supernatant was decanted into 14mL tubes, capped with a rubber stopper, and incubated at room temperature for 30 minutes. Ammonia was quantitated by gas chromatography – mass spectrometry (GC-MS) which can be used for accurately assaying volatile compounds such as ammonia.[37] The GC-MS system consisted of a model 6850 Series II Network GC system (Agilent) and model 5973 Network mass selective system (Agilent). Oven temperature was programmed from 50 degrees (1 min) to 70 degrees (10 degrees / min). 100 μL of culture headspace was withdrawn through the rubber stopper with a syringe and manually injected into the GC-MS. Samples were confirmed as ammonia by comparison with commercially obtained standard, which had a retention time of 1.50 minutes. Ammonia in the headspace was correlated to ammonia in the supernatant by a standard curve. Efficiency is reported as one over the milligrams of ammonia consumed per $10^6$ cells.

**Measurement of abundance and noise values through flow cytometry.** Two gates were used to standardize each cell population. The first gate isolated cells displaying regular morphology based on electronic volume and side-scatter, while the second gate removed non-fluorescent cells from the distribution. This gating method was compared against other methods previously described and the abundance and noise trends observed were consistent between methods.[38, 39] Noise was calculated as the square of the coefficient of variation ($\sigma^2/p^2$) of the distribution[26]. Abundance was calculated as the mean of the distribution. 50,000 events were analyzed to calculate noise for each sample. Noise trends were similar when calculated as the coefficient of variation ($\sigma/p$) and the variance ($\sigma^2$).

**Competition assays and fitness.** The defector strain and a wildtype S288c strain without the *GDH1:GFP* fusion were grown overnight and diluted to $10^3$ cells/mL. The *GDH1:GFP* construct was found to have no fitness effect (data not shown) Cultures were mixed in varying ratios in 2mL of synthetic complete media with 5 g/L ammonium sulfate. Cultures were incubated at 30 degrees with 250 rpm shaking for 48 hours. Cultures were diluted and plated onto YPD-agar and grown for 48 hours. Individual colonies were resuspended in 100μL media and GFP fluorescence was assayed using a Tecan plate reader. 96 colonies were assayed for each competition. Fitness of the wildtype strain is reported as the natural log of the ratio of its final frequency to its initial frequency, $w = \ln(f_{final}/f_{initial})$,[15, 40] such that a values > 0 imply that the wildtype strain increased in frequency versus the defector strain over the competition, while values < 0 imply that it decreased in frequency.

