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► **To cite this version:**

M. K. Nishiguchi, A. M. Hirsch, R. Devinney, G. Vedantam, M. A. Riley, et al.. DECIPHERING EVOLUTIONARY MECHANISMS BETWEEN MUTUALISTIC AND PATHOGENIC SYMBIOSES. *Vie et Milieu / Life & Environment*, 2008, pp.87-106. hal-03246053

HAL Id: hal-03246053

<https://hal.sorbonne-universite.fr/hal-03246053v1>

Submitted on 2 Jun 2021

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DECIPHERING EVOLUTIONARY MECHANISMS BETWEEN MUTUALISTIC AND PATHOGENIC SYMBIOSES

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SYMBIOSIS
MUTUALISTIC
PATHOGENIC
EVOLUTION

ABSTRACT. – The continuum between mutualistic and pathogenic symbioses has been an underlying theme for understanding the evolution of infection and disease in a number of eukaryotic-microbe associations. The ability to monitor and then predict the spread of infectious diseases may depend upon our knowledge and capabilities of anticipating the behavior of virulent pathogens by studying related, benign symbioses. For instance, the ability of a symbiotic species to infect, colonize, and proliferate efficiently in a susceptible host will depend on a number of factors that influence both partners during the infection. Levels of virulence are not only affected by the genetic and phenotypic composite of the symbiont, but also the life history, mode(s) of transmission, and environmental factors that influence colonization, such as antibiotic treatment. Population dynamics of both host and symbiont, including densities, migration, as well as competition between symbionts will also affect infection rates of the pathogen as well as change the evolutionary dynamics between host and symbiont. It is therefore important to be able to compare the evolution of virulence between a wide range of mutualistic and pathogenic systems in order to determine when and where new infections might occur, and what conditions will render the pathogen ineffective. This perspective focuses on several symbiotic models that compare mutualistic associations to pathogenic forms and the questions posed regarding their evolution and radiation. A common theme among these systems is the prevailing concept of how heritable mutations can eventually lead to novel phenotypes and eventually new species.

INTRODUCTION

Because symbiotic systems exhibit a variety of behaviors ranging from mutualistic to pathogenic associations, general evolutionary principles can be expected to emerge from well-studied systems that can address fundamental mechanisms of specificity and recognition (Hirsch *et al.* 2003, Hirsch & McFall-Ngai 2000, Wilkinson & Parker 1996). Additionally, many animal-bacterial mutualisms have been used as models to study colonization and co-evolution, without the interference of tissue necrosis or cell mediated death due to virulence factors that are found in pathogenic associations (McFall-Ngai 2002, Wilkinson *et al.* 1996). Comparing closely related symbiotic bacteria, which have a wide range of host preference, specificity, and virulence, can infer the evolutionary relatedness of each bacterium/virus, as well as origins of pathogenicity islands, horizontal gene transfer of virulence factors, and colonization mechanisms that are shared features among each taxon (Andre *et al.* 2003, Nishiguchi & Nair 2003). Similarities that are associated with both benign and

pathogenic associations can provide necessary information contributing to the basic knowledge of infectious associations, rather than specific disease entities. Therefore, once we understand how shared colonization strategies are used between closely related organisms, we may be able to determine whether virulence is derived from either a benign or more pathogenic form of symbiosis (Cooper *et al.* 2002).

Most symbiosis research has focused on specificity involved in each type of association. Three major stages are recognized that distinguish how each part of the symbiosis is unfolded: (1) the convergence of both host and symbiont, (2) infection and colonization, and finally (3) persistence. The initial stage involves the actual encounter between host and symbiont. Environmentally transmitted (that is, the symbiont is obtained from the environment where the host lives) symbioses entail a multitude of factors that have a major influence upon the actual infectivity of the symbiont and the susceptibility of the host. Initially, the host must be poised to accept the symbiont in its present state. Depending on the type of asso-

