Microbial communities in lepidopteran guts: from models to metagenomics

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Introduction

Most microorganisms live in complex microbial communities. So do their hosts. Eukaryotes have co-evolved with diverse microorganisms on their internal and external surfaces. These microorganisms constitute communities, and the host's fitness may be affected differently by the community than by any single species. Therefore, understanding the structure and function of microbial communities must be integral to the study of host-microbe interactions.

Communities are dynamic assemblages whose stability and functions are governed by dependencies and antagonisms among the members. All microbial communities are dynamic and continually responding to their changing physical environments, but those associated with animals must contend with the vicissitudes of their hosts as well. Host tissues may present attractive surfaces and a rich source of nutrients, but they may also expose the microorganisms to extreme conditions due to pH, toxins in food, or precipitous changes in anatomy, such as the shedding of the very tissue that the microorganisms call home.

Spatial and temporal variation presents challenges to those who study communities as well as the microorganisms that comprise them. Although complexity and change typify the natural environment and are thus central to understanding microbial ecology, it is these very features that microbiologists have striven to minimize in our ecological models. The emphasis on pure culture over the last century of microbiology has removed microorganisms from their communities and focused on their behavior in the biologically simple environment of the Petri dish. Although simple model systems have driven the explosion of knowledge in host-microbe

interactions over the last two decades, the reality of natural communities demands that we direct attention to complex assemblages as well. The recent surge of interest in community ecology coupled with the development of powerful new methods for its study present an unprecedented opportunity to dissect the interactions among microorganisms in communities and elucidate how the function of the entire community determines outcomes for its host.

Global ecological questions

There are several overarching themes that need to be studied to develop a comprehensive view of the function of any community. Key among these are the influence of physical and chemical factors on community structure and function, succession and community development, mechanisms of mutualism, antagonism, and communication among community members, and the robustness of the community when disturbed or invaded.

An appropriate microbial community can provide a powerful model for studying these principles. Some of the basic tenets of ecology have been well-developed in macroecological systems, and microbial ecology can draw on existing that existing knowledge, while others have been difficult to study at the landscape level and may be better suited for developing in microbial models. For example, the keystone species concept has been tested with macroorganisms quite effectively. This is illustrated by the work of Paine in a rocky intertidal zone where he demonstrated that the removal the top predator, the starfish *Pisaster ochraneus*, resulted in the reduction of species diversity from fifteen species to eight. He showed that the starfish maintains diversity by controlling the population sizes of species that outcompete other members in its absence (Paine 1966). The key findings from this seminal work are broadly applicable to a

diverse range of terrestrial and aquatic systems, and a broad range of taxa (Crooks and Soulé 1999), (Gonzalez-Megias and Gomez 2003; Hanke, Nichols, and Coon 1992; Naiman et al. 1994; Navarrete and Menge 1996). This type of study can provide a model for studying microbial community structure and function, and a framework on which to determine whether similar principles govern micro- and macroorganisms living in communities (Andrews 1991).

In contrast with keystone species, our study of biological invasions lacks opportunity for the same type of experimental approaches, because invasions are not conducive to replicated experiments. Biological invasion theory attempts to predict invasion patterns, the nature of successful invaders, conditions conducive to invasion, consequences of invasion, and rates and directions of spread (Shigesada and Kawasaki 1997). The nature of invasions makes it challenging to test theory with a replicated experimental design, as most invasions are unexpected, unwanted, and not studied until after the process has begun. Yet the increasing rate of biological invasions necessitates that more general and predictive theories replace the case-by-case, post-hoc approach on which we currently rely (Kareiva 1996). The field of biological control, however, provides many examples of replicated studies that document the behavior of invaders. Unfortunately, many of the planned introductions of insect biocontrol agents rely on the prior establishment of an earlier invader, and so the applicability of biological control experiences to invasion ecology is uncertain.