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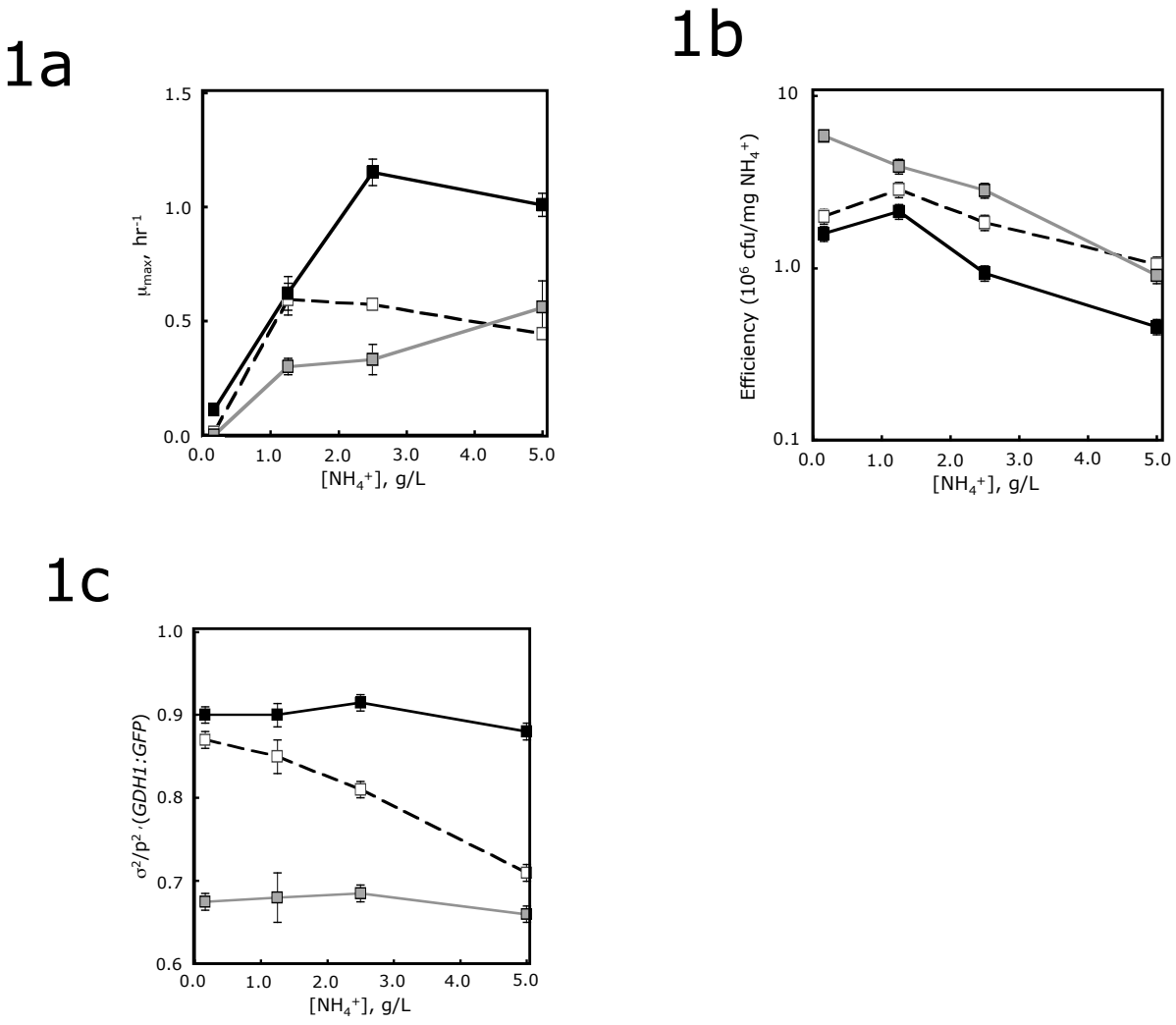


**Figure 1. Characterization of wildtype and mutant strains.** a, growth rate in continuous culture for the wildtype strain (open squares), A strain (black squares, previously selected for high fitness in 5 g/L ammonia), and B strain (gray squares, selected for low fitness in 5 g/L ammonia). Growth was measured using the washout method[24, 25] at each ammonia concentration. The A strain showed the highest growth rate in all concentrations while the B strain was consistently low. The wildtype strain showed rates similar to the B strain at high ammonia and rates similar to the A strain at low ammonia. **b**, ammonia utilization efficiency, as measured by ammonia consumption per biomass. Data is shown on a log scale for clarity. The A strain and B strain show low and high efficiencies, respectively, while the wildtype strain switches between

high and low efficiency as ammonia decreases. **c**, assays of Gdh1p gene expression noise in each strain. The A strain and B strain display high and low noise, respectively, while the wildtype strain displays low noise at high ammonia and high noise at low ammonia. All measurements were performed at least three times and s.d. is shown.

