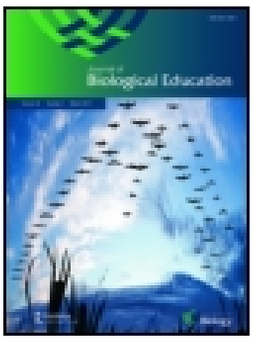

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PRACTICE PAPER

Greenbeards in yeast: an undergraduate laboratory exercise to teach the genetics of cooperation

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ABSTRACT

Recent years have seen a dramatic increase in our understanding of the social behaviour of microbes. Here, we take advantage of these developments to present an undergraduate laboratory exercise that uses the cooperative flocculating behaviour of yeast (*Saccharomyces sp.*) to introduce the concept of inclusive fitness and teach the genetics of cooperation. Students generate their own data using co-cultures of various yeast strains and perform statistical analyses to test whether kin selection or greenbeard effects determine the cooperative flocculating behaviour. The lab has run successfully for two consecutive years in a second year course with some 1, 200 students per year at the University of Toronto, Canada. We discuss the benefits of using microbes to teach social evolution, describe the set-up and learning outcomes of the laboratory exercise, and then outline possible extension and variants of the lab. In addition to providing students with the opportunity to use a model organism to study social behaviour, students are also taught common laboratory skills, such as replica plating and sterile techniques. Ultimately, while the genetics of cooperation has traditionally been taught through computer simulations and evolutionary games, this exercise demonstrates a way to experimentally introduce the topic.

KEYWORDS

Social microbes; yeast; inclusive fitness; kin selection; genetics of cooperation; undergraduate teaching

Introduction

Conflict and cooperation occur at all levels of life, from genes inside genomes to between species in mutualisms (Ågren 2014; Michod 1999; Maynard Smith and Szathmáry 1995; West et al. 2015). Understanding the evolutionary origin and maintenance of cooperation is therefore one of the classic problems in evolutionary biology, as cheating selfish individuals are expected to outcompete individuals performing the costly cooperative behaviour (Bourke 2011; Nowak 2006; Sachs et al. 2004). Empirical insights to the topic have traditionally come predominantly from large mammals and the social insects (Bourke and Franks 1995; Davies, Krebs, and West 2012; Gardner and Foster 2008). Neither system is easily maintained in a teaching lab and the topic of cooperation is therefore often taught to undergraduates either only conceptually or through a series of evolutionary simulations or games (see e.g. Leighton (2014) for a recent example).

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In this paper, we present a laboratory exercise using the biofilm-like cooperative budding behaviour (flocculation) in yeast (*Saccharomyces sp.*) to teach the genetics of cooperation. Students use co-cultures of various yeast strains (i.e. two or more yeast strains are grown together) to test competing hypotheses for the evolution of this cooperative behaviour. The lab has run successfully for two years (2013, 2014) in a second year course with an annual attendance of around 1,200 students at the Department of Ecology and Evolutionary Biology at the University of Toronto, Canada.

Below, we first outline Hamilton's theory of inclusive fitness and describe the flocculating behaviour of yeast. We then walk through the experimental set-up and learning outcomes of the exercise, and finally suggest some possible extensions and variants of the lab. This exercise is unique in that it gives students the opportunity to perform a laboratory experiment to empirically test aspects of social evolution theory. Students are also introduced to common laboratory techniques (e.g. replica plating, sterile techniques) in addition to applying the scientific method to test two competing hypotheses on the evolution of cooperative behaviour. Students generate their own data and perform the appropriate statistical analyses. This whole process engages students in active learning of these evolutionary concepts, which can lead to deeper understanding of this content and greater confidence in their laboratory skills (e.g. Bransford, Brown, and Cocking 1999; Marshall 2007; Simurda 2012).