In an ironic contradiction, invasions by certain insects and microorganisms are responsible for damage to agriculture and forestry, whereas invasions by others, in the form of biological control agents, have been powerful regulators of invasive pests and pathogens. The study of biological

control has informed invasion theory. Biocontrol is is often dependent on successful invasion by a natural predator or parasitoid of an insect pest. Such invasions have been remarkably effective in some cases. For example, in 1888 the citrus tree pest *Icerya purchasi* was successfully controlled by the introduction of *Rodolia cardinalis* (Shigesada and Kawasaki 1997). More recent successes include control of the cassava mealybug and green mite in Africa using parasitoids from South America (Bellotti, Smith, and Lapointe 1999). In addition to reducing populations of pests, invasion by biocontrol agents can also affect native communities adversely, as is illustrated by *Compsilura concinnata,* a parasitoid fly, which was repeatedly introduced to North America to control 13 pests between 1906 and 1986. This biocontrol agent successfully invaded New England and may now be responsible for the decline in the population of the native, nontarget silk moth. (Boettner, Elkinton, and Boettner 2000). Due to this and other examples (Pearson 2000), the potential for further biological control has declined as environmental concerns have risen (Daehler 1997; Schaffner 2001; Simberloff 1996). Once again, the lack of comprehensive theories that can be empirically validated hinders further advances. Similar problems hinder the field of planned introduction of genetically modified organisms. The enormous potential of this approach is well established, as is our ability to test for potentially direct, immediate effects in closed systems. Unfortunately, most adverse effects that have accompanied other technologies have been delayed, indirect, and only manifested under natural conditions (Kareiva 1996).

Microbiology has provided other systems for exploring invasion biology because some microbiological therapies depend on successful invasions. Biological control of plant disease, for example, involves successful establishment and proliferation in a community by a

microorganism, resulting in suppression of plant disease (Cook and Baker 1983; Handelsman and Stabb 1996). Kinkel and Lindow examined the invasion and exclusion abilities of *Pseudomonas syringae* strains on plant leaves. They found that population size alone did not predict a strain's ability to invade a community or to exclude others from it and the characteristics of successful invaders and excluders differed (Kinkel and Lindow 1993).

Invaders to root-associated communities contend with the highly complex community in the rhizosphere. In general, the invader's population size is correlated with biological effects, such as disease suppression, but there are some surprises. Gilbert et al. (Gilbert et al. 1993) found that when *Bacillus cereus* was inoculated onto seeds, its population on the root emerging from the seed diminished rapidly, but it continued to have a global impact on the composition of the microbial community long after it was detectable. The complexity of the rhizosphere community makes it difficult to test the hypotheses that are simple enough to be empirically tractable yet robust enough to incorporate community-plant interactions, and thus, simple models would be useful to track bacterial populations and their lasting impacts on communities through which they pass.

A human gut is an ecosystem in which invasion is of particular interest. The success of gut pathogens depends on their invasive ability. Likewise, probiotics, or bacterial inoculants such as *Lactobacillus* and *Bifidobacterium* spp., might be more effective if they survived and colonized the gut (Bengmark 1998; Goossens et al. 2003; Guarner and Malagelada 2003; Holzapfel et al. 1998) Both pathogens and beneficial bacteria must compete with the indigenous community for attachment sites and nutrients. A healthy gut community is highly resistant to invasion,

providing the “barrier effect” or “colonization resistance” that maintains gut function (Bourlioux et al. 2003; Guarner and Malagelada 2003). But little is known about what makes a microbial community robust to or able to recover from invasion.

There is scant knowledge on which to base the design of successful invaders for probiotics or biological control, or to predict how easily a microbial or macrobiological community will be penetrated, and perhaps altered, by an invader. Developing the right model system will advance microbial and macroecology by providing a context for testing invasion theory. One of our goals is to develop a model system in which hypotheses can be tested rigorously with precisely modulated treatments, controlled variables, and replicated experimental designs.