ciation, the age of the host (McFall-Ngai 1999), fitness, host size (Bates 2000), behavior (Secord 2001), and other additional factors can drastically change the dynamics of the association. At this point, abiotic factors also play an important role in determining whether the conditions for the actual infection can occur during contact. Such factors may include temperature, salinity, pH, ion concentration, geography, and other related micro-climate factors (Hentschel *et al.* 2000, Hirsch *et al.* 2003, McFadden *et al.* 1997, Nishiguchi 2000, Olafsen *et al.* 1993, Secord 2001, Soto *et al.* 2008a, b, Soto & Nishiguchi 2008). Finally, the symbiont has to be in an “infectious state”; if there are other biotic factors that prevent the symbiont from obtaining access to the host or inhibiting the symbiont (such as competition from other bacteria, inability to access the host, or repression of necessary “symbiotic factors” that are required for colonization), then the symbiosis cannot commence (Millikan & Ruby 2002, Nyholm & McFall-Ngai 2004, Silver *et al.* 2007b).

Once the partners have been united, then the onset of the association can begin. Whether it is a mutualistic or pathogenic association, there must be some specificity involved, which targets particular sites of infection or colonization. Most symbiotic associations with prokaryotes have very specific sites where the bacteria either enter the host or subsequently colonize tissue that harbor the symbiotic bacteria (Hirsch *et al.* 2001, Nyholm & McFall-Ngai 2004, Visick & McFall-Ngai 2000). There are also biotic factors that may induce the symbiosis to occur, such as interactions among other bacteria (Nyholm *et al.* 2002, Nyholm & McFall-Ngai 2003), specific chemical signals that may induce the bacteria to aggregate or adhere to specific sites on or around the host (Bassler 1999, Hirsch *et al.* 2003), and the induction of effector molecules that may interfere with host function (Finlay & Falkow 1997, Foster *et al.* 2000, Hueck 1998, Thomas & Finlay 2003). These types of interactions may be the initiation of what becomes either a “beneficial” association, or a deleterious one.

After infection and colonization has occurred, a number of host-mediated responses oftentimes follow. Whether the symbiosis is mutualistic or pathogenic, the partnerships have very similar routes of interplay. For example, many mutualistic and pathogenic bacteria have virulence or symbiosis factors that are only expressed when colonization has successfully occurred. These factors may include specific gene products that enable the symbiont to exploit host nutrients, metabolites or enzymes (produced by the symbiont that are beneficial to the host), or the production of toxins that enable symbiont transfer of nutrients to and from the host (Hentschel & Felbeck 1993, Pak & Jeon 1997, Sandstrom *et al.* 2000, Stabb *et al.* 2001). Genes induced by infection may also be expressed during initial stages to increase the interactions between host and symbiont (Braschler *et al.* 2003, Chun *et al.* 2006, Handfield *et al.* 2000, Lee & Camilli 2000, Smith 1998).

Recently, many of the genes expressed upon symbiosis have been shown to contain regulatory elements that are only expressed when the bacteria have infected the host (Girardin *et al.* 2003, Lee *et al.* 1999, Millikan & Ruby 2003, Young *et al.* 1999). Similarly, the host has a multitude of responses, which are activated upon infection that either select which symbiont maintains the association (Koropatnick *et al.* 2007, Nishiguchi 2002, Silver *et al.* 2007a), or causes morphological or physiological alterations that enhance the partnership (Ben-Haim *et al.* 2003, Downie & Walker 1999, Koropatnick *et al.* 2004, Montgomery & McFall-Ngai 1994, 1995). In the most extreme cases, hosts can be detrimentally affected, with tissue necrosis or death as the end result of the infection. It is the carefully balanced liaison between maintaining a mutualistic association (beneficial), or extension into a pathogenic one that has intrigued scientists to study the similarities/differences between these symbioses, and whether they have independently co-evolved similar mechanisms. Deciphering similar infection mechanisms is also relevant to understanding how organisms can adapt to a specific host environment rapidly, and whether mechanisms such as horizontal gene transfer has some influence on the chimeric nature of such organisms. This perspective will attempt to bring together some common themes of symbiosis in relation to the evolution, radiation, and speciation among different groups of benign and pathogenic microbes. By comparing a number of well studied model systems, we hope to further our understanding of how complex interactions evolve, and whether these “evolutionary innovations (Margulis 1989, Sapp 1989)” can be thought of as a continuum of speciation.

