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NOVEL COOPERATION EXPERIMENTALLY EVOLVED BETWEEN SPECIES

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Cooperation violates the view of "nature red in tooth and claw" that prevails in our understanding of evolution, yet examples of cooperation abound. Most work has focused on maintenance of cooperation within a single species through mechanisms such as kin selection. The factors necessary for the evolutionary origin of aiding unrelated individuals such as members of another species have not been experimentally tested. Here, I demonstrate that cooperation between species can be evolved in the laboratory if (1) there is preexisting reciprocation or feedback for cooperation, and (2) reciprocation is preferentially received by cooperative genotypes. I used a two species system involving *Salmonella enterica* ser. *Typhimurium* and an *Escherichia coli* mutant unable to synthesize an essential amino acid. In lactose media *Salmonella* consumes metabolic waste from *E. coli*, thus creating a mechanism of reciprocation for cooperation. Growth in a spatially structured environment assured that the benefits of cooperation were preferentially received by cooperative genotypes. *Salmonella* evolved to aid *E. coli* by excreting a costly amino acid, however this novel cooperation disappeared if the waste consumption or spatial structure were removed. This study builds on previous work to demonstrate an experimental origin of interspecific cooperation, and to test the factors necessary for such interactions to arise.

KEY WORDS: **Consortia, cross-feeding, *E. coli*, *Salmonella*, spatial structure.**

Cooperation is a problem that has mystified biologists since the original proposal of evolution by natural selection. Natural selection should favor selfish acts, and yet cooperation is evident at all levels of biological organization from genes to societies. A large body of theory has been generated to explain the patterns observed in nature (Sachs et al. 2004; West et al. 2007a), and recently, exciting empirical tests of the theory have begun to emerge (Griffin et al. 2004; MacLean and Gudelj 2006; Ross-Gillespie et al. 2007). These tests largely focus on the maintenance of cooperative traits within a species. However, we lack a clear illustration of the mechanisms necessary for the evolutionary origin of cooperation, particularly between species.

Previous work suggests that several factors are important for the evolution of interspecies cooperation (Trivers 1971; Sachs et al. 2004; West et al. 2007a). When an organism aids an individual of another species it must acquire a direct benefit in return, as it is not feasible to gain inclusive fitness simply through increasing a recipients reproductive potential. Cooperation between unrelated individuals likely depends on (1) reciprocation between partners, and (2) direction of reciprocation to cooperating individuals. This raises several intriguing questions. If it is only advantageous to cooperate if your partner also cooperates, how does the process begin? Furthermore, how can benefits be directed to specific cooperating individuals of another species? Finally, are the conditions that maintain cooperation sufficient for its origin?

Excretion of waste products may provide a mechanism for the initiation of reciprocation (Sachs et al. 2004). Excretion of waste is clearly not a costly process that needs evolutionary explanation, but waste products can often be beneficial for other organisms. For example, some insects benefit from the feces of cows, and bacteria often acquire metabolites from the excretions of other microbes (Schink 1997, 2002). These benefits could provide the foundation for the evolution of cooperation. A user of

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waste products may be selected to help its partner as a way of increasing the waste products received. Such selection could give rise to costly cooperation, that is, costly to the producer but which ultimately benefits the producer by increasing the reciprocation from the partner.

A spatially structured environment may provide a mechanism that directs benefits to cooperating individuals (Griffin et al. 2004; Sachs et al. 2004). Individuals that pay a cost to help their partners will only spread in a population if they get more of the benefits from the partner than do individuals that do not pay the cost of helping. Spatial structure may facilitate the direction of benefits by localizing interactions. In the extreme, spatial structure can create patches that contain just one individual of each species. Patches that contain cooperators will permit more growth than those patches that do not. However, perhaps surprisingly, spatial structure can also lead to the evolution of intensified antagonistic interactions between partners (West et al. 2001), so the effect of spatial structure is not clear.