Model Systems

The right model system is essential to test ecological principles about microbial community structure, function, succession, and robustness. To be serviceable, a model community must be easily maintained and reproduced and have reproducible composition. There should be means to introduce chemicals or organisms into the community by a process that approximates a natural event, and the community should be sufficiently simple in composition to ensure that all of its members can be studied in culture or by genomics and that the communication networks connecting community members can be mapped.

A number of outstanding model systems have been established for microbial community study over the last two decades. Each system is suited best to particular investigations. In the following section, we review a few examples of these communities, the advances they have

afforded, and their limitations. Many of these model systems involve associations of a single species with a host. Although they may not immediately inform our understanding of complex communities, they have established broad principles of host-microbe interactions and microbe-microbe interactions that are applicable to multi-species communities. The rapid progress made in these simple systems has facilitated the next steps involving complex ones.

Table 1. Examples of model systems for studying host-microbe interactions.

Symbiosis	Approximate number of species	Strengths and contributions of system	References
Squid-*Vibrio*	1	Study of signal exchange and colonization; effect of symbiont on host development	(McFall-Ngai 2000; Nyholm et al. 2000; Visick and McFall-Ngai 2000; Visick et al. 2000)
Legume-*Rhizobium*	1	Study of signal exchange with host; effect on host development; quantitative modeling of interstrain competition	(Beattie, Clayton, and Handelsman 1989)
Nematode-*Pseudomonas* and -*Enterococcus*	1	Identification of virulence genes; comparative virulence in other hosts	(Choi et al. 2002; Hendrickson et al. 2001; Jander, Rahme, and Ausubel 2000; Miyata et al. 2003)
Gypsy moth	~11	Relatively simple, multispecies	(Broderick et al. 2004)
Cabbage White Butterfly	~4	Very simple, multispecies	Robinson et al., unpublished
Termite gut	100-200	Study of carbon, nitrogen, and hydrogen cycling within a community; demonstration of functional redundancy in microbial community	(Kane and Breznak 1991; Leadbetter and Breznak 1996; Leadbetter, Crosby, and Breznak 1998; Leadbetter et al. 1999; Lilburn, Schmidt, and Breznak 1999; Lilburn et al. 2001; Nakashima, Watanabe, and Azuma 2002)
Mouse gut	500-1000	Effect of bacteria on immune system development	(Hooper et al. 2003; Stappenbeck, Hooper, and Gordon 2002)
Human oral cavity	~500	Bacterial adhesion and succession	(Paster et al. 2001)

Vibrio-squid. *Vibrio fischerii* and its host, the squid *Euprymna scolopes* have an extraordinary relationship. The bacteria enter and colonize the light organ of the squid where the bacteria emit light at night, providing counterillumination that enables the host to avoid detection by its prey

who would otherwise see the squid's shadow in moonlit waters (McFall-Ngai 2000). The partners substantially affect each others' biology – the bacteria affect development of the squid's light organ and the squid provides a non-competitive niche for the bacteria by preventing colonization by other species (Foster, Apicella, and McFall-Ngai 2000; Visick and McFall-Ngai 2000; Visick et al. 2000). Despite its exotic, perhaps unique, outcome, study of this system has revealed principles in microbial behavior and host-microbe interactions that appear to be common throughout the microbial world.

Study of the squid-*Vibrio* symbiosis led to discovery of quorum sensing, a mechanism by which many bacteria sense the population density of their species and express genes accordingly. Population density is detected by accumulation of acylated homoserine lactones (AHLs) whose structures vary, conferring species specificity. Quorum sensing determines expression of the genes responsible for light emission by *Vibrio fisherii* and regulates genes in other organisms that encode virulence factors, antibiotic production, and other functions that require a high density of cells to be useful to the population (Rice et al. 1999).

The squid-*Vibrio* system has shaped thinking about microbial ecology by illustrating communication and cooperative behaviors among members of a population of a single species (McFall-Ngai 2000; Nyholm et al. 2000). In fact, some microbiologists have argued that a single species can comprise a community based largely on the principle that there is communication among members of a population (Buckley 2003). Community, however, has historically been used in the ecological literature to describe a *multispecies assemblage* (Begon 1990), and therefore in this chapter we will adhere to the traditional definition of community.