Maintaining balance: Mutualistic associations between sepiolid squid and luminescent bacteria

Mutualistic associations between animals and their bacterial partners have been long studied in a number of model systems. A large portion of these studies focus on the evolutionary or ecological effects of how the association initially began, the specificity between host and symbiont, and whether this state of “even exchange” is a peaceful truce or one that requires constant “en garde” between the players. Sepiolid and loliginid squids (Cephalopoda: Sepiolidae and Loliginidae) are unique model hosts in that most species within the family have a monoculture of symbionts; that is, they usually maintain one phylotype or strain of luminescent bacteria (McFall-Ngai 1999, Ruby & McFall-Ngai 1999). These strains are of the family Vibrionaceae (Nishiguchi & Nair 2003, Ruimy *et al.* 1994) and have species that form symbiotic niches (pathogenic and mutualistic) with many eukaryotic partners (Colwell 1984, Guerrero & Nishiguchi 2007, Nishiguchi & Jones 2004). Generally, mutualisms involving *Vibrio* bacteria include the production of luminescence generated from the symbionts; this involves a series

of reactions through genes that are located in the *lux* operon (Nealson *et al.* 1981, Nealson & Hastings 1979, Nealson *et al.* 1970). In squid-*Vibrio* mutualisms, symbionts are housed in a bi-lobed or round light organ (Nishiguchi *et al.* 2004) and luminescence production is controlled by the host in a behavior known as counterillumination (Jones & Nishiguchi 2004). Both loliginid and sepiolid squids are known to contain bacteriogenic light organs (those that contain bacteria that produce bioluminescence), and have evolved a highly regulated sequence of events that produce a tightly coupled symbiosis, which allows both host and symbiont a means of increased fitness (Nishiguchi 2001).

In normal seawater, *Vibrio* bacteria number approximately 1×10^3 - 10^4 /ml of seawater, and specifically, symbiotic *V. fischeri* comprise about 8% of total vibrios present (Jones *et al.* 2007, Lee & Ruby 1992, 1994b). Initially, symbiotic *Vibrio* bacteria must first locate and find the entrance to the light organ. During this time, squid hatchlings are induced by gram-negative bacteria to secrete mucus from this area, which are then recruited from the environment to the sites of infection (DeLoney-Marino *et al.* 2003, Nyholm *et al.* 2002, Nyholm & McFall-Ngai 2003). Once the bacteria are in contact with the mucus, they amass in dense aggregations, and only symbiotic *V. fischeri* dominate the population of bacteria found in the mucus aggregate which eventually infects the host (Nyholm & McFall-Ngai 2003, Nyholm *et al.* 2000). Symbiotic *V. fischeri* begin to increase in population size, and are able to out-compete any competitor (non-native symbiont) during the first 48 hours of colonization (Lee & Ruby 1994a, Nishiguchi *et al.* 1998). It is yet undetermined whether symbiotic vibrios are better adapted to their specific hosts by unique recognition factors (Graf *et al.* 1994, Graf & Ruby 2000, Millikan & Ruby 2002, 2003, Visick *et al.* 2000) or are influenced by abiotic factors such as temperature or nutrient limitation (DeLoney-Marino *et al.* 2003, Graf & Ruby 1998, Nishiguchi 2000, 2002, Soto *et al.* 2008a, b, Soto & Nishiguchi 2008). Such factors have played an important role in related pathogens, such as *Vibrio cholerae*, where outbreaks have been linked to increased water temperatures, or pollution (Colwell 1984).

Recognition and specificity

Since both pathogenic and mutualistic vibrios are found amongst a myriad of other Vibrionaceae genotypes, there are a number of mechanisms that may be responsible for the evolution of recognition and specificity in environmentally transmitted symbiosis (Visick & McFall-Ngai 2000). Theoretical predictions state that most symbiotic associations evolve between two or more competing strains, with partial ordering imposed based on the virulence of a dominant (native) strain being more virulent than a suppressed (non-native) strain (Frank 1996).