A system of two bacterial species was used to test whether our current understandings can be used to evolve novel cooperation. The system involved *Salmonella enterica* ser. *Typhimurium* and an *Escherichia coli* mutant unable to synthesize methionine (met-*E. coli*). A preexisting mechanism that would allow for reciprocation was created by growing the two species in lactose. *Escherichia coli* metabolizes lactose and then excretes costless metabolic byproducts on which *Salmonella* can feed. A method of directing benefits was provided by growing the community on agar plates. Although evolution could have improved the growth of each species independently, a cooperative adaptation arose. *Salmonella* evolved to secrete the amino acid that *E. coli* required. This origin of cooperation was dependent on both a preexisting mechanism of reciprocation and a method of directing benefits.

Materials and Methods

SYSTEM

The study system consisted of *E. coli* and *Salmonella*. The *E. coli* strains used was *E. coli* K12 BW25113 (*rrnB3* Δ*lacZ4787 hsdR514* Δ*(araBAD)568 rph-1*) with a metA knockout. This line was acquired as part of the Keio collection (JW3973) (Baba et al. 2006). To reenable lactose metabolism the *E. coli* was mated for 40 min with *E. coli* HfrH PO1 relA1 thi-1 spoT supQ80 nad57::Tn10. The constructed *E. coli* line achieves no appreciable growth in minimal media in the absence of methionine. The *Salmonella* used was *Salmonella typhimurium* LT2. All lines were grown in M9 minimal media with 10 mL of 0.01 M $CaCl_2$, 10 mL of 0.1 M $MgSO_4$, and 10 mL of 20% sugar (lactose or glucose) per liter.

In lactose minimal media, *Salmonella* feeds on the waste byproducts excreted by *E. coli* (likely acetate), whereas the *E. coli* strain used requires the amino acid methionine. At the start of the study, cultures of the bacteria were unable to grow together because there was insufficient methionine for *E. coli* and thus insufficient sugar byproducts for *Salmonella*.

ACQUISITION OF A METHIONINE EXCRETING *S. TYPHIMURIUM* MUTANT

A methionine-excreting strain of *Salmonella* was selected in a two-step process. First, a chemical technique was used to select for increased methionine production. When this did not give rise to cooperation a selection regime was used to evolve methionine excretion. In the chemical technique, 10^8 cells were grown on a glucose M9 minimal media plate with 1 mg/mL ethionine (Lawrence et al. 1968). A resistant colony was then streaked onto a second ethionine plate. A colony from this second plate was grown overnight in glucose and 10^7 was plated with 10^7 *E. coli* on a lactose M9 minimal media plate. The bacteria were allowed to grow for three days at 37°C, and then cells were scraped off. The scraped sample was vortexed and 100 μL was plated onto a fresh lactose plate. This second plate was allowed to grow for five days, *Salmonella* was isolated from large colonies and tested for cross-feeding of *E. coli* and methionine excretion.

Methionine production of *Salmonella* was measured by HPLC analysis. *Salmonella* samples were grown overnight in glucose minimal media. These samples were then centrifuged at 10 K for 2 min and filtered through a 2 μm filter to remove all cells. Spent media was analyzed by HPLC with a Beckman 7300 Amino Acid Analyzer coupled with System Gold software whose limit of detection is 0.01 μg/mL.

Several lines of evidence suggest that methioine is excreted rather than released by cell lysis. First, cell lysis would lead to the release of all cellular metabolites, but HPLC analysis only detected an increase in methionine in spent media. Second, both bacteria increase in unison with no significant difference in growth curves between species ($t = -0.339$, df $= 18$, $P = 0.93$), in contrast to what has been observed in systems with cell lysis (Shou et al. 2007; Rozen et al. 2009).

TESTS OF DYNAMIC STABILITY

To test the selective benefit of assisting a partner, a methionine-excreting *Salmonella* was competed against nonexcreting wild-type in the presence of met-*E. coli*. Three spatial structure replicates were initiated with 1% methionine excreting mutants and 99% nonexcreters. A total of 10^8 *Salmonella* and 10^8 *E. coli* were plated on lactose M9 plates. Bacteria were allowed to grow for two days at 37°C, whence the cells were scraped off in 3 mL of M9 minimal media. A total of 100 μL of the cell suspension was spread onto a new plate (30-fold dilution). A similar protocol was followed for other experiments on plates, changing only the initial frequency of cooperators or sugar where appropriate.