Rhizobium-legume symbiosis.

The *Rhizobium*-legume interaction is another system involving one microbial species and its host that has dramatically shaped thinking about microbial ecology. Members of the *Rhizobiaceae* family infect the roots of their leguminous hosts and induce the formation of an organ, known as a nodule, in which the bacteria fix nitrogen. Both the bacteria and the plant host produce signals that are interpreted by the other partner, leading to initiation of a new developmental program (Cullimore and Denarie 2003; Long 2001; Peters, Frost, and Long 1986). Legumes release an array of flavonoids that induce the early *nod* genes, which results in the synthesis of Nod factors by the bacterial partner. Nod factors are lipooligosaccharides with chitin-like structures that induce morphological changes in the plant. The signals in this symbiosis are highly specific – in general, each species of bacteria infects one or a few species of legumes, and the flavonoids and Nod factors generally define the host range of the rhizobia. This example of signal exchange differs from quorum sensing in that the exchange of signals is between members of different domains of life, the partners are sensitive to minute concentrations of each others' signals (10^{-6} M), and the events leading to nodulation can be initiated by a single cell (Begum et al. 2001; Faucher et al. 1988).

The *Rhizobium*-legume system has also provided a simple model for studies of bacterial competition. When strains that are able to nodulate a common host are applied together, the proportion of nodules occupied by each strain is often quite different from their representation in the inoculum. The successful competitor establishes a pure culture inside the nodule, making quantitative studies of competition simpler than in a system that remains in an open environment

that is bathed in other bacteria. Quantitative modeling and mutant analysis has revealed an ecological picture of interstrain competition.

These relationships are not linear or simple (Figure 1) and mathematical modeling that describes the relationship has offered insights into the biology (Beattie, Clayton, and Handelsman 1989). Extensive mutant analysis indicates that cell surface features (Bittinger et al. 1997; Handelsman, Ugalde, and Brill 1984; Lagares et al. 1992; Milner, Araujo, and Handelsman 1992; Thomas-Oates et al. 2003), toxin production (Oresnik, Twelker, and Hynes 1999; Robleto et al. 1998) and nutritional competence and many other physiological factors determine competitiveness (Triplett and Sadowsky 1992). Plants differ in their ability to admit certain strains into their nodules (Laguerre et al. 2003; Rosas et al. 1998), demonstrating that each partner plays an active role in microbial competition. As in the *Vibrio*-squid interaction, interstrain competition in the *Rhizobium*-legume symbiosis has revealed mathematical and genetic relationships that likely apply to competition in more complex communities.

Insect-microbe interactions.

The interactions between insects and their microbial symbionts have provided the basis for many principles in symbiosis and have revealed unexpected mechanisms that illustrate how organisms cooperate to perform life functions and gain a competitive edge. The *Buchnera*-aphid symbiosis provides an example of an ancient relationship that has evolved into one of mutual dependence. Analysis of the *Buchnera* genome indicates that the bacterium has lost the capacity to synthesize certain amino acids that it acquires from its host. The *Buchnera* genome has been whittled down to a minimal genome, smaller than genomes of most free-living bacteria. Likewise, the aphid

cannot synthesize and depends on *Buchnera* for other essential nutrients (Moran 2002, 2003; Moran and Mira 2001; Moran et al. 2003; Ochman and Moran 2001; Wilcox et al. 2003).

Other insects depend on their bacterial associates for production of mating (Brand et al. 1975; Brand et al. 1976) and cohesion pheromones (Dillon, Vennard, and Charnley 2002). *Wolbachia* alters reproductive behavior in diverse insects (Plantard 1998, 1999),and an unnamed member of the Cytophaga-Flexibacter-Bacteroides group induces parthenogenesis in parasitoid wasps (Zchori-Fein et al. 2001).

