The Evolutionary Ecology of Model Microbial Communities

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2009

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The Evolutionary Ecology of Model Microbial Communities

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Acknowledgements

This dissertation was substantially improved as a result of extensive discussions with my adviser, Dr. Jim Bull. He is a fantastic scientist and I learned a great deal from him. My committee also played a significant role in shaping the work. Specifically, Dr. Leibold and Dr. Hawkes encouraged me to incorporate ecology into my evolutionary perspective. Lab members provided an invaluable sounding board for ideas, especially Dr. Rick Heineman, Thomas Keller, Rachael Springman, and Dr. Joel Sachs. Numerous friends provided a great deal of emotional support, particularly members of the House of Science, Jeremy Brown, Eric Q Miller, and Cindy Hayes. Finally, I would like to thank my family. They were the first to introduce me to the wonders of science, and have always been supportive even when I decided not to follow their advice.

The Evolutionary Ecology of Model Microbial Communities

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The biological world is complex. Communities contain a multitude of interacting species, while populations contain extensive genetic variation. How much complexity must one consider to understand patterns and processes of interest? When are species interactions and community properties shaped by evolution? Conversely, when is evolution altered by community context? I test these questions in a series of experiments with simple microbial communities. The first data chapter investigates the impact of competition on the evolution of phage resistance in bacteria. This work demonstrates that community context can dramatically alter the evolution of resistance to phage. Next I tested the impact of evolution on assembly of a three species community. I demonstrate that evolution can influence the content of a microbial community by altering the process of assembly. Finally, I investigated the evolutionary origin and maintenance of cross-feeding mutualisms. This work suggests that species interactions can enable novel evolutionary pathways, and that evolution can significantly increase the productivity of cross-feeding communities. Jointly these experiments suggest that consideration of the interplay between ecological and evolutionary forces can provide insight into the complexity of the natural world.

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Chapter 1

Introduction

Introduction

The biological world is complex. A community can consist of millions of species that affect each other through a multitude of interactions. Furthermore each species contains variation in thousands of interacting genes. To make matters worse these systems are dynamic, with parts and interactions changing through time. This raises questions about how much diversity must be considered to understand patterns and processes of interest. When are species interactions and community properties shaped by evolution? Conversely when is evolution altered by community context?

Extensive feedback may exist between species interactions and evolution. Evolution has the potential to modify species interactions and community properties. For example, character displacement could facilitate the coexistence of species, or evolution of a mutualism could dramatically impact community productivity. Conversely, diverse species interactions have the potential to constrain or facilitate the evolution of traits relevant to community properties. For instance, interactions with competitors may constrain the evolution of herbivore defense in a foundation species. While it is easy to make verbal arguments of why such feedback might be critical to consider, we lack empirical tests of these ideas (Haloin and Strauss 2008). Significant questions remain

about the types of feedback that exist, the mechanisms that drive those feedbacks, and the impact that feedback has on community properties.

It may be particularly useful to examine these questions in microbes. Microbes offer powerful systems to test evolutionary and ecological theory because of their rapid generation time and experimental tractability (Elena and Lenski 2003; Jessup et al. 2004). Additionally, microbes are of interest in and of themselves as they drive important processes from global nutrient cycling to human health. The forces driving ecological and evolutionary change in microbial communities remain largely unexplored.

For my dissertation I developed simple microbial model systems to investigate the interplay between species interactions and evolution. I tested how community complexity can constrain or facilitate evolution and how evolution can alter species interactions to change community properties. Below I provide further background on what is known about the interplay between species interactions and evolution. I then discuss the utility of investigating this interplay with microbial model communities. Finally, I introduce the chapters of my dissertation.

Synthesis of Ecology and Evolution

The existence of interplay between evolution and species interactions is not a new idea. Both Darwin and Elton made reference to such interactions in their seminal works, which gave rise to evolution and community ecology respectively (Holt 2005). However, the relationship between ecological and evolutionary forces is often not explicitly taken into account. This lack of synthesis may in part be due to the direction the two fields have

taken recently (Holt 2005). Over the past two decades evolution has become focused on changes at the sequence level, often ignoring the ecological relevance and context of those changes. Meanwhile, ecology has focused on how local interactions determine biodiversity, often excluding consideration of why those interactions exist or how they might change through time. This is starting to change, however, as a slew of recent reviews call for a synthesis of the fields (Urban and Skelly 2006; Johnson and Stinchcombe 2007; Haloin and Strauss 2008; Urban et al. 2008). Below I briefly describe the data that support interplay between evolution and species interactions.

Evolution impacts species interactions

Rapid evolution has been shown to alter pair-wise species interactions. There are several examples of rapid evolution of defense altering exploitative interactions. For instance, in less than ten years evolution of resistance reduced the ability of myxoma virus to control rabbit populations in Australia (Fenner 1983). Additionally, there are multiple cases of consumer-resource interactions changing as a result of evolution. These cases include evolution of host plant preference in *Rhagoletis* and beak morphology in Darwin's finches (Thompson 1998). Thompson has verbally argued that such evolutionary changes occur on the time scale of ecological changes. Hairston et al. (2005) carried this argument a step further and mathematically demonstrated that evolution can in fact be the dominant factor driving ecological properties such as population dynamics.

The impact of rapid evolution on broader community properties is less clear. Data supporting the influence of evolution are based on the impact of genetic differences. Several researchers have demonstrated that the genotype of keystone or foundation species can alter the content of the community that forms around them. For example, Whitham et al. (2003) showed that different genotypes of cottonwood harbor different arthropod communities. In most of these cases the impact is caused by differences in plant defense that arise in different hybrid classes. Additionally, De Meester et al. (2007) demonstrated that differences in resident daphnia genotype can alter the ability of zooplankton to invade. These studies suggest that evolution might alter communities by modulating genetic variation; however, none of the studies actually include evolution.

The impact of rapid evolution on community content and function remains unclear. Can traits that influence community properties evolve rapidly? For example, is rapid evolution capable of altering community assembly?

Species interactions impact evolution

There is a substantial body of literature on how pair-wise interactions can drive evolutionary change in the species involved. This work includes research on character displacement between competitors (Pfennig and Murphy 2000), arms races between predator and prey (Brodie et al. 2002), and arms races between parasite and host (Lively and Dybdahl 2000). Recently researchers have become interested in how patterns of evolution are shaped by molecular details, and how pair-wise co-evolution plays out over geographically separated populations.

There is debate about the extent to which evolution is driven by independent pair-wise interactions versus more complex community interactions (Haloin and Strauss 2008). Community context might alter pair-wise evolution by either changing the genetic variation for a trait (such as through selection on a correlated trait), or by changing the strength of selection on a trait. The extent to which evolution is diffuse (i.e. driven by interactions with multiple species) has been tested in two ways. First, several researchers have tested for the presence of diffuse selection on traits (Haloin and Strauss 2008). Diffuse selection has almost always been observed when tested, though such tests have largely been restricted to plant-herbivore interactions. Second, diffuse evolution has been investigated indirectly by looking at traits in the presence and absence of a third species. For example, it has been shown that the match between pinecone and crossbill beak morphology is broken in populations with red squirrels (Siepielski and Benkman 2004).

It remains unclear how many species interactions must be taken into account to understand trait evolution. What factors influence whether a species will increase its fitness through independent evolution or through multi-species cooperation? When will diffuse selection alter the evolution of pair-wise interactions? Can evolution overcome tradeoffs, and thereby influence the amount of diffuse selection on traits?

Microbial model systems

Experimental evolution with simple microbial communities may be a powerful tool for gaining insight into general properties of the interaction between ecology and evolution. Microbial systems are ideal for studies of evolution and ecology (Elena and

Lenski 2003; Jessup et al. 2004). They enable studies that incorporate incredible spatial and temporal scales. This is critical for understanding how local interactions between individuals impact regional patterns, as well as for understanding how interactions will change through evolutionary time. Additionally, they provide extensive environmental control, thereby facilitating precise tests of biological mechanisms. Microbial systems also provide the ability of replication thus increasing the power of tests. Finally, microbes can be compared to frozen ancestors thus enabling post-hoc analysis. Though there are clearly some differences between microbes and macrobes (most notably sexual reproduction), microbes have provided many key insights into both ecology and evolution. For example, Gause (1934) used microbes to establish the tenet of competitive exclusion, and Avery et al. used microbes to establish DNA as the basic unit of evolutionary heredity.

Additionally, experiments with microbial systems are useful as they provide insight into microbial communities. Microbes govern many important processes from global nutrient cycles (Schmidt et al. 2007) to human health (Dethlefsen et al. 2006). These communities may be particularly prone to feedback between evolution and species interactions as microbes are capable of rapid adaptation and have the potential for extensive interactions. A synthesis of community ecology and evolution may profoundly improve our understanding of microbial communities. Below I discuss the current state of knowledge about the interplay between species interactions and evolution in microbes.

Evolution affects microbial species interactions.

Microbes are involved in many species interactions that may be shaped by evolution. Bacterial populations are often regulated by predation. For instance, 15% of cyanobacteria are killed by phage every day (Suttle and Chan 1994). Experiments have demonstrated that bacteria can readily evolve resistance thereby decreasing the effect of predators (Bohannan 2000). Additionally, competition is likely important to microbes as suggested by the prevalence of allelopathic compounds (Riley and Wertz 2002). Indeed almost every bacterial species studied produces some toxin to kill competitors. The effect of competition may be altered by evolution of toxin production or resistance, as well as by evolutionary divergence of resource use. Finally, bacteria partake in a multitude of social behaviors (West 2007). Bacteria interact with others through the secretion of a wide range of extracellular compounds from signaling molecules to degradative enzymes. These secretions are important for microbial function, as suggested by the fact that intercellular signaling controls 6-10% of all *Pseudomonas aeruginosa* genes (Schuster et al. 2003). Such interactions, mediated by public goods, are notoriously sensitive to destabilization by the evolution of cheaters.

Additionally, evolution has the potential to alter community properties. The catabolic properties of many microbial communities are governed by interactions between multiple species. For instance, the anaerobic degradation of cellulose involves a network of four interacting microbes (Schink 1997). Evolutionary changes in a rate of nutrient flux may dramatically alter community function. Understanding how to improve community function will be particularly useful for industrial applications (Brenner et al. 2008; Wall 2008). Intriguingly, there is suggestive data that microbial community

function may be improved by selection at the level of the community (Swenson et al. 2000a; Swenson et al. 2000b; Williams and Lenton 2007).