To test the effect of mass action, bacteria were added to a 125-mL flask with 10 mL of lactose M9 minimal media. Every 24 h 100 μL was transferred to a new flask (100-fold dilution). Three replicates were carried out with initial frequencies of 99.99% methionine excreters and 0.01% nonexcreters.

After every passage, the number of *E. coli* and *Salmonella* were determined by plating on LB plates with X-gal. To determine the frequency of excreters and nonexcreters, 30 Salmonella colonies were stabbed onto a lawn of *E. coli* on a lactose plate with X-gal. If an isolate was an excreter a blue colony formed on the plate, otherwise no colony appeared.

Results

At the start of the study, cultures of the bacteria were unable to grow together (Fig. 1, left). A specific selection regime was used to evolve cooperative methionine excretion in *Salmonella*, thereby allowing community growth.

EVOLUTION OF *SALMONELLA* WITH HIGH METHIONINE EXCRETION

HPLC measurements indicated that initially *Salmonella* excreted very low levels of methionine (0.005 ± 0.002 mM methionine in overnight glucose culture). A two-step process was used to acquire cooperative *Salmonella*. First, an established chemical technique was used to select overproduction of methionine. Resistance to the methionine-analog ethionine has been shown to cause a constitutive expression of the methionine pathway (Lawrence et al. 1968). It was anticipated that selection on ethionine plates would be sufficient to create cooperative *Salmonella*, but methionine excretion levels were no higher than ancestral *Salmonella* as measured by cross-feeding assays (Fig. 1, middle) and HPLC.

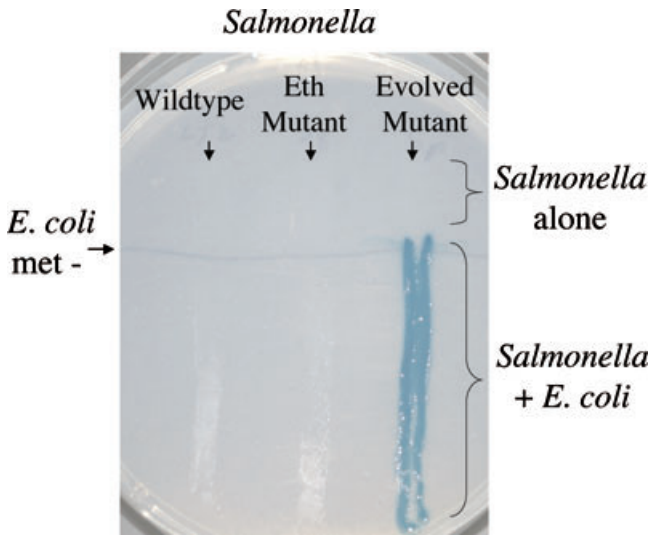

Figure 1. **Cross-streaks of the three types of *Salmonella* across *E. coli*. *E. coli* was streaked horizontally across the plate. *Salmonella* was then streaked vertically from top to bottom. "Wild-type" indicates the initial *Salmonella typhimurium*. "Eth mutant" indicates the ethionine-resistant mutant. "Evolved mutant" indicates the methionine-excreting mutant that arose on plates with *E. coli* and was used in experiments. The blue line is bacterial growth where the methionine-producing *Salmonella* was streaked across *E. coli*.**

An indirect selection method was then used to select for increased methionine excretion by *Salmonella*. Lactose minimal plates were seeded with 10^7 each of met-*E coli* and ethionine-resistant *Salmonella* and allowed to grow for three days at 37°C. The three-day plate contained little visible growth, but was scraped and an aliquot was spread on a new plate. After five days on the second plate, several large colonies appeared, containing both *E. coli* and *Salmonella*. The *Salmonella* in these colonies were a mutant that excreted high levels of methionine thus enabling the *E. coli* to grow. Assays of methionine levels in spent media confirmed an approximate 15-fold increase (0.08 ± 0.02 mM) in methionine excretion by these *Salmonella* mutants (Fig. 1; Methods). High excretion mutants arose twice in 10 replicates (multiple colonies forming on the second plate within a replicate were conservatively deemed one evolutionary origin as they could have come from a single mutant on the first plate). The second mutant performed identically in cross-feeding assays, but was not measured with HPLC.