Theoretical and empirical studies of cooperation

For the last half century, Bill Hamilton's concept of inclusive fitness (Hamilton 1963, 1964) has been the dominating framework for the study of the evolution of cooperation and–although not without critics (see Birch and Okasha (2015) for a recent overview)–modelling organisms as if they strive to maximise their inclusive fitness has enjoyed great empirical success (Bourke 2011; Davies, Krebs, and West 2012; Gardner and West 2014). There are two ways to maximise inclusive fitness. The most common way is if the costly cooperative behaviour is directed towards individuals likely to share the gene(s) for cooperation, i.e. close relatives, or kin. As a consequence, the process is often referred to as kin selection (Maynard Smith 1964). However, Hamilton also recognised that in some cases what matters is not the average relatedness across the genome, but the relatedness at the locus for the social behaviour in question. In his classic 1964 paper, Hamilton performed a thought experiment where he imagined a gene (or set of tightly linked genes) that did three things:

(1) It creates a noticeable phenotypic label.
(2) It allows individuals with this label to identify other individuals with the same label.
(3) It causes individuals to behave favourably towards other individuals as long as they share this label.

Later, Richard Dawkins (1976) imagined a situation where a gene like this may give the individual carrying it a green beard and make that individual cooperate with other individuals carrying the beard; ever since, these kinds of genes are known as greenbeards (Gardner and West 2010). Dawkins and others were quick to point that greenbeards were likely to be rare in nature, both because of their genetic architecture but also because they are likely to be outcompeted by 'false beards' that carry the beard but do not engage in the cooperative behaviour (Biernaskie, Gardner, and West 2013). Greenbeards are indeed rare, but not unheard of (West and Gardner 2010). In addition to the yeast *FLO1* example used in this exercise, examples include the *Gp-9* gene in the fire ant *Solenopsis invicta* (Keller and Ross 1998) and the *csa* gene in the slime mould *Dictyostelium discoideum* (Queller et al. 2003).

Microbes are increasingly being used in the study of social evolution (Mitri and Richard Foster 2013; Monds and O'Toole 2009; West et al. 2007). Like any study system, they bring with both advantages and disadvantages. Their easy maintenance and short generation times has meant that empirical insights from studies of microbes have successfully been translated into practical teaching tools for a variety of evolutionary questions, including how natural selection leads to local adaptation (Delpech 2009), the process of adaptive radiation (Green et al. 2011), and the origin of multicellularity (Ratcliff et al.

2014). The social behaviour of microbes obviously differs from that of many mammals, particularly humans where morality and intentions play key roles (El Mouden et al. 2012; Wilson 2015). Insights from experiments on microbes may not necessarily translate into other social organisms, and vice versa, but the evolutionary logic is the same. A benefit of using social microbes is that they offer insights to the genetic basis of social behaviour, information that has traditionally been difficult to obtain from the classic animal study objects of social behaviour (Foster, Parkinson, and Thompson 2007; Mitri and Foster 2013; Nadell, Xavier, and Foster 2009; Van Dyken and Wade 2012). Thus, whereas microbes will tell us nothing about the role of culture in social behaviour, they offer a way around the notoriously thorny issue of nature versus nurture by only focusing on the former. Here, we combine these advantages in one laboratory exercise.

Cooperative flocculation in yeast

While the short generation time and ease of lab cultivation and genetic manipulation have meant that yeast (*Saccharomyces sp.*) has long been a model organism for a wide variety of biological questions, its suitability for studies of social behaviour has only recently been fully appreciated (Wloch-Salamon 2014). For example, in response to several environmental stressors thousands of yeast cells may come together to form large clumps ('flocs') similar to bacterial biofilms. Indeed, this flocculation behaviour has been used for centuries in the brewing industry to help remove excess yeast from alcohol (Speers 2012), but the behaviour is typically missing in lab strains.

The various aspects of yeast flocculation have been subject to extensive study (reviewed in e.g. El-Kirat-Chatel et al. 2015; Verstrepen, Reynolds, and Fink 2004; Vidgren and Londesborough 2011). In a recent experiment, Smukalla et al. (2008) identified the flocculating gene (*FLO1*), as a green beard locus in *S. cerevisiae*. The *FLO1* locus produces a transmembrane protein that allows yeast cells to bind to one another. Cells that share the 'cooperative' *FLO1* allele flocculate together while those that do not have the cooperative allele are more often excluded from flocs. Moreover, Smukalla et al. (2008) also showed that this cooperative behaviour provides protection from various environmental stressors, including anti-microbial substances and ethanol, as cells on the inside of the floc are physically protected against the external environment. However, this protection comes with a fitness cost as cells on the inside are also shielded against accessing oxygen and nutrients. This is illustrated by growing flocculating and non-flocculating cells under normal (non-stressful) conditions; non-flocculating grow four times faster than flocculating cells (Smukalla et al. 2008). The *FLO1* system therefore offers an excellent opportunity for students to test why cells flocculate and what aspect of inclusive fitness theory best explains the cooperative behaviour.