There are many interesting questions about the effect of evolution on microbial species interactions. Why is phage resistance not global? How does interspecific cooperation between microbes arise? What is the best way to select for improved community function?

Species interactions affect microbial evolution

Species interactions likely influence the evolution of many traits of interest in microbes; however, studies of microbial evolution have been dominated by the tradition of using monocultures to investigate molecular mechanisms. The few studies that test the impact of ecological factors have included only limited interactions. Several researchers have investigated how intra-specific competition drives niche differentiation (Rainey and Travisano 1998; Dykhuizen and Dean 2004; Blount et al. 2008) and bacteriocin prevalence(Kerr et al. 2002). Similarly, there is increasing interest on cooperation in microbes. Behaviors from swarming (Strassmann et al. 2000; Velicer and Yu 2003) to iron scavenging (West and Buckling 2003) are being investigated, though again largely in single species. The best interspecific work has investigated predator-prey dynamics. A large body of literature exists about the evolution of phage-bacteria systems (Bohannan 2000). Surprisingly, however, there have been almost no studies on how these pair-wise interactions are affected by additional species (Brockhurst et al. 2006). Several

interesting studies of multi-species interactions have emerged recently. Hansen et al. (Hansen et al. 2007) demonstrated that selection of a two-species community in a biofilm lead to the evolution of an exploitative interaction. Goldman and others have demonstrated that evolution of a four species community alters population densities (personal communication).

Clearly a plethora of questions remain about how microbial evolution is influenced by species interactions: How does metabolic cooperation shape the evolution of metabolism? How prevalent is diffuse selection in microbial communities? How much of the complexity of microbial communities must be considered to understand evolution of traits of interest?

Conclusion

My dissertation describes three studies that utilize model microbial communities to investigate the interplay between species interactions and evolution. The dissertation opens with a chapter investigating the impact of competition on the evolution of phage resistance. This work suggests that community context can dramatically alter the evolution of resistance to phage. Next I tested the impact of evolution on assembly of a three species community. I demonstrated that evolution can influence the content of a microbial community by altering the process of community assembly. Finally, I investigated the evolutionary origin and maintenance of cross-feeding mutualisms. This work suggests that species interactions can enable novel evolutionary pathways, and that evolution can significantly increase the productivity of cross-feeding communities.

The chapters of my dissertation illustrate cases in which considering greater biological complexity provided key insight into process of interest. They also elucidate mechanisms through which microbial communities are altered by the interplay between evolution and species interactions. Continued work linking ecological and evolutionary forces is likely to improve our understanding of the complexity of the biological world.

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Chapter 2

Phage impact in two-species bacterial communities

Abstract

A long history of experimental work has shown that addition of bacteriophages to a monoculture of bacteria leads to an only temporary depression of bacterial levels. Resistant bacteria usually ascend to abundance, even if they have reduced growth rates relative to phage-sensitive bacteria. This rise in bacterial counts occurs even if the phages evolve to overcome bacterial resistance. We consider that the generality of this result may be limited to monocultures, in which the resistant bacteria do not face competition from bacterial species unaffected by the phage. As a simple case, we investigated the impact of phages attacking one species in a two-species community of bacteria. In the absence of phages, *Escherichia coli B* and *Salmonella typhimurium* were stably maintained during daily serial passage in glucose minimal media (M9). When either of two *E. coli*-specific phages (T7 or T5) was added to the mixed culture, *E. coli* went extinct or was maintained at densities orders of magnitude lower than before phage introduction. This depression in numbers occurred even though *E. coli* with phage achieved high levels in monoculture controls. In contrast, the addition of a phage that attacked only *Salmonella* (SP6) led to only transient decreases in bacterial frequency. These results suggest that phages can sometimes, though not always, provide long-term suppression of target bacteria.

Introduction

Bacteriophage are predators of bacteria. Soon after the discovery of phage in the 1910s scientists attempted to use them as agents to cure bacterial infection – phage therapy (d'Herelle 1926, Eaton and Bayne-Jones 1934). By the 1940s, phage therapy was considered a failure in the West and was abandoned in favor of antibiotics, although some Eastern European countries nurtured the technology and kept it to the present (Summers 2001, Merril et al. 2003).

Western interest in phage therapy is undergoing a revival (Summers 2001, Merril et al. 2003, Levin and Bull 2004). The greatest interest lies in using phage to treat infection, a technique that may face considerable economic hurdles because of the enormous cost of clinical trials coupled with the typically narrow host range of most phages. It has also been proposed that phage might be applied environmentally to depress bacterial abundance before they cause infection (Huff et al. 2002, Nakai and Park 2002). The advantage of this approach is that there should be fewer regulatory concerns and perhaps none of the medical complications associated with administration of phage to a person; both of these advantages should translate into a greatly reduced cost of implementation (Goodridge and Abedon 2003).

A critical obstacle for any form of phage therapy is the evolution of bacterial resistance to the phage. When large continuous cultures of bacteria have been treated with phages, resistance invariably evolves and bacteria return to nearly their former

numbers (Chao et al. 1977, Levin and Lenski 1985). This outcome has been obtained (i) despite a demonstrable fitness cost to phage resistance in many bacteria (Lenski and Levin 1985, Lenski 1988), and (ii) even when multiple rounds of phage evolution allow the phage to enter an evolutionary arms race with bacteria (Buckling and Rainey 2002, Mizoguchi et al. 2003).

These experiments throw doubt on the idea that release of phages will suppress levels of target bacteria. Yet the experiments share one major limitation: they have all been conducted with single species of bacteria (usually *E. coli*) growing essentially in monoculture (Lenski and Levin 1985, Lenski 1988, Buckling and Rainey 2002, Mizoguchi et al. 2003). Outside of the laboratory, bacteria rarely exist in monocultures, but rather typically exist in complex microbial communities. We hypothesize that the ascent of phage-resistant bacteria could be profoundly affected by competition from other microbial species in ways that cannot be anticipated from monocultures.

There is some support for this idea from studies of cyanobacteria in natural environments. Several studies found that cyanobacterial densities can be significantly reduced by phage (Proctor and Fuhrman 1990, Suttle and Chan 1994, Hennes *et al* 1995). However this result is somewhat contentious as one study found that cyanobacteria are largely resistant and therefore unaffected by phage (Waterbury and Valois 1993).

In the study presented here we make a well-controlled attempt to address the effect of interspecies competition on bacteriophage control of bacterial population. A pair of bacterial species was maintained during serial transfer in minimal medium. Phages

specific to one host were introduced to observe the impact on the target species given interspecific bacterial competition.

Methods

Bacterial strains and bacteriophages

The bacteria used were *Escherichia coli B* (designated *E. coli*) and *Salmonella enterica* serovar *Typhimurium* ATCC 14028S (*Salmonella*). The two lytic phages T7 and T5 were used as *E. coli*-specific phages. T7 binds to the heptose residues in the LPS (Goldberg et al. 1994), while T5 binds to the outer membrane transporter protein FhuA (Hantke and Braun 1978). Competition assays suggest that resistance to T7 comes at a high cost to competitive ability while resistance to T5 has little or no cost (Lenski and Levin 1985). Phage SP6 was used to attack *Salmonella*. SP6 is a lytic phage in the same family as phage T7 but does not attack *E. coli*. The primary binding site of SP6 is the O-antigen (Scholl et al. 2004). Little is known about the cost of resistance to SP6.

Design

Flasks were set up with one of the following combinations: (i) a monoculture of one bacterium, (ii) a community of *E. coli* and *Salmonella*, (iii) a monoculture of one bacterium plus a phage specific to it, or (iv) a community of *E. coli* and *Salmonella* plus one phage.

Bacteria were grown in 125 ml flasks containing 10 mL of M9 minimal medium with 0.2% glucose (Miller 1972). Flasks were inoculated with approximately 10^6 *E. coli*

and/or *Salmonella* from stocks grown overnight in minimal media. The cultures were grown with aeration in a shaking incubator at 37° C, 175 rpm for 24 hours. After 24 hours, 1 μl was transferred to a new flask that contained 10 ml of fresh media. For trials in which *E. coli* was being challenged with phage, 10^3 pfu of T7 or T5 were added to the day-1 flask after 11 hours of bacterial growth. The delay in phage addition was necessary to keep *E. coli* from going extinct. For trials in which *Salmonella* was being challenged, 10^6 pfu of SP6 were added to the first flask at the same time as the bacteria (no delay was necessary). All trials lasted 5 days (4 transfers) or until an extinction occurred.

When T7 was used to challenge *E. coli* in the presence of *Salmonella*, *E. coli* density dropped profoundly. This drop made it difficult to maintain T7 at sufficient densities to transfer into the next flask. In one trial, 100 μl of the previous culture was passaged between flasks to overcome this obstacle (instead of the usual 1μl). This increase in transfer volume did not cause any noticeable change in the population dynamics as compared to the other trials. In a second trial, 10^6 T7 were added every day to the flasks containing *E. coli* and phage (1μl passages). Again this caused no noticeable change in the population dynamics as compared to other trials.

Bacterial density was measured at the end of each 24hr period by plating on X-gal LB plates (40 μl of 20 mg/ml X-gal/plate, LB broth by weight: 1% NaCl, 1% tryptone, 0.5% yeast extract). *E. coli* colonies turn blue in the presence of X-gal, as the *lacZ*-encoded beta-galactosidase of *E. coli* hydrolyzes X-gal. *Salmonella* colonies remain white as they lack the enzyme. Additionally, 10^6 pfu of a phage specific to one

bacterium were spread on plates to kill one of the bacterial species. Phage densities in the experimental flasks were determined by plating medium from a flask on a lawn of sensitive cells. For both bacteria and phage, the lower limit of detection was 10^3/ml; below this level an organism was unlikely to be transferred to the next flask. The densities reported represent the final density reached by the bacteria or phage each day after 24 hours of growth.

Bacterial-resistance assays

Streak tests were used to test for phage resistance. Approximately 10^7 phage were spread in a line across the center of an LB plate and dried. A bacterial colony was then touched with a sterile loop and streaked orthogonally across the phage deposit. A control of sensitive bacteria was also streaked on each plate. Colonies were scored as sensitive if the line of cells stopped at the line of phage, or scored as resistant if the line of cells showed no change as it crossed the line of phage.