Most of the arthropod symbioses involve highly specific interactions, often between one bacterial species and one insect. There are a few examples of multispecies communities, such as the associates of the hindgut of termites. Degradation of complex carbohydrates and fixation of nitrogen form the metabolic foundation for the community's function (Bakalidou et al. 2002; Breznak and Canale-Parola 1972; Breznak and Pankratz 1977; Breznak and Kane 1990; Breznak et al. 1973; Leadbetter and Breznak 1996; Leadbetter et al. 1999; Nakashima, Watanabe, and Azuma 2002). The intricacies of managing the carbon and nitrogen economy of the hindgut provides evidence for interdependence and coevolution among the members of the community. A combination of ultrastructural studies, microbiology, and microelectrode measurements indicates that the hindgut is delicately structured to maintain gradients of oxygen and hydrogen, and the microbial community is spatially arranged to take advantage of and maintain the chemical gradients (Brune 1998). Perhaps the most ecologically intriguing aspect of the termite hindgut community is the functional redundancy. The community contains numerous and diverse spirochetes, for example, that fix nitrogen (Lilburn, Schmidt, and Breznak 1999; Lilburn

et al. 2001). A future challenge in microbial ecology is to determine the role and significance of functional redundancy in communities and the termite hindgut provides an ideal venue for such studies.

The termite system offers one of the most powerful existing models for studying nutritional interactions among community members. The exchange of energy and elements is well-understood and establishes principles that are likely repeated in other microbial communities. However, its relative complexity prevents a complete genomic analysis of the community members and will make it challenging to construct a comprehensive map of its communication networks.

Microbial communities in mammals.

The human oral cavity has been the site of intense study of microbial succession, which has revealed a delicately orchestrated sequence of colonization events that lead to reproducible and consistent three-dimensional community architecture. Cleaned teeth or enamel chips in a normal mouth provide the vehicle for tracking the succession of community membership (Kolenbrander et al. 2002). The early colonizers are predominantly streptococci, which attach directly by adhesions to the salivary receptors on the pellicle that coats the tooth surface in mixed species biofilms (Palmer et al. 2003). A mixture of species then attaches to the streptococci, forming a complex, multispecies biofilm. A member of the mixed biofilm is *Fusobacterium nucleatum*, which provides the platform to which diverse late colonizers attach (Kolenbrander et al. 2002). In addition to the surface contact, the bacteria communicate with diffusible signals of the type 2 quorum sensing class of inducers (Blehert et al. 2003). The spatial and temporal organization of

the oral community is both shaped by and contributes to community function and impact on the host, thereby providing an impressive model system for relating structure and function.

Gnotobiotic, or germ-free, animals have provided the basis for some elegant and informative studies of host-microbe interactions. Gnotobiotic animals are raised from offspring are delivered by Cesaerean section and raised in sterile conditions. This approach simplifies experiments and definitively isolates the role of one organism. For example, in a stunning piece of work, (Hooper et al. 2003) have shown that a member of the mouse gut microflora, *Bacteroides thetaiotaomicron,* induces the normal development of the mouse immune system. They implicated angiogenins, which have been associated previously with tumor-associated angiogenesis, in innate immunity. The angiogenin, Ang4, is secreted into the gut lumen and has bactericidal activity against intestinal microbes. Ang4 expression is induced by *B. thetaiotaomicron*, a predominant member of the gut microflora. Moreover, another angiogenin, Ang1, is found in the circulatory system in mice and humans and exhibits microbicidal activity against systemic bacterial and fungal pathogens, suggesting that they contribute to systemic responses to infection (Hooper et al. 2003; Stappenbeck, Hooper, and Gordon 2002) The gnotobiotic mouse system has revealed intricacies of bacterial communication with the mammalian immune system that would have been essentially impossible to pinpoint in a more complex microbial environment.

To address the next level of complexity in host-microbe interactions, we need model systems that offer analytical power comparable to that provided by gnotobiotic plants and animals but have utility for understanding interactions among community members.