Ten indirect selection replicates were also initiated with wild-type *Salmonella*. No evolution of high methionine excretion was observed in these cases. This suggests that the ethionine treatment facilitated the evolution of methionine excretion.

METHIONINE EXCRETION IS COSTLY

To determine whether methionine excretion impaired *Salmonella* fitness, mutant *Salmonella* were competed against wild-type *Salmonella* in acetate minimal media. In these conditions, *E coli* were absent and the *Salmonella* grew according to their intrinsic metabolic abilities. Any fitness effect of methionine excretion would lead to reduction in growth of methionine excreters and therefore an increase in the frequency of wild-type *Samonella*. In liquid, the wild-type swept from an initial frequency of 2% to near fixation in one transfer, a selection coefficient (s) of -0.43 ± 0.05 for methionine excretion. The selection coefficient of methionine excreters in comparison to nonexcreting ethionine mutants is -0.37 ± 0.06. This result suggests that there was a cost associated with ethionine resistance, and an additional cost was associated with methionine excretion.

The apparent cost of methionine excretion distinguishes *Salmonella's* excretion from that of *E. coli*. *E. coli*'s excretion is beneficial for the bacteria independent of other species, whereas *Salmonella*'s excretion clearly is not. I use the term cooperation to describe *Salmonella's* excretion as it benefits another species, and is not beneficial to *Salmonella* in the absence of interspecific

feedback. This definition of cooperation as an adaptation that is selected because it helps a recipient follows West et al. (2007b).

COOPERATION IS SUPERIOR IN A STRUCTURED ENVIRONMENT

The evolutionary fate of cooperative versus noncooperative *Salmonella* was tested in a structured environment. *E. coli* and *Salmonella* were plated together on lactose minimal plates at a density of 5×10^7 each. Initially, the *Salmonella* population consisted of 99% wild-type and 1% cooperative methionine excreters. Over four transfers (approximately 20 generations), cooperative methionine excreters spread through the population to greater than 80% (Fig. 2A). Coincident with the increase in cooperators, the density of bacteria on the plates after 48 h increased by more than 15-fold (Fig. 2E). This result demonstrates that, on lactose plates, the fitness cost of high methionine excretion by *Salmonella* is overcome by the fitness gained from receiving more food from enhanced *E. coli* growth.