Adsorption tests were used to assay for partial phage resistance in some bacteria that tested sensitive in streak tests. Cells from a single colony were grown in a 10 ml culture under the same conditions as in experimental trials. The density of cells was monitored with a Klett-Summerson photoelectric colorimeter. Once a flask reached a turbidity value of 60 (~10^7 cells/ml), a sample was plated to determine the cell density (C) and 10^6 phage were added to the flask. Five minutes after the phage were added, a sample was mixed in top agar with sensitive cells and plated to measure the total number

of phage (N_0). A 1 ml sample was also centrifuged for 1 minute to pellet bacteria and adsorbed phage; the supernatant was plated on a lawn of bacteria to determine the number of unadsorbed phage (N_U). The adsorption rate (k) was estimated from the equation $k = -\ln(N_U/N_0) / Ct$ where t = 5 minutes. Adsorption assays were run for one isolate of *E. coli* from each of the three trials in which *E. coli* was maintained in community with T7, as well as for two isolates of the original *E. coli* stock.

Resistant-cell competition assay

To determine the cost of resistance to T7 and T5, resistant *E. coli* were competed against *Salmonella* in the absence of phage. The resistant *E. coli* were obtained as colony isolates from monocultures grown in the presence of phage. Colonies were screened to ensure that they remained resistant and were free of phage. Competition trials were initiated with 10^4 of each bacterium and then followed the same passaging protocol as above.

Analysis

Standard t-tests were used to compare cell density in different treatments. Analyses treating days as independent data points were compared to analyses treating days as non-independent data points. Under the assumption of independence, daily values were used for analysis, while under the assumption of non-independence 5-day means were used. The two methods agreed qualitatively (though not quantitatively) about significance in all but one case. Both p-values are reported; day-independent p-values appear first.

Results

Without phage

In monoculture the bacteria attained a density of 10^8 - 10^9 bacteria/ml within the first day and then returned to this density again each day for the duration of the trial (fig. 2.1 A, B). *E. coli* reached a grand mean $\log_{10}$ density of 9.12 (± 0.04) bacteria/ml over 6 trials (table 2.1). *Salmonella* reached a grand mean log density of 8.81 (± 0.07) bacteria/ml over 3 trials (table 2.1).

In communities of both bacteria and no phage, bacterial densities followed roughly the same pattern as in monocultures (fig. 2.1 A, B). Within the first day each bacterial species reached log densities of 8-9 and then rose to this density every day thereafter. *E. coli* had a grand mean log density of 8.45 (± 0.06) bacteria/ml over 5 trials (table 2.1). *Salmonella* had a grand mean log density of 9.00 (± 0.08) bacteria/ml over 4 trials (table 1). In community, therefore, *E. coli* attained approximately 1/5 the mean density it attained in monoculture, a significant reduction ($p < 0.001$, $p<0.001$). *Salmonella* density, in contrast, was slightly higher in community than in monoculture; the increase is marginally significant ($p = 0.04$) if days are treated as independent and insignificant ($p = 0.18$) if days are treated as non-independent.

In view of the result that the density of *Salmonella* was not reduced in the presence of high *E. coli* densities, we tested whether either bacterium was feeding on a metabolite of the other species. Monocultures of each bacterium were maintained at saturation for one day in the original medium. The spent medium was filtered and

inoculated with the same bacterium or the other bacterium (filters were rinsed in sterile water first to remove soluble chemicals that might have affected growth). No substantial difference was observed between growth rates when the bacteria were inoculated into their own spent media or spent media of the other species (data not shown). The same outcome applied in media from bacteria maintained at saturation for three days before filtering.

Additionally we tested the ability of each bacterium to invade a population of the other species. Two-species flasks were set up in which one bacterium was started at a density 4 orders of magnitude lower than its competitor. When initially rare, *Salmonella* rose to a mean log density of 8.03 for 5 days (1 trial). When *E. coli* was initially rare, in one trial it attained a mean log density of 7.03 for 5 days. However, in two later trials, *E. coli* remained at a density below 10^5 cells/ml and was driven to extinction on the fifth day. These data suggest that Salmonella is able to invade a population even when rare, but that *E. coli* is sometimes unable to enter a population if put at a numerical disadvantage.

E. coli challenged with T7

In monoculture, phage T7 had little long term effect and *E. coli* rose to high densities (fig. 2.2 A). *E. coli* reached a grand mean log density of 8.19 (± 0.20) bacteria/ml every day over 5 trials (table 1), less than 1 order of magnitude below the monoculture density in the absence of phage, though the difference is highly significant ($p < 0.001$, $p=0.007$). Ten colonies from day 2 in each of two trials all tested as T7

resistant. However, some sensitive cells must have been present as T7 was maintained for 5 days at a grand mean log density of 8.03 (± 0.25).

In contrast, when competing with *Salmonella*, *E. coli* was not able to reach high density when challenged with T7. *E. coli* went extinct in 3 of 6 trials, twice on the first day and once on the second day. In one trial T7 went extinct on day 2, but even in the subsequent absence of the phage, *E. coli* did not reach a log density higher than 5.0 in the remaining 3 days. In two trials *E. coli* and phage were maintained for 5 days by passaging 100 μl daily or adding phage daily (as per Methods). In these two trials *E. coli* was maintained at low levels with a grand mean log density of only 4.37 (±0.26) (table 2.1, fig. 2.2 B). In the trial in which 100 μl was passaged, T7 was lost on the sixth day but *E. coli* levels stayed low until the trial was ended on day 7. Cells remained sensitive to T7 for the 3 trials in which *E. coli* was maintained (10 of 10 colonies from day 2 of each trial tested sensitive by streak test). Adsorption tests on an isolate from each of these three trials failed to detect any reduction that could serve as an alternative to outright resistance.

T7-resistant *E. coli* showed a competitive cost initially, however this cost was compensated with time. When resistant *E. coli* obtained after 8 hours exposure to T7 was then competed against *Salmonella* in the absence of phage, *E. coli* was driven to extinction after three days. In a trial with *E. coli* exposed to T7 for 24 hours, *E. coli* survived for 5 days but never got above a density of 10^5 cells/ml. *E. coli* that had grown with T7 for 5 days, rose to a density of 10^7 within the first day.

E. coli challenged with T5

In monoculture, T5 had little effect on *E. coli* densities, as was seen for T7 (fig. 2.3A). *E. coli* with T5 reached a grand mean log density of 8.36 (± 0.24), less than 1 order of magnitude below the density of *E. coli* in the absence of phage, though again the difference is significant ($p = 0.003$, $p < 0.001$). In a resistance test 90% (27/30) of the *E. coli* colonies were resistant to T5 on day 3. T5 densities peaked on day 2 and then went extinct on day 4 in all trials.

In another parallel to the T7 results, T5 had a strong impact on *E. coli* densities within the *E. coli-Salmonella* community. In three trials, *E. coli* was driven to extinction, once on day 2, once on day 4 and once on day 5. In these trials T5 was maintained as long as *E. coli* was present. In three other trials, *E. coli* was maintained at low levels with a grand mean log density of 6.34 (± 1.56). This density is significantly lower ($p = 0.008$) than the density of *E. coli* in community without phage (fig. 2.3B). T5 was lost once on day 4, and twice on day 5. In all of these cases *E. coli* density remained low even in the absence of phage. Surprisingly, cells all tested as sensitive to T5 (10 of 10 colonies from day 3 of each trial).

To ensure that our community results were not an artifact of T5 resistance never arising in the population we ran two further trials. In these trials, *E. coli* was allowed to evolve resistance to T5 for three hours before *Salmonella* was added. *E. coli* was maintained for five days at a grand mean log density of 5.88 (± 0.83), not significantly different from the other community trials ($p = 0.23$). Half of colonies on day three tested as resistant.

E. coli showed no competitive cost to T5 resistance in the absence of phage. In a trial initiated with *E. coli* that had grown with phage for 8 hours, *E. coli* rose to 10^7 within a day and was maintained for 5 days. At the end of the 5 days the *E. coli* still tested as resistant to T5.

Salmonella challenged with SP6

In monoculture *Salmonella* reached high densities whether SP6 was present or absent (fig. 2.4A). In the presence of the phage, *Salmonella* had a grand mean log density of 8.58 (± 0.13) over 3 trials (table 2.1), not significantly different from the mean log density of *Salmonella* alone ($p = 0.07$, $p = 0.18$). In resistance tests from day 3 of the three trials, all 30 colonies tested as resistant. However, some sensitive cells must have been present as SP6 was maintained in all trials at a mean log density of 6.56 (± 0.45).

Salmonella reached high density in the presence of SP6 even in competition with *E. coli* (fig. 2.4B). In competition SP6 reduced the density of *Salmonella* for two days, but by the third, *Salmonella* was back up to densities equivalent to those achieved in the absence of phage. *Salmonella* had a grand mean log density of 8.14 (± 0.19) over 3 trials, significantly lower than *Salmonella* in the absence of phage ($p < 0.001$, $p = 0.01$), but a reduction in density of less than 1 order of magnitude. In resistance tests from day 3 of the three trials, all 30 colonies tested as resistant. However, some sensitive cells must have been present as SP6 was maintained in two trials at a grand mean log density of 8.03 (± 0.27). In one trial, phage were lost on the third day and added back on the fourth. The

fifth day, phage reached a log density of 6.49, and there was no obvious change in the pattern of *Salmonella* density.

Discussion

On the basis of previous work there is justifiable doubt on the usefulness of phages as control agents of bacteria in the environment. However, the previous experimental work was done in monoculture conditions, thereby excluding interactions with other bacterial species unaffected by the phage (Lenski and Levin 1985, Lenski 1988, Buckling and Rainey 2002, Mizoguchi et al. 2003). Competition between bacterial species may impact the effectiveness of phage therapy because phage resistance can decrease the competitive ability of bacteria (Lenski and Levin 1985, Lenski 1988). Our results indicate that the presence of a second bacterium drastically reduces the density of the focal bacteria in some cases, while in other cases the presence of a second bacterium has little effect. Below we compare our results to those obtained in monoculture and then discuss an explanation for the patterns that we observed.

Previous work suggests that *E. coli* resistance to T7 imparts a large cost in monoculture. In the presence of phage, some sensitive cells are maintained, suggesting a tradeoff between resistance and competitive ability (Chao et al. 1977, Lenski and Levin 1985). Furthermore, in the absence of phage, resistant cells are quickly driven out of the population by the sensitive cells (Lenski and Levin 1985).