Approaches to community study

There are few models that approximate the natural events involved in colonization by a multispecies community as effectively as the tooth biofilm system. In other systems, far more disruptive approaches have been used. Much of microbial community ecology in host-microbe studies is predicated on the "scorched earth" approach, which involves ridding the environment of all its microorganisms and then adding them back singly or in small groups. Although this method has revealed much about interactions of hosts with one microbial species, the resulting community is too different from a natural community to provide a meaningful basis for analysis.

Many powerful approaches in microbiology involve the removal of one element from an otherwise normal biological system. In genetics, for example, we use mutant analysis to isolate the role of a single gene, and in the study of metabolism, we use inhibitors or mutants to isolate the role of an enzyme in a pathway. There are few tools available for microbial ecology that approximate this degree of rigor or yield arguments with the power of those established through genetics and biochemistry. We propose that a method that selectively removes a single species from a community will provide a powerful and precise strategy to understand community interdependencies. As we cannot reach in and remove members of a species in a microbial community by hand as can be done with starfish, for example, we must develop alternative strategies.

Lepidopteran gut community

Our goal is to describe the species diversity in a community and then determine the role of each member of the community in the health of the host and in the stability and function of the community. To be tractable for these studies, the community needs to be readily available and reproducible, portable and contained, readily manipulated by addition of chemicals and organisms, and of relatively simple composition. Communities that meet these criteria reside in the midguts of the lepidopteran insects, such as the gypsy moth.

Overall approach

The overall approach is to remove each species individually from the midgut community and study the resulting effect on the host and the community. We will measure effects on resistance to disease and toxins, development, and fecundity in the host, and nutritional status and robustness of the microbial community following reduction of the population size of one member. Robustness is comprised of resistance, stability, and resilience. Resistance is the ability of a community to maintain its structure upon challenge by an invader, stability is the ability to return to its original structure, and resilience is the rate of return to original structure (Begon 1990).

Microbial diversity in lepidopteran midguts

We have characterized the microbiota in the larval midguts of the gypsy moth by culturing and culture-independent methods. When fed on sterilized diet, the community was comprised of 10 members (Table 2), 7 of which were culturable. Most of the members of the community belong to the _-Proteobacteria and Firmicute phyla.

Table 2. Bacterial phylotypes identified by culturing and culture-independent analysis of 3rd instar gypsy moth midguts based on 16S rRNA gene sequence analysis.

Sequence ID	Bacterial Division	Genus	Database matches	Population or Presence
		(≥ 95% identity)	**(≥ 98% identity)**	Artificial diet
Identified by culturing and culture-independent analysis				
NAB1	γ-*Proteobacteria*	*Pseudomonas*	*P. putida*	1.8×10^5
NAB3	γ-*Proteobacteria*	*Enterobacter*	uncultured soil	4.8×10^6
NAB4	γ-*Proteobacteria*	*Pantoea*	*P. agglomerans*	2.5×10^5
NAB7	low G+C Gram-positive	*Staphylococcus*	*S. lentus*	8.1×10^4
NAB8	low G+C Gram-positive	*Staphylococcus*	*S. cohnii*	2.9×10^6
NAB9	low G+C Gram-positive	*Staphylococcus*	*S. xylosus*	1.3×10^6
NAB11	low G+C Gram-positive	*Enterococcus*	*E. faecalis*	1.5×10^8
Identified by culture-independent analysis only				
NAB16	α-*Proteobacteria*	*Agrobacterium*		+
NAB17	γ-*Proteobacteria*	*Enterobacter*		+
NAB20	low G+C Gram-positive			+

[a] Cultured: numbers represent the average population of phylotype based on colony morphology in 20 individual larvae per treatment (cfu/ml gut material), Non-cultured: + indicates presence in guts of insects in diet treatment.
[b] Cytophaga/Flavobacteria/Bacteroides

The gypsy moth is a generalist that feeds on 300-500 plant species that contain a diverse array of allelochemicals. Therefore, we determined the community composition when the larvae were fed foliage from various tree species. The microbial composition of midguts differed substantially among larvae feeding on sterilized artificial diet, aspen, larch, white oak, or willow. A culturable *Enterococcus* spp., and an *Enterobacter* species that was culturable from some guts and not other, were both present in all larvae, regardless of feeding substrate.