It has also been observed that similar mechanisms exist between mutualistic and pathogenic associations, which facilitate successful colonization of the eukaryotic host (Hentschel *et al.* 2000, Reich & Schoolnik 1994, 1996). It has been commonly observed that both mutualistic and pathogenic bacteria “acclimatize the symbiotic niche”, where they have evolved regulatory mechanisms which induce the host to be more accommodating to their arrival. This is important to the selection of more and more “adapted” strains, since those that cannot colonize or persist in the symbiosis will not be successful. Mechanisms which govern this adaptability in the squid-*Vibrio* mutualism include quorum sensing (Gilson *et al.* 1995, Lupp *et al.* 2003), two component regulatory mechanisms (Darnell *et al.* 2008, Geszvain & Visick 2008, Husa *et al.* 2007, Visick & Skoufos 2001), cell signaling (Stabb *et al.* 2001), and the ability of differential adhesion to a particular host light organ (Hensey & McFall-Ngai 1992, Stabb & Ruby 2003). These mechanisms can also be found in closely related *Vibrio* pathogens, such as *V. cholerae* and *V. parahaemolyticus*, which have similarly related mechanisms that enable strains to infect and colonize their eukaryotic hosts (Colwell 1984, Mekalanos 1985, Nishibuchi & Kaper 1995, Reich *et al.* 1997, Reich & Schoolnik 1994, 1996, Sechi *et al.* 2000). Obviously, there are many more genes that are regulated both at onset and during colonization (Crookes *et al.* 2004, Davidson *et al.* 2004, Doino Lemus & McFall-Ngai 2000, Kimbell *et al.* 2006), and future research will aid in understanding how those mechanisms are similar among various symbiotic strains, whether they are pathogenic or mutualistic in origin.

Evolutionary consequences of environmentally transmitted symbiosis

Earlier studies of *Vibrio* symbionts and their sepiolid squid hosts have indicated that phylogenetic patterns of cospeciation exist among allopatric populations residing in the Indo-West Pacific (Kimbell *et al.* 2002, Nishiguchi 2001, Nishiguchi *et al.* 1998). Along with this, a competitive hierarchy was observed among symbionts that was congruent to host and symbiont phylogenies (Nishiguchi 2002, Nishiguchi *et al.* 1998). These congruencies demonstrated that native symbionts had a competitive advantage over non-native symbionts. It also suggests that all light organ symbionts tested had evolved independently from a free-living *Vibrio* strain, not from other host taxa living in the same environment. Since these allopatric populations have shown strain specificity (and possible speciation) among the Vibrionaceae found in environmental seawater, this brings to question whether sympatric populations are able to evolve the same specific mechanisms for recognition. More recent evidence has suggested that *Vibrio* strains are adapting at a much faster rate than their host squids, and are able to migrate large

distances via “leap frogging” between host populations (Jones *et al.* 2006). This generates doubt as to whether environmentally transmitted symbioses are strictly evolving with each other, but rather have ecological factors that also drive the symbiosis (Dunlap *et al.* 2007). Since sympatric symbionts oftentimes lack host fidelity or display host fidelity but use multiple hosts (Berlocher 1998, Lynch 1989, Nishiguchi 2000), examining patterns of cospeciation may help us understand whether speciation among prokaryotic partners is influenced by their direct environment (their host) or other extrinsic factors (Boucher & Stokes 2006). Previous work determining whether pathogenic *Vibrio* strains have a common ancestor with mutualistic strains show no clear pattern of a single radiation within the group (Nishiguchi & Nair 2003, Ruimy *et al.* 1994). Consequently, some patterns exist between symbiont strains that have common or related host species (Browne-Silva & Nishiguchi 2008, Nishiguchi & Nair 2003). Although similar mechanisms exist between mutualistic and pathogenic *Vibrio* strains, many of those mechanisms have been co-opted for other functions that may not induce tissue necrosis or other pathogenic interactions which result in damage or death of the host (Colwell 1984, Reich & Schoolnik 1994). Future studies will hope to enlighten whether multiple lineages of pathogenic and mutualistic genotypes of *Vibrio* have evolved under specific conditions, or, if they have been able to horizontally transmit any of these genes for infection and colonization. This would enable multiple strains of *Vibrio* to have similar genotypic mechanisms of infection, but because they are in a different genetic background, are incapable of expressing complete virulence (similar to *E. coli*, see below in later section). Determining if these chimeras exist, and whether this is another mechanism of pathogen evolution, still needs further attention.