If the cost of resistance is manifested the same way in environments with competitors, then *E. coli* levels should be suppressed, because sensitive *E. coli* are killed

by T7 when abundant and resistant *E. coli* are at a competitive disadvantage with other bacteria. Our results agree with these predictions and prior observations. In monoculture both *E. coli* and phage were maintained, and resistant *E. coli* were abundant. In community *E. coli* was either driven to extinction or kept at low density by the phage; resistant cells were never observed. In the two trials in which phage were lost, *E. coli* levels remained low presumably because of *E. coli*'s difficulty in increasing when greatly outnumbered by *Salmonella*.

The suppression of *E. coli* in community with T7 was not solely due to frequency effects however, as our competition assay demonstrates that resistance to T7 decreases *E. coli*'s ability to compete with *Salmonella*. In time, *E. coli* apparently underwent compensatory evolution that decreased the cost of resistance to T7. Evidently, when faced with immediate competition from *Salmonella*, resistant *E. coli* were out competed before they had the chance to evolve compensation. In view of these results, *E. coli* may have fared somewhat better in population sizes larger than ours, where compensatory evolution would have occurred more quickly.

Resistance to phage T5 in *E. coli* is thought to have no fitness cost, since T5-resistant *E. coli* are stably maintained with sensitive *E. coli* in the absence of T5. Lenski and Levin (1985) demonstrated in monoculture with T5 that resistant *E. coli* sweep to fixation and T5 is driven extinct. This lack of a cost of resistance to T5 leads to the prediction that in both monoculture and community, resistant *E. coli* should sweep to fixation and attain normal *E. coli* densities thereby driving T5 extinct.

Our results in monoculture follow these predictions, but those in community contradict them. In monoculture, resistance swept through the population by the third day, *E. coli* rose to high densities and T5 was driven to extinction. In community *E. coli* was driven to extinction or maintained at low levels and resistance was infrequent. This was a surprising result, particularly in light of the fact that resistance to T5 had no obvious cost in our system. The effect of T5 is likely due to *E. coli*'s inability to increase in a community when rare. *E. coli* numbers were initially reduced by T5, and then depending on the degree of initial reduction, *E. coli* was maintained at low levels or went extinct. Furthermore, at low density *E. coli* dynamics were apparently dominated by competition rather than phage predation, so T5 resistance had little benefit and hence was rare.

Little is known about the cost of *Salmonella* resistance to phage SP6. The fact that SP6 was maintained in monoculture suggests that resistance to SP6 comes at a cost in monoculture. This cost of resistance did not seem to affect the competitive ability of *Salmonella* in community, however, as *Salmonella* rose to high density even when SP6 and *E. coli* were present.

The community results from *E. coli* and *Salmonella* are contrasting, therefore: *E. coli* is controlled by phage (even a phage with no cost of resistance) while *Salmonella* is not. The mechanisms that cause this variation in effect are unclear. One explanation for the disparity is differences in community interactions. *E. coli* appears to experience competition from the community, as is evident from the reduction of *E. coli* densities in community. *Salmonella*, in contrast, experiences little competition and may even be

facilitated by the community. The difference in competition may make *E. coli* less robust in this community and hence more sensitive to insults. Under such a scenario the effect of phage would vary with the composition of each community. Alternatively, the variation in phage effect may be due to more intrinsic differences between *E. coli* and *Salmonella*. More work with bacterial communities will be necessary to understand the mechanisms of phage effect.

Our data provide a mixed message for the potential utility of phage therapy in environmental contexts. The presence of a bacterial community may significantly enhance the effectiveness of phage therapy, though it will not do so in all cases. This mixed message parallels the mixed results found in the few studies in natural systems, providing support for both camps of the cyanobacterial debate (Proctor and Fuhrman 1990, Hennes *et al.* 1995, Waterbury and Valois 1993). Further studies are needed to gain an understanding of how community competition influences bacteria/phage dynamics. For example, how does the composition and complexity of a community influence the evolution of phage resistance? Our results indicate that phage can significantly decrease long-term bacterial densities in bacterial communities.

Acknowledgements

We thank S. Abedon, H. Brussow, A. Dean, D. Dykhuizen, and members of the Dykhuizen lab for helpful comments and suggestions. The work was funded by a Houston Livestock Show and Rodeo Fellowship to WRH and NIH (GM57756 JJB).

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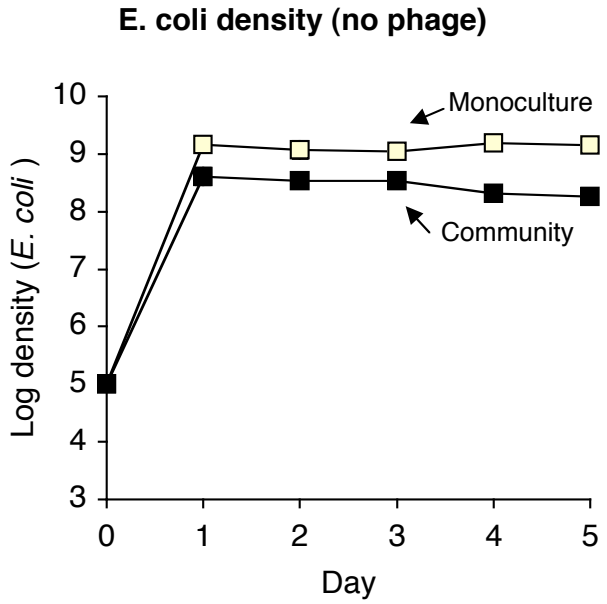
Table 2.1 - log cell densities

	Monoculture	Community
E. coli		
No Phage	9.12 (± 0.04) N = 6	8.45 (± 0.05) N = 5
with T7	8.19 (± 0.20) N = 5 *	Extinct N = 3 4.37 (± 0.26) N = 2 *
with T5	8.36 (± 0.24) N = 3 *	Extinct N = 3 6.34 (± 1.56) N = 3 *
Salmonella		
No phage	8.81 (± 0.07) N = 3	9.00 (± 0.08) N = 4
with SP6	8.58 (± 0.13) N = 3	8.14 (± 0.19) N = 3 *

Table 2.1 - Grand mean log densities of *E. coli* and *Salmonella*. The community column lists the density of the focal bacteria (the bacteria attacked by the phage). Asterisks denote a significant reduction from the corresponding no phage control from the same column. Standard errors are in parentheses.

Figure 2.1 – Bacterial mean density by day in the absence of phage. Data are the log densities reached after 24 hours growth. (A) *E. coli* density in monoculture (open squares, N=6) and in two-species culture (filled squares, N=5). (B) *Salmonella* density in monoculture (open squares, N=3) and two-species culture (filled squares, N=4). Error bars represent standard errors.

A



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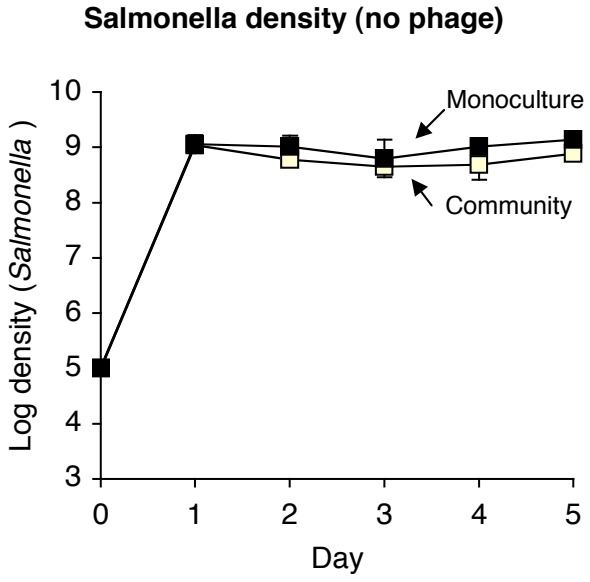
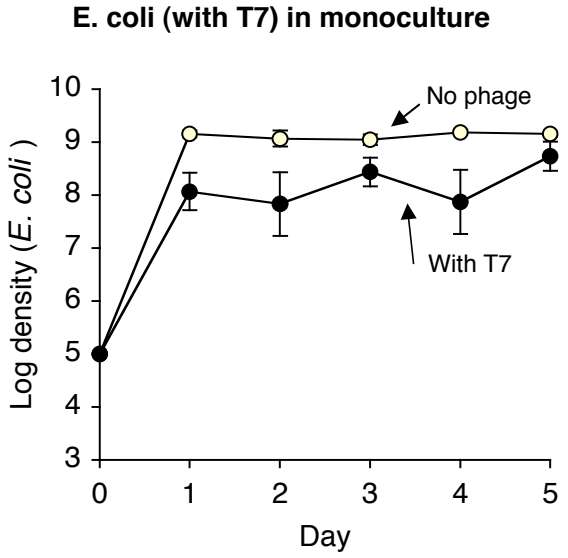


Figure 2.2 – *E. coli* mean densities when cultured with phage T7. Data are the log densities reached after 24 hours growth. (A) *E. coli* density in monoculture with T7 (filled circles, N=5). (B) *E. coli* density in two-species culture with T7 (filled circles). E (T7) in two-species culture is the mean of the two trials in which *E. coli* and T7 were maintained. Each figure also shows bacterial densities in controls in which phage were absent (open circles, from fig. 1). Error bars represent standard errors.