Manipulation of community composition

Antibiotics. The methods we are using to reduce targeted populations in a community cover a range of specificities. Antibiotics, for example, are not species-specific, but by using various drugs of different specificities, the effect on a given species might be isolated. Larvae fed on antibiotics in various combinations, display numerous signs of reduced health. For example, a cocktail of antibiotics including tetracycline reduces larval survival by 50% over the first 20 days after hatching, and the surviving larvae weigh $1/10^{th}$ of the untreated control larvae. Tetracycline alone has a similar effect on development, whereas gentamicin, rifampicin, and penicillin have no effect on survival or growth.

Another antibiotic, zwittermicin A, dramatically increases larval sensitivity to the insecticidal toxin produced by *Bacillus thuringiensis,* although this antibiotic has no measurable effect on the larvae by itself (Figure 2). Zwittermicin A alters the population size of more than one member of the gut community; thus it is not possible to assign the effect on toxin sensitivity to any one member of the community. Gypsy moth larvae fed on aspen leaves have substantially altered gut

communities, and also show enhanced sensitivity to *B. thuringiensis* and greater susceptibility to virus infection (Hunter 1993; Lindroth 1999). In aggregate, these results suggest that a change in the gut microbiota may stunt larval growth, stall development, and make the larvae more sensitive to pathogens and toxin. The results do not elucidate the role of any one bacterium or group of bacteria, however, because all of the treatments affect more than one member of the gut community. To test the hypothesis that a member or members of the gut community contribute to larval development and protect larvae from pathogens requires methods that isolate the effects of the members of the community. One approach to assigning functions to specific organisms, is to feed bacteria to the treated insects to determine whether any of the normal residents of the gut rescues the host. However, methods to selectively remove members from the normal community are also essential because they will generate fewer secondary effects and be less disruptive to overall community function.

Phage. Bacteriophage, or phage, are viruses that inject into bacteria a nucleic acid genome that directs synthesis of viral components, leading to the production of more phage particles. Some phage are highly lytic, completing their replication cycle by weakening the bacterial cell wall, which results in cell lysis and release of phage particles. Some phage can release hundreds of infective particles from one infected cell. Their rapid life cycles and high reproductive rates make phage excellent agents to reduce bacterial populations. As agents of bacterial destruction, phage have advantages over antibiotics, such as their high degree of host specificity and amplification over time (Sulakvelidze, Alavidze, and Morris 2001). The host range of most phage is limited to a single species and most are selective for certain strains within a species. The host selectivity and explosive killing exhibited by many phage have drawn attention to them

as therapeutic agents to cure infectious disease. This idea was introduced by d'Herelle in 1917 and pursued in collaboration with his colleagues in Georgia (former USSR), although it did not gain attention until recently in the U.S. Early experiments by d'Herelle and others claimed high survival rates of people treated with phage to control dysentery and cholera, but the experiments were not designed with the rigor of modern experimentation; thus the data, however intriguing, must be interpreted cautiously. A combination of political prejudices and the discovery of antibiotics diverted attention from phage therapeutics in Western medicine until recently (Summers 2001); (Sulakvelidze, Alavidze, and Morris 2001). The last few years have seen renewed interest in phage, as the antibiotic resistance crisis impels microbiologists to find new (or rediscover old, in this case) solutions to infectious disease. Recent reports present rigorous and highly successful in vivo tests of phage to control infectious disease. One study showed that mice infected with *E. faecalis* were cured by a single injection of a phage that reproduces in that bacterium. Even mice that were already moribund had a 50% survival rate when treated with the phage, compared with 0% of the untreated ones (Biswas et al. 2002).