Plant Symbioses: Altered States

Associations between eukaryotes and microbes exist as commensalisms, mutualisms, and parasitisms, with some well-known human pathogenic organisms, such as *Vibrio cholerae* or *Helicobacter pylori*, living as either commensals or parasites depending on the host (Lipp *et al.* 2002, Merrell & Falkow 2004). Rhizobia, some of the best-studied plant-associated bacteria, live as commensals on non-host plants and as mutualists with their legume hosts (Foster *et al.* 1983, Schwieger & Tebbe 2000). As mutualists, they induce, when soil nitrogen is in short supply, the formation of nitrogen-fixing nodules on the roots of their legume host (Lum & Hirsch 2003). This interaction involves signal exchange between the symbiont partners to ensure recognition and specificity. Rhizobia may also live as parasites in nodules (Denison & Kiers 2004, Kiers *et al.* 2003) although Timmers and coworkers (Timmers *et al.* 2000) defined this mildly parasitic interaction

as a saprophytic state. In any case, rhizobia appear to traverse the commensal-mutualist-parasitic continuum (Denison & Kiers 2004, Hirsch 2004).

Does understanding rhizobia’s ability to exhibit alternative life styles (Denison & Kiers 2004) help us better understand rhizobial evolution? Rhizobia have been portrayed as “refined parasites”, nodulation being described as a “beneficial plant disease” (Djordjevic *et al.* 1987, Vance 1983). Although many of the early stages of rhizobial entry into legume host cells resemble pathogenic events on plant hosts see references in McKhann & Hirsch 1994, Mithöfer 2002, rhizobial species are not related to plant pathogenic bacteria other than *Agrobacterium*. Indeed, except for *Sinorhizobium meliloti*, most rhizobial species are only distantly related to *Agrobacterium* (Goodner *et al.* 2001, Wood *et al.* 2001).

Moreover, *Agrobacterium* is an atypical plant pathogen; it does not elicit a disease in the classic sense, but rather a transformed tumor. In general, only a minimal amount of cellular damage occurs in tumors or in aborted or senescent nodules in contrast to most plant pathogen-elicited host defense responses (McKhann & Hirsch 1994, Mithöfer 2002). Nevertheless, several investigators have proposed that when the nitrogen-fixing symbiosis fails, rhizobia elicit a hypersensitive response (HR), the classic symptom of disease resistance.

Rhizobia is not a pathogen

Although rhizobia may initially live as parasites (or saprophytes) upon host entry or within ineffective nodules, they differ significantly from plant pathogenic bacteria, particularly those pathogens involved in cultivar-dependent host resistance, where the parallels to rhizobia-legume association are often drawn. In gene-for-gene resistance, the product of a dominant *R* (resistance) gene from the host was originally defined as a protein interacting with the product of the corresponding *avr* (avirulence) gene of the pathogen. The complementary combination of *R* and *avr* genetic backgrounds, i.e. recognition, would then bring about resistance via the HR, which is characterized by rapid, localized host cell death thereby limiting the pathogen’s growth. In contrast, non-recognition brings about the disease. This is the exact opposite of what happens in the *Rhizobium*-legume symbiosis (Table I). However, the gene-for-gene model is much more complicated than when it was originally proposed and subsequently applied to the rhizobial-legume interaction. The *R* and *avr* players are constantly evolving—the pathogen to avoid detection and the plant to recognize the pathogen. Moreover, numerous proteins are involved in resistance, associating in multi-protein resistance systems that can yield new complexes as a result of rearrangement, recombination, transposon activity, and genome shuffling (see references in Friedman & Baker 2007). In addition, the *avr* gene products, also known as pathogen effectors, are now

believed to suppress basal resistance or PAMP-mediated immunity (PMI) and elicit effector-triggered immunity (ETI), i.e. the HR (Jones & Dangl 2006, Zipfel 2008).