A



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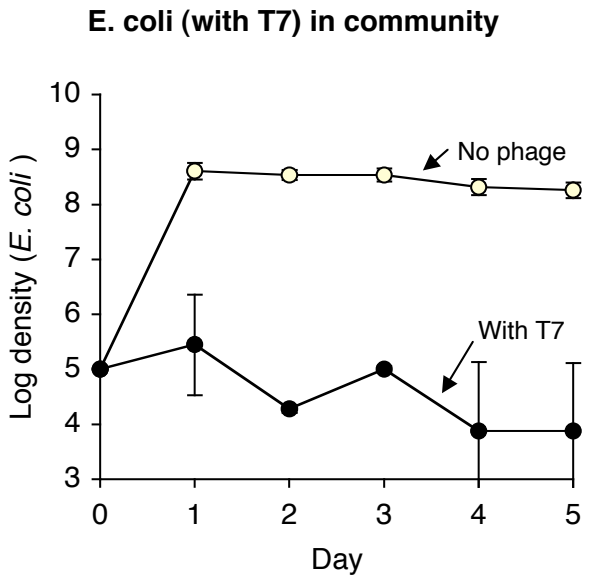
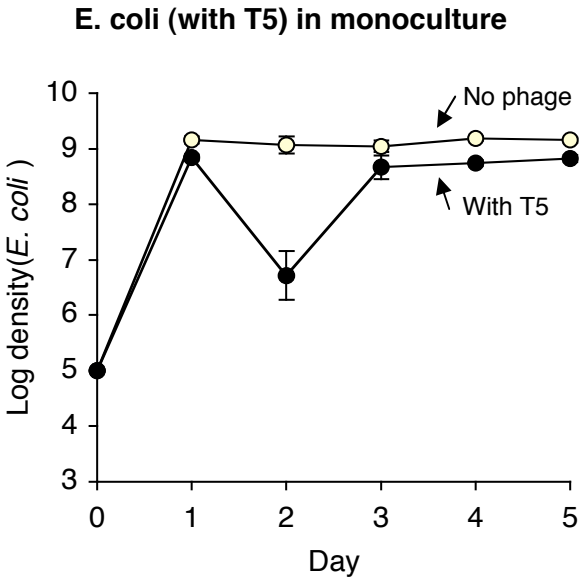


Figure 2.3 – *E. coli* mean densities when cultured with phage T5. Data are the log densities reached after 24 hours growth. (A) *E. coli* density in monoculture with T5 (N=3). (B) *E. coli* density in two-species culture with T5. E (T5) in two-species culture is from the three trials in which *E. coli* was maintained for 5 days. Each figure also shows bacterial densities in controls in which phage were absent (open circles, from fig. 1). Error bars represent standard error.

A



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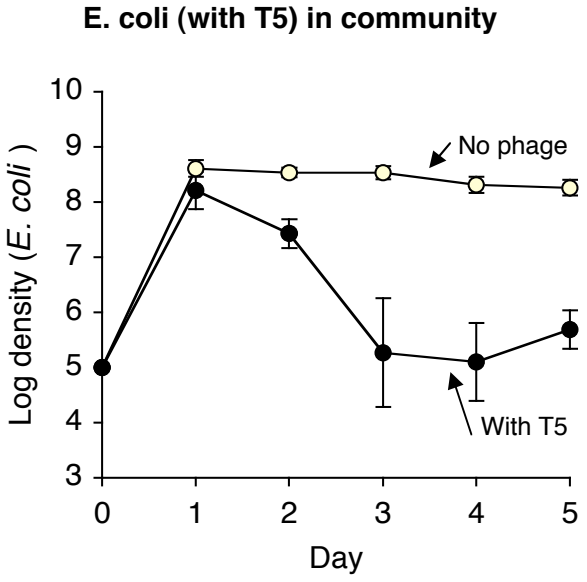
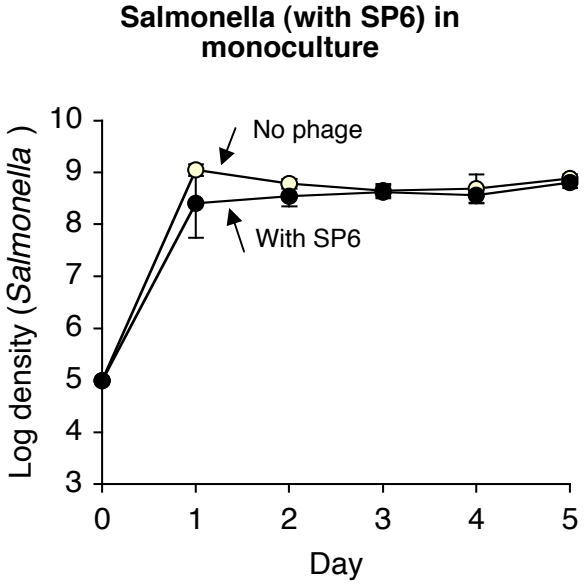


Figure 2.4 – *Salmonella* mean densities when cultured with phage SP6. Data are the log densities reached after 24 hours growth. (A) *Salmonella* density in monoculture with SP6 (N=3). (B) *Salmonella* density in two-species culture with SP6 (N=3). Each figure also shows bacterial densities in controls in which phage were absent (open circles, from fig. 1). Error bars represent standard error.

A



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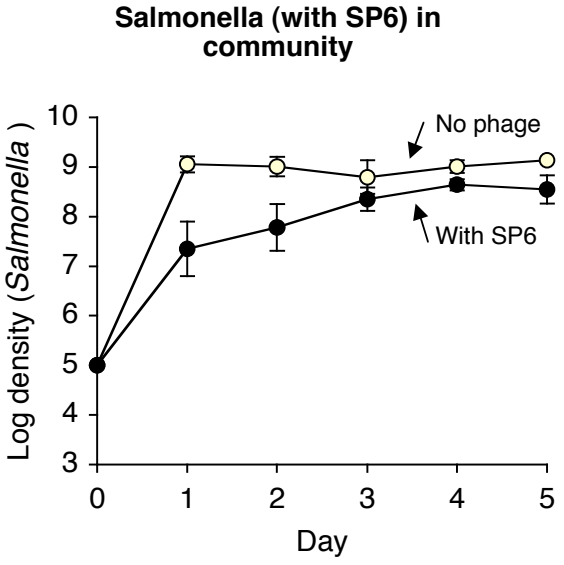

Chapter 3

Evolution alters community assembly in an experimental system

Abstract

Understanding the content of communities is a central goal of ecology. Traditionally, investigations of community assembly have included just ecological forces. However, recent data suggest that rapid evolution can alter species interactions. I test the impact of microevolution on the community assembly of three microbes: *Escherichia coli*, *Salmonella typhimurium* and the coli-specific bacteriophage T7. Initially, a three-species community can not be assembled, as *E. coli* is driven extinct by the combined forces of competition and predation. However, the three species can coexist if *E. coli* has adapted in the presence of T7 before *Salmonella* is added to the community. Evolution alters community assembly by enabling *E. coli* to overcome the cost of predator resistance. Evolution is directly observed and the impact of this evolution is demonstrated on assembly of a multi-species community. This study therefore, supports the view that microevolution can alter basic ecological processes.

Introduction

Though the interplay between ecology and evolution has been recognized since Darwin's description of the tangled bank (Darwin 1859; Holt 2005), many have suggested that explicit consideration of evolution is not necessary to explain ecological

patterns of interest (Holt 2005). This perception is changing, however, as there is increasing interest in regional patterns, and growing evidence that evolution can act over short timescales (Hairston *et al*. 2005; Holt 2005; Urban & Skelly 2006; Urban *et al*. 2008). For example, it is widely accepted that macroevolution shapes the patterns of species diversity and trait dispersion observed in island biogeography and community phylogenetics (Johnson & Stinchcombe 2007). Additional work has demonstrated that microevolution can alter ecological processes such as predator-prey cycles (Yoshida *et al*. 2003). Several recent reviews have argued that integrating evolution will substantiallyimprove our understanding of ecology even at the community level (Urban & Skelly 2006; Johnson & Stinchcombe 2007; Haloin & Strauss 2008; Urban *et al*. 2008). Yet despite this enthusiasm the spectrum of ecological processes that is likely to be shaped by short-term evolution remains unclear.

Community assembly is one area for which the impact of evolution is unknown. Community assembly, the process by which abiotic factors, species interactions and stochastic forces determine the content of a community, is central to an understanding of community ecology. The rules that drive this sorting process have traditionally been viewed as unchanging (Diamond 1975). Some have argued that the equilibrium community that develops can be altered by changing the order of species introductions (Chase 2003; Warren *et al*. 2003); however such changes are explained as arising from ecological priority effects rather than evolutionary change. If, however, species are able to adapt rapidly, the rules about how species can be assembled into communities may in fact be evolutionarily dynamic. Several lines of evidence support this notion. Work on

adaptive radiations suggests that species can evolve in just a few generations to fill niches they initially did not (Losos *et al*. 1998; Gillespie 2004; Fukami *et al*. 2007; Herrel *et al*. 2008). Additionally, it has been demonstrated that genetic differences among individuals of one species can alter other species' abilities to invade a community (Whitham *et al*. 2003; Crutsinger *et al*. 2006; Johnson *et al*. 2006; De Meester *et al*. 2007). Finally, there are many examples of evolution altering the interaction between species pairs (Lively & Dybdahl 2000; Pfennig & Murphy 2000; Brodie *et al*. 2002; Yoshida *et al*. 2003). These previous studies have either observed evolutionary change, or observed changes in community assembly. To date no study has monitored evolutionary change and demonstrated its impact on the assembly of a multi-species community.

Here I test whether evolution changes the community assembled from three interacting species: *Escherichia coli*, *Salmonella enterica,* and the bacteriophage T7. *E. coli* and *Salmonella* are ecologically similar bacteria that compete for resources. T7 acts as a predator on *E. coli* (though not *Salmonella*) and selects bacteria that evolve resistance. In a confirmation of previous work, *E. coli* is driven extinct if naïve strains of the three species are combined (Harcombe and Bull 2005). Phage-sensitive *E. coli* are killed by T7 and phage-resistant *E. coli* are competitively excluded by *Salmonella*. However, if *E. coli* is allowed to adapt to the presence of T7 before *Salmonella* is added, then the previously unattainable three-species community can be assembled.

Materials and Methods

Strains

The bacteria were the common laboratory strains *Escherichia coli* B and *Salmonella enterica ser. Typhimurium*. The phage used was wildtype T7, an *E. coli*-specific phage that binds to heptose residues in the lipopolysacharide (Goldberg 1994). All lines were grown in M9 minimal medium with 0.1 mM $CaCl_2$, 1 mM MgSO4, and 0.2 % glucose. Experiments were carried out in 10 mL of media in well-mixed flasks incubated at 37° C.

Design

Flasks were inoculated with *E. coli, Salmonella* and T7. Bacteria from freezer stocks were grown overnight and then added to flasks at a density of ~10^5 each. Bacteria were allowed to grow for 7 hours before 10^3 phage were added to ensure that *E. coli* population size was large enough for phage-resistant mutants to arise. The community in each flask was grown for 24 hours before a sample was transferred to a new flask (100 fold dilution). Serial transfers were halted at the end of 5 days. Densities of *E. coli*, *Salmonella*, and T7 were determined by selective plating at the end of each growth period (Harcombe & Bull 2005).