Plant pathologists have also used phage to protect plants from infection by bacterial pathogens ; (Flaherty et al. 2000); (Vidaver 1976) and found that virulent phage interfere with establishment of a bacterial biocontrol agent (Keel et al. 2002), indicating that phage exert influences on population structures outside the controlled conditions of the laboratory. Of particular interest to us is the fact that d'Herelle first observed phage in cultures of a pathogen that caused an epizootic infection of locusts in Mexico in 1909 (d'Herelle 1926). Thus, observations about the role of phage in bacteria associated with insects date back to the very beginning of phage biology.

The history of phage biology and ecology suggests potential for their use in ecological studies. Their specificity makes them carefully addressed "letter bombs" that will destroy only one component of a community and remain biologically invisible to the rest. The resistance problem encountered in previous studies can be addressed either by using the phage for short-term studies or by combining more than one phage, in which case the frequency of a doubly resistant mutant is lower than the number of bacteria in the population to be addressed.

Bacteriophage exhibit more specificity than antibiotics, infecting only certain members of a species. The advantage of phage is that they can reduce bacterial populations by 4-8 orders of magnitude in vitro. A disadvantage is the difficulty in isolating phage for bacteria that we do not yet know how to culture. A concern with phage was that they might not be able to infect in a gut, especially the gypsy moth gut, which has an average pH >12. Variations and combinations of methods will likely lead to the best tools that incorporate phage into the study of community ecology.

Functional connections among community members – cultured and uncultured

A long-ignored aspect of community ecology is the uncultured majority. Most bacteria in environmental samples are not culturable by standard methods. Therefore, to understand the structure and function of microbial communities, we must include the uncultured bacteria in our analyses. Community structure can be analyzed by PCR amplification of 16S rRNA genes from DNA directly extracted from the environmental sample. DNA isolated from environmental samples can also be used for functional genomics by cloning into a suitable vector that replicates

in a culturable host. This approach, termed metagenomics, has provided insight into uncultured communities in soil, seawater, sponge tissue, and the human oral cavity (Beja et al. 2000; Courtois et al. 2003; Diaz-Torres et al. 2003; Handelsman et al. 2003; Henne et al. 1999; Rondon et al. 2000; Schleper et al. 1997; Stein et al. 1996).

We are characterizing the cultured and the as-yet-unculturable bacteria in the gypsy moth midgut. We have constructed highly redundant libraries from DNA extracted directly from the gut bacteria that have not been subjected to culturing (Figure 3). Preliminary studies indicate that clones in these libraries express novel functions that have not been found among the cultured bacteria. A major focus of this work is to identify molecules that play a role in communication among bacteria – both culturable and unculturable – in the gut environment. This aspect of host-community interactions will add a new dimension to our understanding of the interactions of animals with their associated microorganisms.

Conclusion

The study of the impact of communities on their hosts is at a new intersection. New tools are available for the dissection of communities, and the knowledge of interactions of single species with their hosts lays a strong foundation for the study of multispecies communities and their hosts. Application of molecular methods that address both the uncultured and cultured members of the communities, computational approaches to model quantitative events, and diverse biological and chemical approaches to perturb communities will produce an understanding of the complex networks that maintain the structure of the community and govern its influence on the host.

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Figure Legends

Figure 1. *Rhizobium* competition. When multiple strains compete for infection of leguminous hosts, one strain often dominates in the nodules. The quantitative modeling that has been applied to intrastrain competition may be useful in understanding multispecies communities (Beattie, Clayton, and Handelsman 1989).

Figure 2. Synergy of zwittermicin A and Bt toxin. Zwittermicin A potentiates the activity of the insecticidal toxin produced by *Bacillus thuringiensis* (Broderick et al. 2000).

Figure 3. Metagenomics provides a means to access the genomes of as-yet-unculturable microorganisms by direct extraction of their DNA from mixed communities (Handelsman 2003; Handelsman et al. 1998; Schloss and Handelsman 2003).