Basal resistance, also known as cultivar-independent, or non-host resistance (Jones & Dangl 2006, Thordal-Christensen 2003, Zipfel 2008), commonly occurs between plants and pathogenic fungi or bacteria. Molecules derived from infectious microbes, such as flagellin, chitin, small peptides, glycans, and lipopolysaccharide (LPS) (“elicitors” in the plant pathology literature), are now known as Pathogen-Associated Molecular Patterns (PAMPs) or Microbe-Associated Molecular Patterns (MAMPs) and have been shown to interact with proteins named Pattern Recognition Receptors (PRRs) to generate innate immunity and disease resistance (Janeway 1989). Leucine-rich repeat receptor-like kinases (LRR-RLKs) in plants resemble the animal PRRs, e.g., *Drosophila* TOLL. Similar to animal innate immunity, a PRR (FLS2 protein from *Arabidopsis*) interacts with a PAMP (a 22 amino-acid epitope from bacterial flagellin) (Gómez-Gómez & Boller 2000), triggering a MAP kinase signaling cascade with similarity to signal transduction pathways in insects and mammals (Asai *et al.* 2002). Recognition between PRRs and PAMPs enables plants to develop resistance to many pathogens. Interestingly, although a peptide derived from flagellin from a pathogenic bacterium is recognized by FLS2 and triggers a host response, the conserved peptide, if derived from *Rhizobium*, does not bind to FLS2 or activate flagellin recognition (Felix *et al.* 1999). This indicates that the host perceives pathogenic versus symbiotic signal molecules (Symbiont-Associated Molecular Patterns; Hirsch 2004) differently. It further argues for the existence of PRR-type proteins that recognize these SAMPs. However, very few have been identified.

An important exception is Nod factor and the proteins that recognize it. Whereas plant pathogen-produced chitin, an *N*-acetylglucosamine polymer, serves as an elicitor of host defense. Nod factor, a substituted *N*-acetylglu-

cosamine oligomer produced by rhizobia is vital for the establishment of the legume-rhizobial symbiosis (Table I). Recognition of the cognate Nod factor occurs via receptor proteins that interact with this SAMP in some yet unknown way. These proteins are essential for nodule development. NORK/SymRK (Nodule Receptor Kinase/Symbiotic Receptor Kinase) was one of the first receptor proteins in the nodulation pathway to be identified and cloned (Endre *et al.* 2002, Stracke *et al.* 2002), but so far there is no evidence for Nod factor binding to NORK/SymRK. Putative Nod factor binding proteins, predicted on the basis of genetics have been identified (Limpens *et al.* 2003, Madsen *et al.* 2003, Radutoiu *et al.* 2003). These proteins are serine/threonine RLKs with extracellular LysM domains and could be considered PRRs (Zipfel 2008). LysM domains are found in proteins involved in peptidoglycan and chitin binding, suggesting that these proteins bind Nod factor, but the critical experiments have not yet been performed. Additional downstream genes in the Nod factor-signaling pathway have been cloned and are the focus of many studies concerning nodule development.

In any case, rhizobia are not perceived as pathogens even when they lack the appropriate Nod factor to be recognized by plant receptor proteins. Moreover, if either cheaters or the wrong rhizobia enter the nodule, they elicit neither a disease nor an HR.

What happens when the symbiosis fails?

When the *Rhizobium*-legume symbiosis fails, an ineffective (Fix⁻) nodule is the result. It is usually smaller than an effective (Fix⁺) nodule, and white or green in color due to either the lack or degradation of leghemoglobin (Hirsch *et al.* 1992). Depending on the stage where the symbiosis fails, the nodule may show a histological organization similar to an effective nodule, but the bacteroid zone (zone III) of the nodule may be reduced in size.

Table I. - Similarities and differences between selected symbiotic and pathogenic bacteria-host interactions.