Replicate communities were started with T7, *Salmonella*, and either (i) naïve phage-sensitive *E. coli,* (ii) naïve phage-resistant *E. coli*, or (iii) evolved phage-resistant *E. coli*. Naïve phage-resistant *E. coli* were created by growing *E. coli* with T7 for 2 days (1 passage) so that the bacteria evolved resistance but did not have time to evolve compensation for the cost of this resistance. The evolved *E. coli* were created by allowing the bacteria to adapt to the presence of T7 for 14 transfers (~ 90 generations). Three

replicate communities were set up with each of the naïve *E. coli* types, and four replicate communities were set up for each of two lines of evolved *E. coli* (8 evolved communities).

Results

Naïve *E. coli*

Communities initiated with naïve phage-sensitive *E. coli* collapsed to monocultures of *Salmonella* within 2 days (Fig 3.1 A). *E. coli* fell below the limit of detection ($<10^3$) in the first day in all replicates. Subsequently, the absence of prey led T7 to drop out of the community. *Salmonella* reached a density of approximately 10^9 each day across all replicates. These findings agree with previous work (Harcombe & Bull 2005).

E. coli may have been driven extinct solely as a result of predation. To test this possibility three replicate communities were initiated with just *E. coli* and T7. In the absence of *Salmonella*, *E. coli* evolved resistance to T7 and attained a density above 10^7 in all trials (data not shown). Previous work demonstrated that *E. coli* can also survive if grown with just *Salmonella* (Harcombe & Bull 2005). These combined results demonstrate that the extinction of *E. coli* in the three-species community is a result of both predation and competition together.

Salmonella could have influenced *E. coli* extinction either by somehow limiting the acquisition of resistance mutations (e.g. keeping population size low) or by out-competing resistant mutants once they arose. To distinguish between these possibilities,

three replicate communities were initiated with phage-resistant *E. coli*. The three-species communities initiated with phage-resistant *E. coli* followed a pattern similar to the communities initiated with phage-sensitive *E. coli,* though resistant *E. coli* and T7 persisted a day longer (Fig 3.1 B). This result suggests that *E. coli* is driven extinct in three-species communities by the two mechanisms of T7 killing phage-sensitive *E. coli* and *Salmonella* out-competing phage-resistant *E. coli*.

Evolved *E. coli*

After phage-resistant *E. coli* were propagated with T7 for two weeks secondary evolution made it possible to assemble a three-species community (Fig 3.1 C, Fig 3.2 B). In the three-species community *E. coli* and *Salmonella* each reached a mean log density of 10^8-10^9 each day while T7 densities fluctuated around 10^6. The persistence of T7 was a result of either partial phage resistance or rare *E. coli* mutants that reverted back to phage-sensitivity. The survival of *E. coli* in the three-species community is consistent with *E. coli* evolving to compensate for the cost of phage resistance.

Trials were ended after 5 days (~30 generations) to study the effect of *E. coli* compensatory evolution without *Salmonella* evolution. The slight decrease in *E. coli* density on day five perhaps suggests that evolution could continue to alter the content of the community.

Discussion

There is debate over the extent to which evolution influences ecological patterns of interest. To investigate this topic I tested the impact of evolution on the community assembly of three microbes. Initially *E. coli* was driven to extinction in a community with a predator (T7) and a competitor (*Salmonella*). This occurred whether the naïve *E. coli* were initially sensitive or resistant to phage, indicating that the extinction was driven not by a lack of resistance mutations, but rather by the inferior competitive ability of resistant mutants. However, following adaptation in the presence of the predator, *E. coli* was able to coexist with T7 and *Salmonella*.

At a mechanistic level, the data are consistent with *E. coli* evolving to compensate for the cost of resistance to a phage. This result is unsurprising, as compensatory evolution for costs is well documented in microbes (Lenski 1988; Schrag *et al*. 1997), and in eukaryotes (Pischedda & Chippindale 2005). However, the impacts that compensatory evolution had on the community were less predictable. The compensatory evolution could have only effected intra-specific competition, and had no influence on *E. coli*'s inter-specific competition with *Salmonella*. Alternatively, the compensatory evolution could have been insufficient to change the outcome of competition between the two bacteria. Finally, adaptation to the presence of phage might have lead to the exclusion of T7. In these cases compensatory evolution would not have enabled the assembly of a novel three-species community.

The results bear on several questions in ecology. First there is debate over the existence of tradeoffs between resistance and competitive ability. Many studies find that resistance to a predator or parasite is accompanied by reduced competitive ability (Brodie & Brodie 1999; Yoshida *et al*. 2004), but many do not (Bergelson & Purrington 1996). These differences are usually attributed to static differences in the details of the systems; however the data here suggest that evolution can alter the cost of resistance. Similarly there has been substantial debate over how the joint effects of predation and competition shape communities (Chase *et al*. 2002). Again, observed outcomes are usually attributed to system-specific differences in the way that predation alters the ratio between intra-specific and inter-specific effects. My data demonstrate that the interplay between predation and competition can change not just across systems, but also across time.

The primary significance of this work is to show that experimental evolution can alter the assembly of a multi-species community (Fig 3.2). There are multiple mechanisms by which evolution might alter community content so it is perhaps surprising that a prior experimental demonstration of this principle is lacking. Indeed, multiple lines of evidence across disparate taxa suggest that assembly can be influenced by evolution. First, the genotype or genotypic variation of a population can alter community composition. For example, De Meester *et al*. (2007) demonstrated that the ability of zooplankton species to invade a community was influenced by the genotype of resident *Daphnia*. Additionally, work in community genetics has shown that plant genotype can determine the arthropod community that assembles on that plant (Whitham *et al*. 2003; Crutsinger *et al*. 2006; Johnson *et al*. 2006). Though these experiments included no

evolution, they strongly suggest that genetic differences arising from microevolution have the potential to alter community assembly. Furthermore, patterns of adaptive radiation in anoles (Losos *et al*. 1998) and spiders (Gillespie 2004) suggest that evolution can change which species fill available niches in a community. In an experiment with *Pseudomonas,* Fukami *et al*. (2007) showed that adaptation of a resident genotype altered the niches filled by an immigrating genotype. This work was done with genotypes of a single species; however it certainly supports the assertion that adaptive radiations can alter how communities are assembled. Finally, Loeuille and Leibold (2008) demonstrated that evolution and dispersal can interact to alter food web structure in simulated metacommunities.

Here, evolution altered the ecological assembly of a simple microbial system. The ability to change the stable equilibria of microbial communities has many applications for human health (Dethlefsen *et al*. 2006) and industry (Brenner *et al*. 2008; Wall 2008). Additionally, as noted above, several lines of evidence suggest that evolutionary processes can shape the content of eukaryotic communities. Evolution is likely to play a key role in shaping community content as climate changes (Parmesan & Yohe 2003) and more invasive species are introduced (Lau 2006). Continued work will be necessary to understand the variety of mechanisms, and contexts in which evolution influences the composition of communities.

Acknowledgements

I thank J. Pantel, M. Leibold, J. Bull, P. Harcombe, and G. Smith for comments on the manuscript. Support for supplies was provided by NIH GM 57756 to J. Bull.

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Figure 3.1 Community dynamics.
Average log density of *E. coli* (solid squares), *Salmonella* (open circles) and T7 (triangle on dashed line) at the end of each 24 hour growth. Error bars represent the standard deviation of the log densities. (A) Average of the three communities initiated with naïve phage-sensitive *E. coli*. (B) Average of the three communities initiated with naïve phage-resistant *E. coli*. (C) Average of the eight communities initiated with evolved *E. coli*.