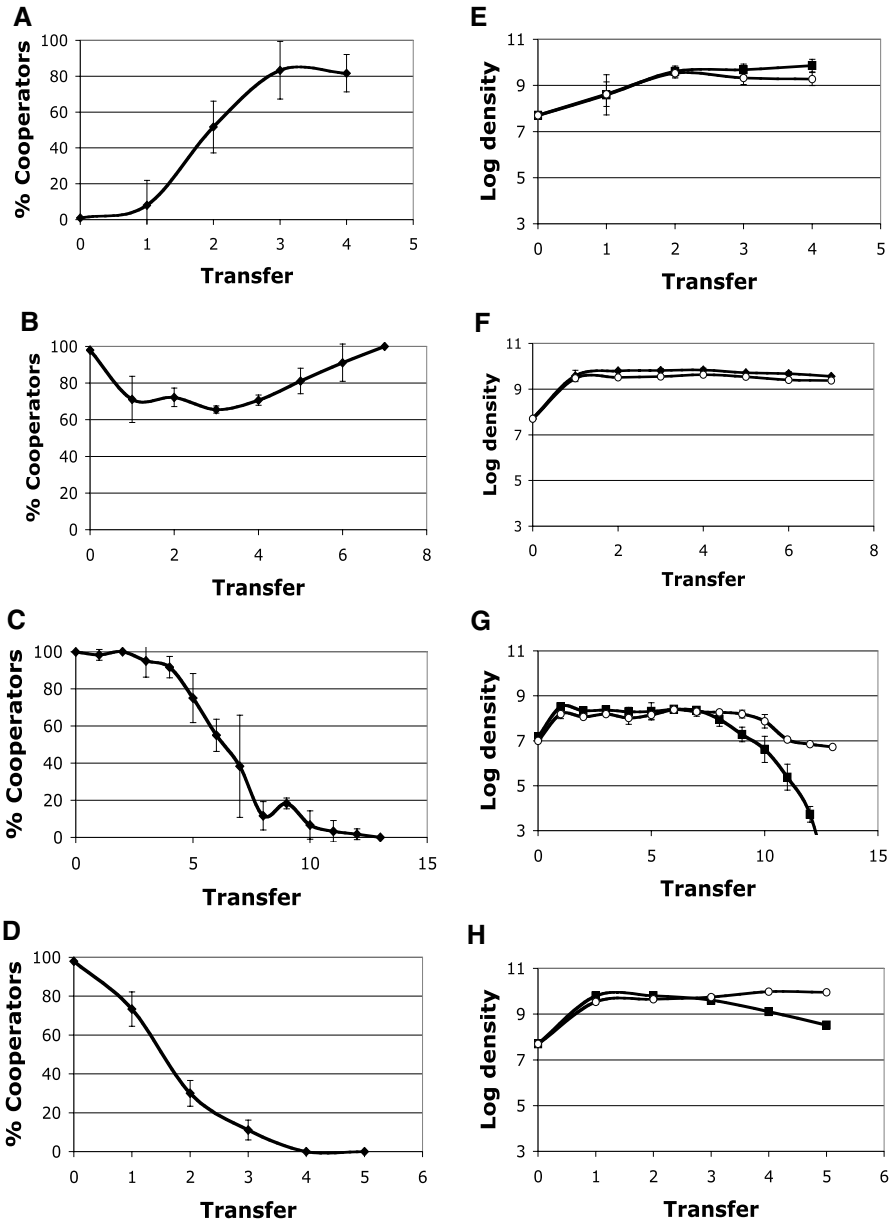

Figure 2. Dynamics of the system with variation in reciprocation and spatial structure. Graphs A–D are the percentage of cooperators in the *Salmonella* population. Graphs E–H are the log density of *E. coli* (filled squares) and *Salmonella* (open circles). A and E are the results from communities grown on lactose plates when cooperators were initially rare. B and F are the results from communities grown on lactose plates when cooperators were initially common. C and G are the results from communities grown on acetate plates. D and H are the results from communities grown in lactose flasks with no spatial structure. Error bars represent the standard deviation.

The rapid increase in excreter frequency demonstrates that cooperation can arise from rare mutants. To test the strength of selection when cooperative mutants dominate, *E. coli* was spread on lactose plates with a *Salmonella* population that consisted of 98% cooperators and 2% wild-type. Surprisingly, the wild-type increased to 30% in the first growth phase; however, it subsequently decreased in frequency (Fig. 2B). On one plate wild-type decreased to 7% by transfer six and then the plate became contaminated. On two plates, wild-type dropped below the level of detection (<3%) by the seventh transfer. When grown with 100% cooperators *E. coli* reaches a density of 5×10^9. The initial invasion of wild-type suggests that selection dynamics may differ when bacteria make the transition from liquid to plates. The ensuing apparent fixation of cooperation illustrates the selective advantage of cooperators in structured environments. Though it should be noted that the selective advantage is likely influenced by cell density (Bull and Harcombe 2009).