	<i>Rhizobium</i> -legume cell	Plant pathogen-host cell (cultivar dependent)	<i>Brucella</i> -mammalian cell
<i>Recognition</i>	Invagination of bacteria into membrane-bound compartment. Nodule development. Sustained infection.	Symptoms of host defense response, including localized accumulation of phenolics, PR proteins (a hypersensitive response). Lack of sustained infection.	Invagination of bacteria into membrane-bound compartment. Sustained Infection. Disease.
<i>Non-recognition</i>	No response or minimal symptoms of a host defense response. Lack of sustained infection.	Sustained infection. Disease.	Lack of sustained infection. Little or no disease.

Some nodules completely lack differentiated bacteroids (see Perotto *et al.* 1994), and in the indeterminate nodule type, a discrete, persistent nodule meristem may not be maintained (Hirsch *et al.* 1992).

Earlier, we had suggested that rhizobial entry into the plant cell was mechanistically more similar to how a pathogen such as *Yersinia* invades a mammalian cell (McKhann & Hirsch 1994). What is the evidence to support this hypothesis? There are surprising parallels between rhizobial entry into its host and that of a mammalian pathogen, such as *Salmonella* and *Yersinia*, which enter their hosts by membrane-mediated endocytosis. *Sinorhizobium meliloti*, a nitrogen-fixing endosymbiont, and *Brucella abortus*, which affects a broad spectrum of mammals, are both enclosed in acidified, host-derived membrane compartments, yielding either a populated nodule cell or a chronically infected mammalian cell. To establish this membrane-compartmentalized state, both bacteria employ the products of *bacA* genes, which are 68.2% identical between them (LeVier *et al.* 2000). The BacA protein has been shown to affect lipid-A fatty acids, which make up LPS (Ferguson *et al.* 2004). In a *bacA* mutant-elicited infection, the membrane compartments are not maintained, most likely because lipid A is altered. This results in the subsequent collapse of the infection in either the legume or mammalian cell (Table I). In this instance, the defective LPS functions as a PAMP, which is recognized by a specific PRR in the host (Ferguson *et al.* 2004). Recognition brings about innate immunity in the infected animal, or the legume version thereof in the case of the *Rhizobium bacA* mutant. The identity of a PRR that recognizes the defective LPS in either the animal or plant cell remains unknown.

By contrast, wild-type LPS must be recognized as a SAMP, which interacts with its corresponding PRR (Hirsch 2004). Alternatively, the SAMP and the PAMP could bind to the same receptor in an agonist/antagonist type of interaction (Mithöfer 2002), but trigger different downstream signal transduction pathways. Thus, when the nitrogen fixing symbiosis fails, because of the production of defective LPS or other PAMPs, a type of defense reaction akin to that observed in type I non-host resistance or in the mammalian cell may ensue. In any case, the plant response is not an HR, which requires a much higher threshold of defense response (Jones & Dangl 2006).

How do legumes differentiate friend (Rhizobium) from foe (pathogen) or cheater rhizobia (non-nitrogen fixers)?

For cheaters that enter nodules but do not fix nitrogen, recognition signals may protect against rhizobia that are not host-capable, but they do not protect the host from rhizobia that produce the correct Nod factor but are mutated in *nif* (nitrogenase) or other critical metabolic genes. Host-induced sanctions are proposed to select against freeloaders or cheater rhizobia, and experimental evi-

dence supports this (Denison & Kiers 2004). However, many field nodules are likely to have multiple inhabitants, both friends that fix nitrogen and cheaters that do not. Under these conditions, host sanctions are unlikely because the plant cannot discriminate between the nitrogen fixer and non-fixer, especially if a sufficient level of fixed nitrogen is produced for the plant's survival.