Learning objectives

The goal of this laboratory exercise is for students to learn aspects of inclusive fitness theory and practical lab techniques with microbes. Students are second year undergraduates, having previously taking one first year evolution course. Students therefore come into this lab having previously learned the basics of evolution, natural selection and drift, speciation, and population and community ecology. The lectures of this second year course build on this material and also introduce the evolution of social behaviour, including altruism, and how evolution can be studied at molecular level using genetic and genomic tools. To get the most out of this laboratory exercise, students should have a familiarity with the above topics as well as some experience with basic lab skills such as the use of micropipettes. Knowledge of yeast as a study system is not required. Other laboratory exercises in this same course experimentally examine directional selection on flowering time in an angiosperm and the maintenance of mutualism in a legume–rhizobium symbiosis.

This exercise is based on the research of Smukalla et al. (2008), which showed that *FLO1* behaves as greenbeard locus. We use three strains of yeast: two that are the same species (*S. cerevisiae*), but that do not share the same genotype at *FLO1* greenbeard locus, and one strain of another species

(*S. paradoxus*) that has the same genotype at the *FLO1* locus as one of the *S. cerevisiae* species. Whereas the genome of *S. paradoxus* does not normally contain a *FLO1* ortholog, and the species does not flocculate, flocculation can be conferred by genetically transforming the strain to carry the *FLO1* locus. See Smukalla et al. (2008) for details of the transformation. We ask students to predict which strains should cooperate based on both kin selection and the fact that two of them share the cooperative green beard allele and then test the resulting two hypotheses. By the end of this laboratory exercise, students should be able to:

(1) Compare and contrast how kin selection and greenbeard selection may lead to the evolution of cooperation
(2) Perform serial dilutions to allow replica plating
(3) Use replica plating to assess the abundance of different yeast strains grown in co-culture
(4) Perform statistical tests to assess the significance of the results

Lab protocol

Materials

- Yeast strain A–*Saccharomyces cerevisiae FLO*$^{-}$ (non-flocculating)
- Yeast strain B–*Saccharomyces cerevisiae FLO*$^{+}$ (flocculating); G418 antibiotic-resistance
- Yeast strain C–*Saccharomyces paradoxus FLO*$^{+}$ (flocculating); G418 antibiotic-resistance; Ura^{-} (can grow in the absence of uracil)
- Plates with YPD (i.e. yeast, peptone, and dextrose) growth media (2 per pair of students)
- Plates with YPD media + G418 antibiotic (2 per pair of students)
- Uracil dropout (Ura^{-}) plates (2 per pair of students)
- P200 pipettors and tips (1 per pair of students)
- Sterile water
- 250 mM EDTA
- Vortex
- 2 mL microcentrifuge tubes for making serial dilutions (3 per pair of students)
- Materials for plating yeast: alcohol burner, alcohol, bacteria spreader
- Sterile velvet for replica plating (2 per pair of students)
- Replica plating apparatus (1 per pair of students)

Methods

The lab is separated into three sections to allow time for the yeast to grow on the different types of media. The full lab instruction and procedure given to students is available in Supplemental Document 1.

Lab 1 (45–60 min)

Students work in pairs and are provided with a single tube of liquid media containing an equal mix of all three strains of yeast that have been growing together for 24 h at 30 °C in an incubator. There should be a visible floc in the bottom of the tube approximately 5 mm in diameter. Once they receive a tube of co-culture, students prepare a sample from the floc and the supernatant above the floc (i.e. the non-floc). To plate the non-floc, students are asked to remove 20 µL of the liquid above the floc and dilute it in 1.98 mL of sterile water (creating their first dilution). It is worth asking the students why they need to perform this dilution step. Students then plate 30 µL of this dilution on a YPD plate and label it.

Next, the students prepare the flocculating cells for plating. First they pour off the liquid media into a waste container while leaving the floc in the tube. They then add 100 uL of sterile water and rinse the tube to remove any contaminating cells from the surface of the floc. Care should be taken in this step, because if the floc is not washed thoroughly then the results can be very hard to interpret. The water is then removed from the tube again, leaving only the floc. The students perform a second rinse. In order to dissolve the floc, 200 µL of 250 mM EDTA is added to the tube, and it is vortexed. Vortexing needs to be done repeatedly over 5 min to get the floc to fully dissolve into a milky liquid.