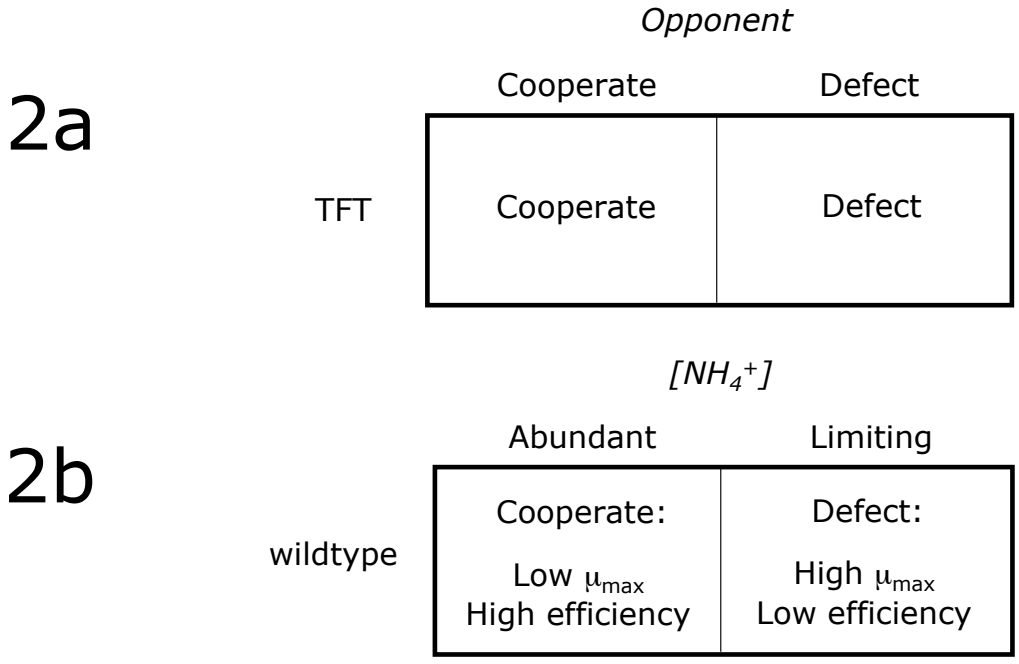


**Figure 2. Conceptual model of TFT-like strategy in the wildtype strain. a**, TFT strategy in the idealized Prisoner's Dilemma game. After cooperating in the first round, the TFT player does exactly as its opponent did in the last round. **b**, growth and ammonia efficiency strategy in the wildtype strain described here. In high ammonia the strain shows altruistic behavior with high utilization efficiency at expense of growth rate. In low ammonia the strain shows cheater behavior with high growth rate and low efficiency of ammonia utilization.

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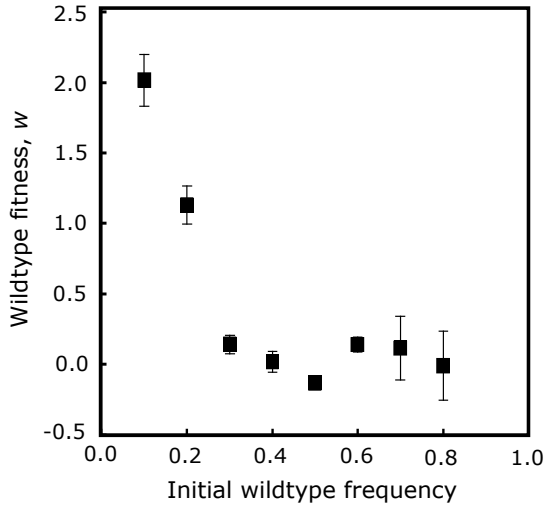


**Figure 3. Direct competitions between the wildtype and defector strains.** The frequency-dependence of the wildtype strain in competition with the defector strain (B strain) was measured by direct competition in batch culture. Relative frequencies were measured after competition and fitness is reported as w, the natural log of the ratio between final and initial wildtype frequency (Methods). At low initial frequencies (< 0.3) the wildtype strain showed w > 1 indicating that it was able to invade the defector population. In contrast, the defector strain was unable to invade a large population of the wildtype strain (wildtype frequency > 0.5) indicated by the non-negative w values for wildtype in those competitions. All measurements were performed in triplicate and s.d. is shown.