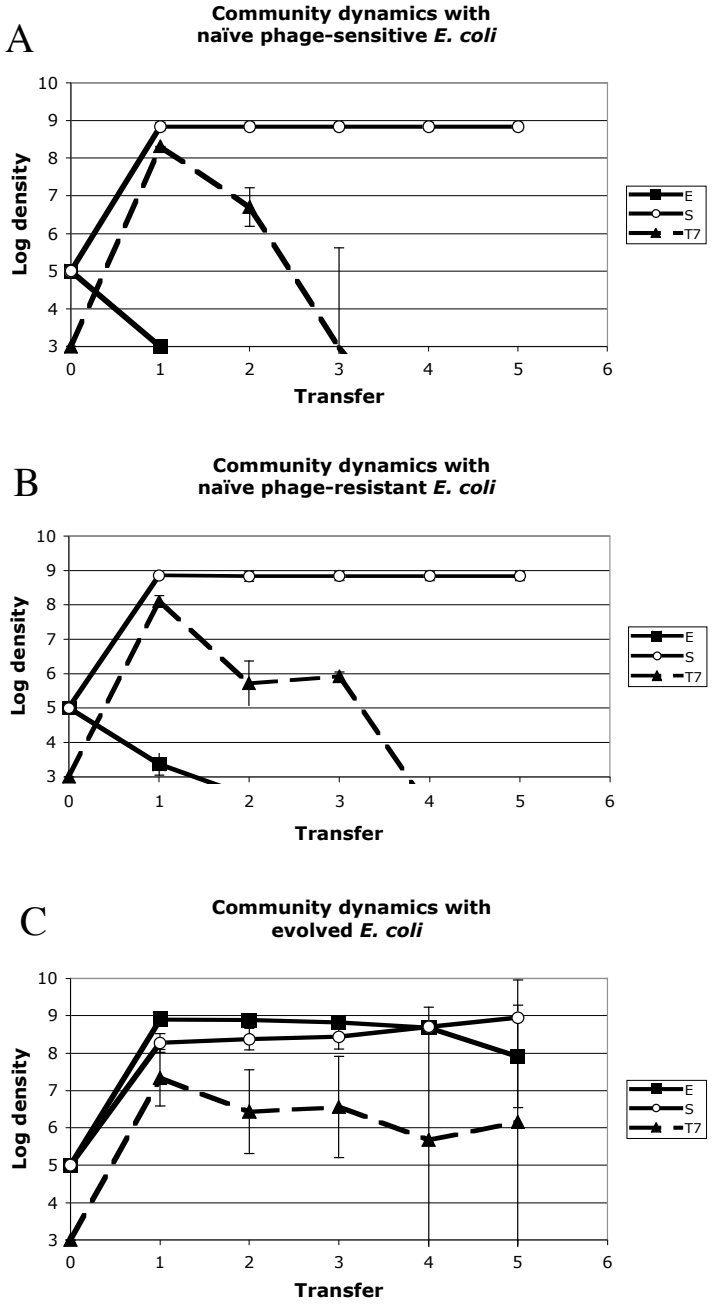
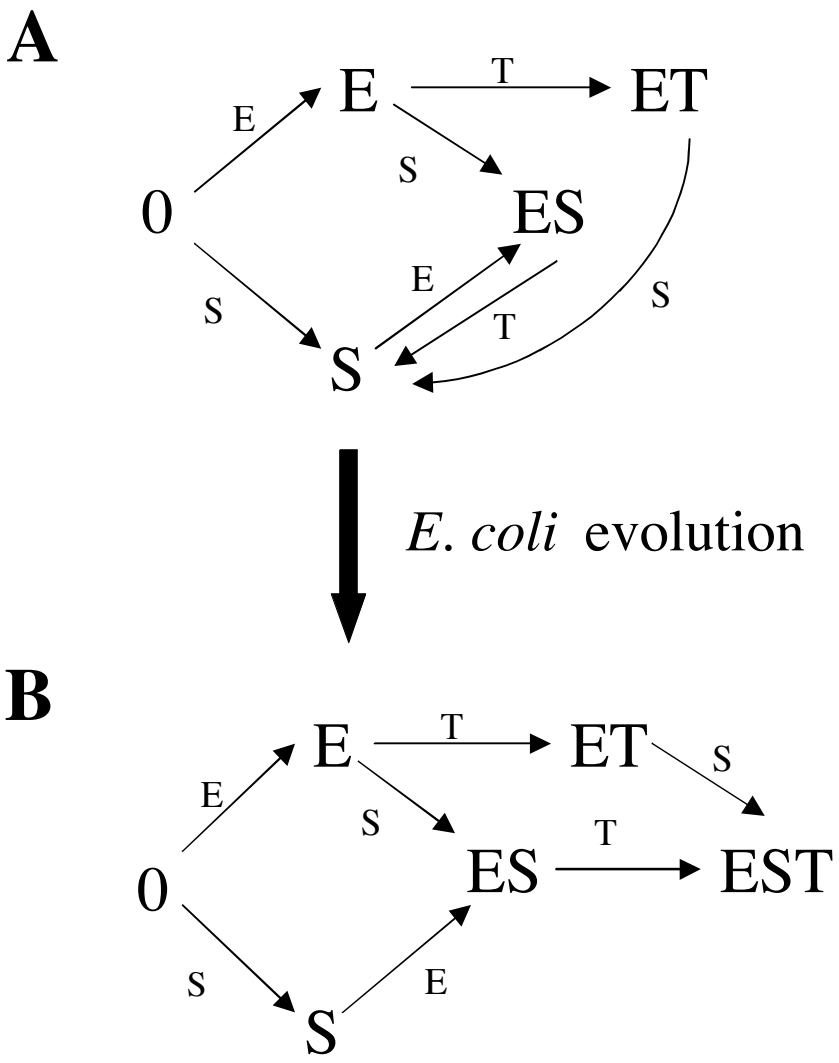


Figure 3.2 Assembly graphs
Estimations of the community assembly graphs for the three species *E. coli* (E), *Salmonella* (S), and phage T7 (T). (A) Community assembly with naive *E. coli*. (B) Community assembly with evolved *E. coli*. Small letters next to the arrows indicate the species addition that causes the observed change in state.



Chapter 4

Experimental evolution of novel cooperation between species

Abstract

Cooperation violates the view of "nature red in tooth and claw" that prevails in our understanding of evolution, yet examples of cooperation abound (Sachs et al. 2004; West et al. 2007a). Most work has focused on cooperation within a single species through mechanisms such as kin selection (Strassmann et al. 2000; Velicer and Yu 2003; West and Buckling 2003; MacLean and Gudelj 2006). The factors necessary for the evolutionary origin of aiding another species have not been experimentally tested. Here I demonstrate that cooperation between species can be evolved in the laboratory if i) there is preexisting reciprocation or feedback for cooperation, and ii) 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 provide the most complete demonstration of the factors necessary for the evolutionary

origin of interspecific cooperation, and represents experimental evolution of a multi-species solution to growth limitation.

Introduction

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 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). Cooperation likely depends on i) reciprocation between partners, and ii) 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 not just to another species, but also to specific cooperating individuals within that species? Finally, are reciprocation and direction of benefits necessary for the evolutionary origin of cooperation?

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. These benefits could provide the foundation for the evolution of cooperation. A user of 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 i.e., 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 (Chao and Levin 1981; West et al. 2001), so the effect of spatial structure is not clear.

I demonstrate that with reciprocation and direction of benefits one can experimentally evolve novel cooperation between species. I used a two species system involving *Salmonella enterica ser. Typhimurium* and an *Escherichia coli* mutant unable

to synthesize methionine (met- *E. coli*). A pre-existing mechanism that would allow for reciprocation was created by growing the two species in lactose. *E. coli* metabolizes lactose and then excretes costless metabolic byproducts on which *Salmonella* feeds. A method of directing benefits was provided by growing the community on agar plates. The result was the evolution of costly, cooperative methionine excretion by *Salmonella*. I show that in the absence of either requirement cooperation disappears.

Results

In lactose minimal media *Salmonella* feeds on the waste byproducts excreted by *E. coli* (likely acetate), while the *E. coli* strain used (met- *E. coli*) requires the amino acid methionine. At the start of the study, cultures of the bacteria were unable to grow together (fig 4.1, left) because there was insufficient methionine for *E. coli* and thus insufficient sugar byproducts for *Salmonella*. 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±.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 constitutive expression of the

methionine pathway (Lawrence 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. 4.1, middle) and HPLC.

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 3-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. 4.1; Methods). High excretion mutants arose twice in ten 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 wildtype *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 wildtype *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 wildtype *Samonella*. In liquid the wildtype swept from an initial frequency of 2% to near fixation in 1 transfer, a fitness coefficient (s) of roughly 0.45 for methionine production. This result is consistent with *Salmonella* experiencing a cost for methionine excretion. It should be noted that the methionine-excreting genotype may carry multiple mutations and hence the reduced fitness of this genotype may not be strictly due to 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. In contrast, *Salmonella*'s excretion is costly in the absence of other species. 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 inter-specific feedback. This definition of cooperation as a behavior that is selected because it helps a recipient follows West *et al*. 2007 (West et al. 2007b).

Cooperation is superior in a structured environment

The evolutionary fate of cooperative versus non-cooperative *Salmonella* was tested in a structured environment. *E. coli* and *Salmonella* were plated together on lactose

minimal plates at a density of 5 x 10^7 each. Initially the *Salmonella* population consisted of 99% wildtype and 1% cooperative methionine excreters. Over 4 transfers (approximately 20 generations), cooperative methionine excreters spread through the population to greater than 80% (fig. 4.2 A). Coincident with the increase in cooperators, the density of bacteria on the plates after 48 hours increased by more than 15 fold (fig 4.2 E). 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.

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% wildtype. Surprisingly, the wildtype increased to 30% in the first growth phase; however, it subsequently decreased in frequency (fig 4.2 B). On one plate wildtype decreased to 7% by transfer six and then the plate became contaminated. On two plates wildtype dropped below the level of detection (<3%) by the seventh transfer. When grown with 100% cooperators *E. coli* reaches a density of 5 x 10^9. The initial invasion of wildtype 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.

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 sugar 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 4.2 D), accompanied by a reduction in *E. coli* density (fig. 2 H). A qualitatively similar pattern was observed on glucose plates. This data supports 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 between species (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 .01% wildtype. Over 20 passages wildtype *Salmonella* spread to apparent fixation at the expense of cooperative

methionine excreters (fig 4.2 C). Over the course of the experiment *E. coli* densities decreased from 3.2 x 10^{8} to below the limit of detection (fig 4.2 G), as expected with the loss of cooperation in *Salmonella*. In communities with 100% cooperative *Salmonella*, *E. coli* densities reach 4 x 10^{8}. These result 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). Though theory exists for how such interaction 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 that interspecies cooperation can be evolved in the laboratory. It was also shown that this cooperation depends on both a pre-existing mechanism for reciprocity, and on the direction of benefits to cooperating partners. If either of these is removed cooperation is selected against.

This is a unique demonstration of experimental evolution of novel interspecies cooperation. 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. Each trial was initiated with 10^{7} *Salmonella* and carried out

for only one transfer (~10 generations). Furthermore, the mutations were of large enough effect to be observed in the assays. *Salmonella* cooperators were only isolated if they led to the formation of visible mixed species colonies. Other *Salmonella* may have increased methionine production, but not enough to cause visible colony growth. The two-step selection process may have facilitated the evolution of cooperation. It is likely that the chemical treatment selected for genotypes that aided the evolution of cooperation on plates, as cooperation never arose from wildtype *Salmonella*. This suggests that combining engineering and evolution may be a useful tool for acquiring bacteria with desired traits. However, the way that selection acted on the available genetic diversity was independent of the engineering step. Indeed, the principles underlying the observed evolution of interspecific cooperation apply to all systems.

It is 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. Such a solution would have been readily detected in the selection regime, but was never observed. A multi-species solution to enhancing growth was favored by the genetic details of this system; however, even with this bias enhancing growth through altering species interactions was only possible in specific conditions. This work 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). However, these

studies have not included evolutionary dynamics. Additionally, several studies have demonstrated that cooperation within a species can be maintained with spatial structure (Griffin et al. 2004; MacLean and Gudelj 2006). Velicer and Yu (Velicer and Yu 2003) illustrated the origin of novel cooperation within a species by evolving swarming in *Myxococcus xanthus*. Finally, several studies have demonstrated the evolution of reduced conflict between species. Several studies have shown that parasites can be selected to cooperate by reducing harm to their hosts (Bull et al. 1991). More similarly to this study, Sachs and Bull (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 it able to grow alone. This work demonstrates an intriguing alternative to the evolution of cooperation. My study builds on this previous research by evolving novel cooperation between species. Interspecific cooperation differs from intra-specific cooperation because cooperation between species is not based on processes involving shared genes, such as kin selection.