The cells from the floc are at a much higher density than those in the supernatant since they are suspended in less liquid; these cells must be diluted more so that a reasonable number of colonies grow on the plates. The students do two 1:99 serial dilutions (20 µL sample to 1.98 mL sterile water) to eventually end up at a 10^{-4} dilution of the floc cells. 30 µL of this final dilution is then plated on YPD media. Both non-floc and floc plates are then placed for a minimum of 24 h at 30 °C in an incubator to allow the yeast to grow.

At the end of the lab the students are given a worksheet (Figure 1; Supplemental Document 2) illustrating the different plates they are using and are asked to write in their expected results under various scenarios. For example, if all three strains are present in equal proportion in the non-floc sample and 30 colonies grow on the YPD plate (Strains A, B, and C) then 20 should grow on the G418 plate (Strains B and C) and 10 should grow on the Ura$^-$ plate (Strain C only). Note that the astute student may realise that these outcomes assume that all three strains of *Saccharomyces* grow at the same rate. This assumption is most likely violated and it is a fair criticism of this method. This issue can be used as a good discussion point at the end of the lab for what may be affecting unexpected results or how the experiment could be improved. Alternatively, a haemocytometer may be used to count individual cells in each sample and the calculations can then be adjusted for differential growth rates.

Similarly if only Strains B and C are present in the floc sample the there would be 30 colonies on the YPD plate (Strains B and C), 30 colonies on the G418 plate (Strains B and C), and 15 on the Ura$^-$ plate (Strain C only). The students are asked to fill out this sheet for each prediction (kin selection or green beard).

Lab 2 (30 min)

In this second lab, the students take the plates they made in Lab 1 and replica plate them onto the other media types. Students are given back the plates they created in Lab 1, as well as two plates each of G418 media and Ura$^-$ media. On each plate (including their original YPD plate) they draw a mark on the side of the plate. They align this mark on the plate with the mark on the replica plating apparatus so that all of the plates have the same alignment. Next, they put a sterile velvet on their replica plater, open up their YPD plate, and gently press the velvet (so that the marks on the plater and plate align). Without changing the velvet, they press the velvet into one of the G418 plates and one of the Ura$^-$ plates in the same orientation. They do this for both the floc and non-floc samples, changing the velvet between the two samples. These plates are once again left to grow for a minimum of 24 h at 30 °C.

Lab 3

In the final lab, students count the number of colonies on each plate and calculate the contribution of each *Saccharomyces* strain to the floc and non-floc (Figure 2). To determine whether the flocculating behaviour is primarily driven by kin selection or a greenbeard locus, students compare if the proportion of colonies of each strain is different from the expected equal representation of all strains on each plate. For a suggested statistical test of this, see Supplemental Document 3.

Once all students have collected their data, the data are pooled and a class average for the proportion of each strain within and outside of the floc is calculated (Figure 3; see Supplemental Document 4 for an example of a full class data-set). The two strains that carry the *FLO*$^+$ allele are more abundant in the floc than in the non-floc, whereas the strain that does not carry the *FLO*$^+$ allele is more abundant