COOPERATION REQUIRES RECIPROCATION

To determine the importance of a preexisting mechanism of reciprocation, the two species were grown on acetate plates. Acetate plates remove the reciprocal benefit of *E. coli* to *Salmonella*, as *Salmonella* consumes the carbon source directly and does not rely on *E. coli* waste products. In the absence of waste consumption, the cooperative *Salmonella* mutant decreased from 98% to <5% in four transfers (Fig. 2D), accompanied by a reduction in *E. coli* density (Fig. 2H). A qualitatively similar pattern was observed on glucose plates.

These data demonstrate that the spread of cooperative *Salmonella* was not the result of adaptation to plate growth, but rather a function of their cooperation. Additionally, the data support the theory that costly interspecies cooperation is dependent on a mechanism of reciprocation (Trivers 1971; Foster and Wenseleers 2006; Bull and Harcombe 2009). As waste production is costless, it may often serve as a foundation for the evolution of cooperation, particularly between species in which nutrient requirements often differ (Sachs et al. 2004).

COOPERATION REQUIRES SPATIAL STRUCTURE

Spatial structure may facilitate the preferential direction of benefits to cooperators by creating patches that localize interactions between individuals (Sachs et al. 2004; Foster and Wenseleers 2006; West et al. 2007a; Bull and Harcombe 2009). Patches that contain cooperators will engender more growth and hence more reciprocity than those patches that do not. To determine the importance of reciprocity being directed to cooperators, the two species were grown in well-mixed flasks, an environment that does not allow for direction of benefits. *E. coli* and *Salmonella* were started at a frequency of 5×10^7 each in flasks of lactose minimal media. Initially the *Salmonella* population consisted of 99.99% cooperative methionine excreters and 0.01% wild-type. Over 20 passages wild-type *Salmonella* spread to apparent fixation at the expense of cooperative methionine excreters (Fig. 2C). Over the course of the experiment, *E. coli* densities decreased from 3.2×10^8 to below the limit of detection (Fig. 2G), as expected with the loss of cooperation in *Salmonella*. In communities with 100% cooperative *Salmonella*, *E. coli* densities reach 4×10^8. These results support the notion that in well-mixed flasks, cooperators share the benefits of reciprocation globally and hence cooperation does not evolve.

Discussion

Nature is rife with examples of interspecies cooperation, from endosymbiosis to plant–pollinator interactions (Sachs et al. 2004; West et al. 2007a). These interactions all depend on some form of reciprocity between partners (Trivers 1971; Foster and Wenseleers 2006). Although theory exists for how such interactions might arise (Trivers 1971; Sachs et al. 2004; Foster and Wenseleers 2006; West et al. 2007a; Bull and Harcombe 2009), we lack empirical tests of this theory. Here, it was shown for the first time that novel interspecies cooperation can be evolved in the laboratory and that theory correctly predicts the necessary conditions.

Salmonella's excretion of methionine fits standard definitions of interspecies cooperation: a behavior that aids an unrelated organism, and that is selected because of its effect on that organism (West et al. 2007b). *Salmonella* benefits from increased carbon as a result of excreting methionine, but those benefits are mediated through the *E. coli*. Interactions with the *E. coli* are important to consider for several reasons. First, at a mechanistic level focusing on the species interactions explains why selection leads to methionine excretion as opposed to some other adaptation. It also explains the different evolutionary trajectories observed in lactose liquid, lactose plates, and acetate plates. These environments provide similar abiotic resources, but differ fundamentally in the way they cause species to interact. Finally, the interspecific nature of the interaction demonstrates that the benefit of cooperation is not accrued through aiding shared genes in relatives, though it should be noted that similar situations can arise between unrelated individuals of the same species.

It is remarkable that interspecies cooperation could be selected so easily once the necessary conditions were understood. Two independent origins of cooperation were observed in 10 trials with relatively few bacteria and each origin occurred after only one transfer (~10 generations). The ease of this adaptation was likely augmented by initial chemical treatment of *Salmonella*, suggesting that combining engineering and evolution may be a useful tool for acquiring bacteria with desired traits. However, the cooperation was not the result of engineering; ethionine-resistant cells did not excrete methionine and hence were no more cooperative

than wild-type *Salmonella*. The mutation for cooperative excretion arose by random mutation and then evolved as the result of a specific selective regime. Indeed, the principles underlying the observed evolution of interspecific cooperation should be broadly applicable to any system.