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 2008). For many applications it will be useful to construct novel communities to carry out a function (Brenner et al. 2008; Wall 2008). Such constructed communities often will not grow well as demonstrated by the initial community growth reported here, and by Shou et al. (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. The demonstration that costly interspecies cooperation requires mechanisms of reciprocation, and of directing benefits applies to all systems. Further research will be needed to understand how these requirements are fulfilled in the many natural examples of interspecific cooperation.

Experimental Procedures

Strains

E. coli K12 BW25113 (*rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)568 rph-1*) with a metA knockout was acquired as part of the Keio collection (JW3973) (Baba et al. 2006). To re-enable lactose metabolism the *E. coli* was mated for 40 minutes 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. *Salmonella typhimurium* LT2 was used. All lines were grown in M9 minimal media with 10 mL of 0.01 M CaCl2, 10 mL of 0.1M MgSO4, and 10 mL of 20% sugar (lactose or glucose) per liter.

Acquisition of a methionine excreting *S. typhimurium* mutant

To select for a methionine producing strain of *Salmonella*, 10^8 cells were grown on a glucose M9 minimal media plate with 1 mg/mL ethionine [6]. 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 5 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 over-night in glucose minimal media. These samples were then centrifuged at 10K for 2 minutes 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 .01 μg/mL.

Tests of dynamic stability

To test the selective benefit of assisting a partner, a methionine producing *Salmonella* was competed against non-producing wild type in the presence of met- *E. coli*. Three spatial structure replicates were initiated with 1% methionine producing mutants and 99% non-producers. A total of 10^8 *Salmonella* and 10^8 *E. coli* were plated on lactose M9 plates. Bacteria were allowed to grow for 2 days at 37° C, whence the cells were scraped off in 3 mL of M9 minimal media. 100 μL of the cell suspension was

spread onto a new plate. 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 hours 100 μL was transferred to a new flask. Three replicates were carried out with initial frequencies of 99.99% methionine producers and 0.01% non-producers.

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 producers and wild type, 30 Salmonella colonies were stabbed onto a lawn of *E. coli* on a lactose plate with X-gal. If an isolate was a producer a blue colony formed on the plate, otherwise no colony appeared.

Acknowledgements:

I thank J. Bull for extensive discussion; I. Molineaux, C. Earhart, L. Forney and I. Matsumura for insights to choosing appropriate cross-feeding bacteria; R. Heineman, T. Keller, C. Marx, C. Hawkes J. Strassman and L. Meyers for comments on the manuscript; D. Greig and two other reviewers for extensive feedback. I am grateful to National BioResource Project (NIG, Japan): E. coli for providing the KEIO collection of mutant bacteria. I am funded by an NSF GRFP. Support for supplies was provided by NIH GM 57756 to J. Bull.

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Figure 4.1

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. Wildtype indicates the initial *Salmonella* typhimurium. Eth mutant indicates the ethionine resistant mutant. Evolved mutant indicates the methionine excreting mutant that arose on plates and was used in experiments. The blue line is bacterial growth where the methionine producing *Salmonella* was streaked across *E. coli*.

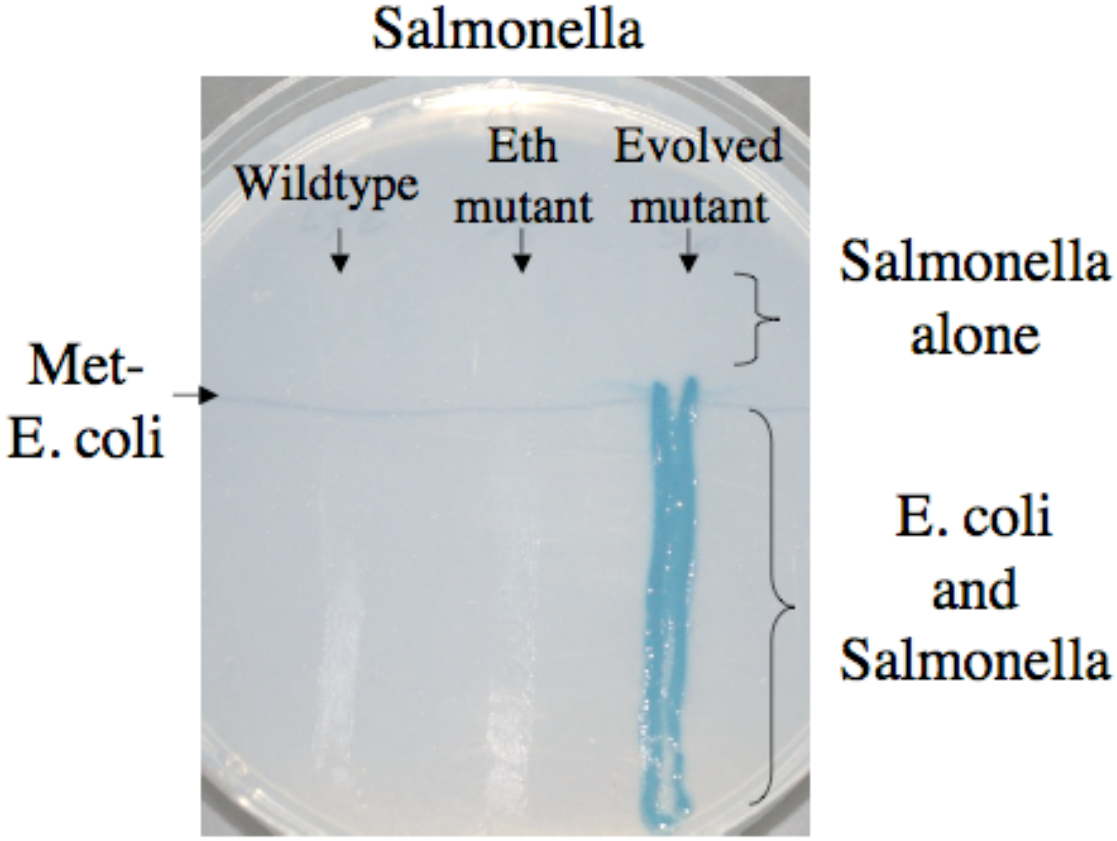
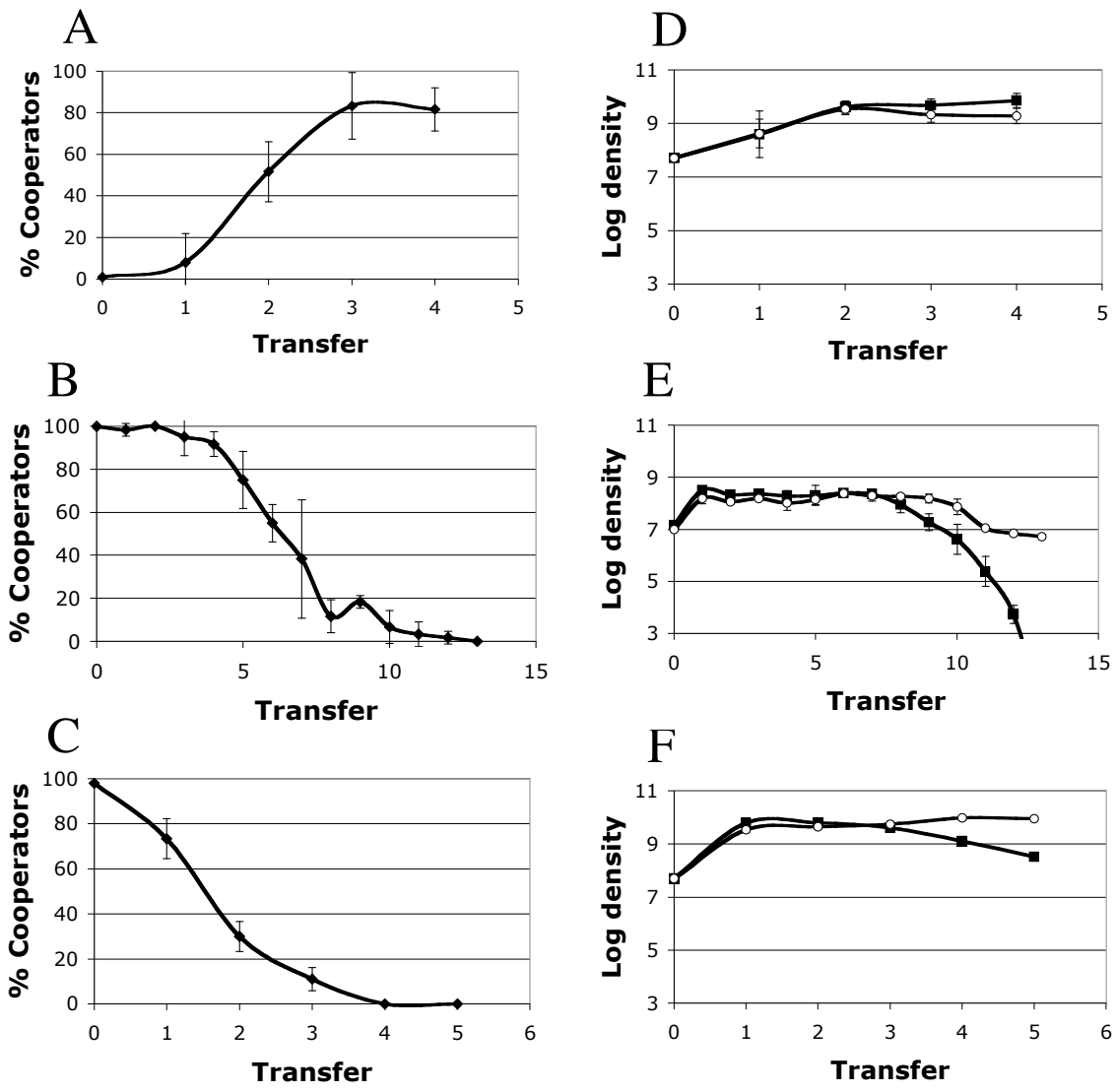


Figure 4.2
Dynamics of the system with variation in reciprocation and spatial structure. Graphs A, B, C and D are the percentage of cooperators in the *Salmonella* population. Graphs E, F, G and H are the log density of *E. coli* (filled squares) and *Salmonella* (open circles). A and E are the results 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. C and F are the results from communities grown in lactose flasks with no spatial structure. Error bars represent the standard deviation.



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