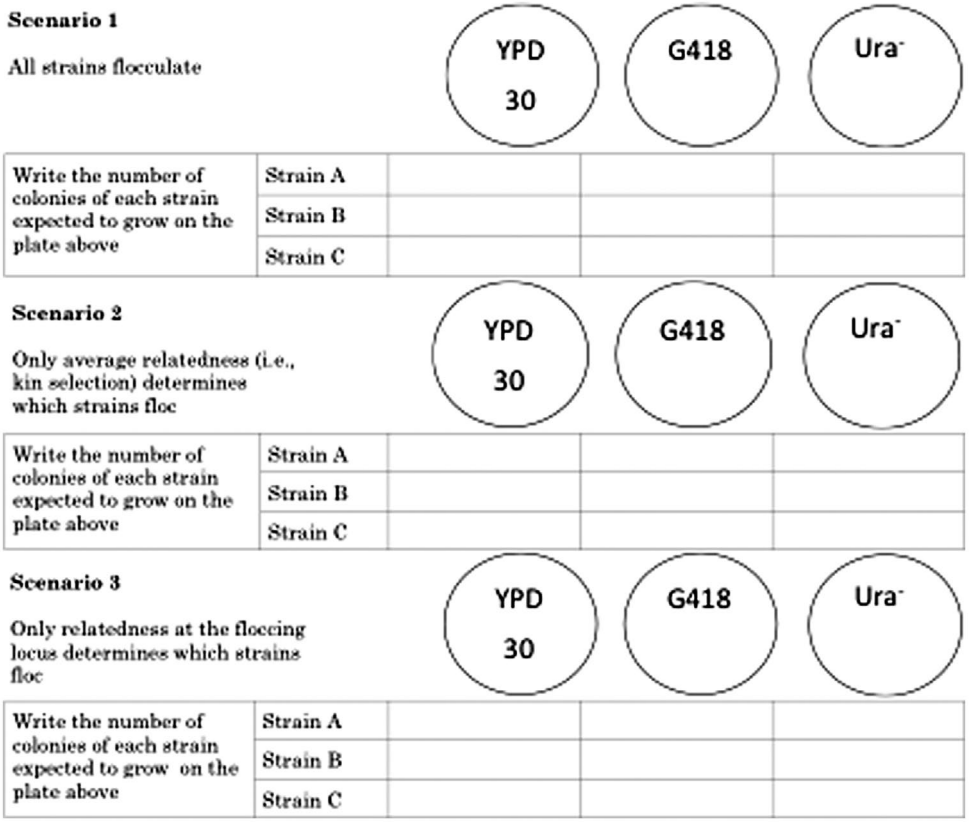

Figure 1. Worksheet where the students predict outcomes based on competing hypotheses. Each scenario assumes 30 colonies grow on the YPD media and that all strains present in the floc are present in equal proportions. This worksheet helps students understand how the replica plating actually tells us which strains are present in a sample and how we can tell what strain each colony is. In this worksheet strain A is *S. cerevisiae FLO*$^-$, strain B is *S. cerevisiae FLO*$^+$ and strain C is *S. paradoxus FLO*$^+$. The full worksheet can be found in the Supplemental Document 2.

in the non-floc than the floc. These results thus support the hypothesis that it is relatedness at the *FLO1* locus that determines which strains flocculate and that the cooperative flocculating behaviour in *Saccharomyces* is driven by a greenbeard mechanism.

It is likely that there are some *FLO-* yeast that grow on the floc plates. Two possibilities that students often point out are that either the non-flocullating yeast are merely contaminants, perhaps they did not wash their floc enough before dissolving the floc, or, alternatively, that some 'cheating' individuals have entered into the floc to gain the benefits of its protection without the cost of having to produce the necessary proteins themselves. Discussing this latter possibility with the students helps them understand some ways that cooperation in a system like this can break down.

Possible extensions

This exercise can be extended to introduce a number of additional topics. First, since this lab generally takes a 'genes' eye view' of evolution (Bourke 2011; Dawkins 1976) it offers an opportunity to introduce other evolutionary issues that lend themselves to this perspective. One example is the study of genetic conflict. Indeed Hamilton himself recognised an intimate relationship between his inclusive models and the study of selfish genetic elements, genes that can promote their own transmission at the expense of other genes in the genome (Hamilton 1967; Burt and Trivers 2006; Ågren 2013) and