It is also interesting to note that cooperation arose before a single species solution. *E. coli* or *Salmonella* could have evolved enhanced growth independent of the other species. Although such a solution would have been readily detected in the selection regime, it was never observed. Presumably this is because the genetic details of the system made the multispecies solution easier to evolve (i.e., fewer mutational steps were required for *Salmonella* to excrete methionine than to acquire lactose metabolism). However, the loss of cooperative mutants in liquid illustrates that mutlispecies solutions are only feasible under specific conditions. This work therefore provides insight into when species interactions are likely to determine how evolutionary problems are solved.

Work by others provides some interesting parallels to the research described here. Several studies have demonstrated that it is possible to engineer mutually reciprocating systems (Shendure et al. 2005; Shou et al. 2007; Kim et al. 2008). Although these studies do not include evolutionary dynamics they demonstrate other ways to create cooperative systems. Additionally, several studies have demonstrated that cooperation within a species can be maintained by spatial structure (Griffin et al. 2004; MacLean and Gudelj 2006). Velicer and Yu (2003) even illustrated the origin of cooperation within a species by evolving swarming in *Myxococcus xanthus*. These studies have critically improved our understanding of intraspecific cooperation, though as their results were largely dependent on shared genes they did not directly address cooperation between nonkin. Finally, several studies have demonstrated the evolution of reduced interspecific conflict. For example, parasites can be selected to reduce harm to their hosts (Bull et al. 1991). More similarly to this study, Sachs and Bull (2005) worked with two distinct viruses that were mutually dependent, but competed for hosts. They demonstrated that one virus co-opted the necessary genes from its partner into its own capsid, making the virus able to grow alone. This work demonstrates an intriguing alternative to the evolution of cooperation. The current study builds on all of this previous research to demonstrate the first experimental evolution of novel cooperation between species.

The work reported here is particularly relevant to the evolution of microbial interactions. Bacteria often obtain metabolites from the excretions of other microbes (i.e., cross-feed) (Schink 1997, 2002); indeed this may be one reason that so many bacteria cannot be cultured in isolation. Previous work has shown that selection can drive bacteria to specialize on the byproducts others excrete (Helling et al. 1987; Rosenzweig et al. 1994; Turner et al. 1996). The current study demonstrates that feeding on byproducts can form the foundation for the evolution of cooperation. Thus selection can not only drive bacteria to specialize on unused waste but can also drive bacteria to produce costly resources to increase the growth of community members. Interestingly, specialization on byproducts can decrease in structured environments (Saxer et al. 2009) (but see Habets et al. 2006; Krone and Guan 2006), whereas here cooperative metabolite provisioning increased when the environment was structured. It will be intriguing to test how contrasting effects on byproduct and cooperative cross-feeding influence the total diversity of microbial metabolite exchange.

The ability to easily turn cooperation between bacterial species on and off may be particularly useful to industry. Communities of bacteria are used industrially for everything from food production to energy generation (Wall et al. 2008). For many applications, it will be useful to construct novel communities to carry out a function (Brenner et al. 2008; Wall et al. 2008). Such constructed communities often will not grow well as demonstrated by the initial community growth reported here, and by Shou et al. (2007). If communities contain waste consumption interactions, my results provide a mechanism for dramatically improving growth and function of the community. Furthermore, the ability to eliminate a community by selecting against cooperation may prove useful for constraining community activity to specific times or places.

Selecting microbes under laboratory conditions is a powerful technique for gaining insight into the evolutionary process (Elena and Lenski 2003). The demonstration that costly interspecies cooperation requires mechanisms of reciprocation, and of directing benefits should be broadly applicable across systems. Further research will be needed to understand how these requirements are fulfilled in the many natural examples of interspecific cooperation.

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