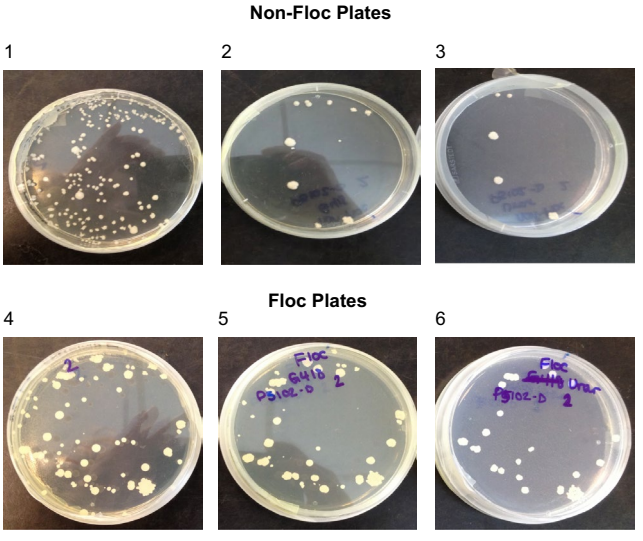

Figure 2. Example set of replica plates grown using the lab protocol. Colonies 1–3 were plated from the non-flocculating sample, whereas colonies 4–6 were plated from the flocculating sample. Colonies 1 and 4 were plated on YPD media (all strains grow on this media). Colonies 2 and 5 were replica plated on G418 antibiotic media (only the two strains with the flocculating allele (*S. cerevisiae FLO*$^{+}$ and *S. paradoxus FLO*$^{+}$ grow on this media). Colonies 3 and 6 were replica plated on uracil dropout media (only the *S. paradoxus FLO*$^{+}$ strain grows on this media).

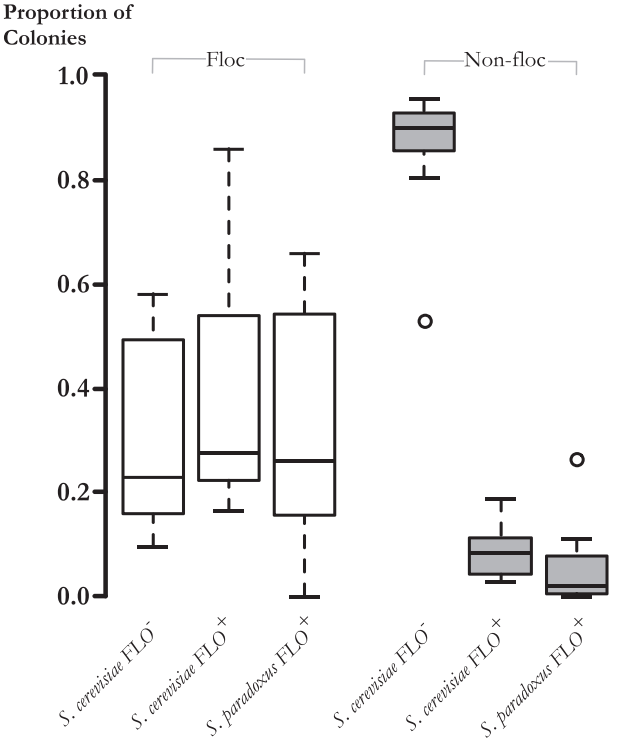

Figure 3. Boxplot showing the proportions of colonies belonging to each strain grown from either the floc (white boxes) or supernatant (grey boxes; non-floc) data are from 12 sets of plates (one lab of 24 students). Raw data can be found in Supplemental Document 4.

there has been much debate whether greenbeard genes should be placed in this category (Biernaskie, West, and Gardner 2011; Helanterä and Bargum 2007; Ridley and Grafen 1981). In one year (2013), we asked students to read papers on different examples of selfish genetic elements (e.g. transposable elements, selfish plasmids, genomic imprinting, and B chromosomes). In class, students formed groups so that each person in the group had read a different paper and explained the different systems to one another. As a group they then explored similarities and differences between the different examples to find common features of selfish genetic elements. Finally, as a class we then discussed whether or not greenbeards should be considered a kind of selfish genetic element.

Second, the cost of the flocculating behaviour can be examined. While strains in the floc enjoy greater protection against several environmental stresses, they suffer a cost through a reduction in growth rate relative to non-floc strains (Smukalla et al. 2008). By following the fate of flocculating and non-flocculating strains under several environmental conditions, the lab can therefore also be extended to study trade-offs between cooperative and uncooperative behaviour and to discuss under what ecological conditions cooperation may break down and 'cheaters' can be expected to spread in a cooperative system.

Conclusions

The past decades have seen a dramatic improvement in our understanding of the social lives of microbes. In this paper, we demonstrate how these insights can be translated into practical teaching tools to study the concept of inclusive fitness and the genetics of cooperation. Moreover, they also receive exposure to common laboratory skills such as sterile technique, serial dilutions, and replica plating. Ultimately, while the genetics of cooperation has traditionally been taught through computer simulations and games, this lab offers a way to experimentally introduce the topic.

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