In general, recognition of friend versus foe is still incompletely understood in plants. Many of the analyses regarding cultivar-dependent and culture-independent host resistance have been performed in *Arabidopsis*, which does not establish mutualistic associations with nitrogen-fixing rhizobia or with phosphate-acquiring mycorrhizal fungi. It is assumed that the legumes exhibit the same patterns of host resistance as *Arabidopsis* does, and some PRRs have been identified (see references in Zipfel 2008). In addition to cultivar-dependent and culture-independent host resistance, plants deal with pathogens by stopping them at their borders, employing such physical barriers as waxy, extracellular layers and rigid cell walls. Recently, root cap border cells have been evoked as a first line of defense against pathogenic organisms. Martha Hawes and her colleagues have unequivocally demonstrated that the pea root tip is protected from infection, and that this resistance is closely associated with the presence of border cells (Gunawardena & Hawes 2002). By attaching to border cells, fungi and other potential pathogens are discharged from the root surface leaving the root tip free of infection as the border cells are sloughed off. If the integrity of the root cap and border cells is breached, the root tip becomes infected. Experiments suggest that pea seed lectin, which is one of ca. 100 proteins detected in the root cap secretome, is responsible for agglutinating the pathogenic microbes and triggering the innate immunity pathway components, which are released into the secretome (Wen *et al.* 2007).

Interestingly, lectin has long been known as a recognition molecule in the *Rhizobium*-legume symbiosis (see references in Hirsch 1999). The original lectin recognition hypothesis was based on the strong correlation between the ability of legume host-produced lectin to bind to rhizobia that nodulated that host. Lectins are found in the right place, in roots and especially in root hairs, the sites where rhizobial bind. The recognition hypothesis was bolstered by the findings that transgenic legumes carrying an introduced lectin gene from another host were nodulated by the other host's rhizobia, but only if there was a match in Nod factors (Díaz *et al.* 1989, van Rhijn *et al.* 1998, 2001). Rhizobia are able to overcome the plant's basic lines of defense and can penetrate root hair cell walls and enter into root cells via infection threads initiated by endocytosis. Does the interaction between rhizobia and host-produced lectin somehow lead to the masking of determinants that would normally signal the presence of a foe? Or does lectin agglutination of rhizobia lead to a critical mass of bacterial cells that produce sufficient Nod

factor to overcome the host's recalcitrance to infection? The answers are so far unknown.

Final comments

Although there are numerous parallels between how rhizobia cells associate with their legume host versus how a plant pathogenic bacterial species interacts with its host, the outcomes are significantly different (Table I). It may be useful to focus on the parallels between virulent plant pathogens and benign plant symbionts, but it is extremely important to remember that incorporating information from such highly derived plant-microbe interactions into an evolutionary context can produce misleading conclusions. Cultivar-dependent host resistance is a tightly intertwined evolution of both host and pathogen; one changes to overcome the continual challenge by the other (Friedman & Baker 2007, Jones & Dangl 2006). Similarly, most of the *Rhizobium*-legume symbioses that have been investigated in detail are the narrow-host range ones between rhizobia and legume plants that are highly selected for agronomic performance. Many are highly specific with only one or two legume species nodulated by a particular rhizobial strain. The only broad host range rhizobial species that has been thoroughly investigated is *Rhizobium* NGR234, which nodulates ca. 50% of all legumes (Puepke & Broughton 1999). Interestingly, some rhizobia including NGR234 (Marie *et al.* 2001, Viprey *et al.* 1998) use a Type 3 secretion system (T3SS), a protein injection system that is a hallmark for pathogenic bacteria, both of plants (Gürlebeck *et al.* 2006) and animals (Trosky *et al.* 2008). Proteins secreted through the T3SS by NGR234 make possible this rhizobial species' broad host range (Marie *et al.* 2001). More emphasis needs to be placed on symbioses that occur in nature, particularly those within the Caesalpinioideae, the most primitive group subfamily of the Fabaceae. For some of these legumes, no true nodules are formed although infection threads develop and nitrogen is fixed (de Faria *et al.* 2000).

It is also important to consider other models of prokaryotic-eukaryotic interaction in addition to plant pathogens. Pathogens that exhibit the stealth mode of assault into their mammalian hosts may be particularly good models for the rhizobia-legume symbiosis. Studies of *bacA* in *S. meliloti* and *B. abortus* discussed here have already shown a connection in how the infection is established. Like rhizobia, *Vibrio cholerae* occupies many ecological niches including multiple hosts, one to which it attaches and the other which it invades (Lipp *et al.* 2002). The difference between a competent pathogen or symbiont and an incompetent one is the expression of a set of virulence or symbiotic genes in association with the host.

Merrell & Falkow (2004) have argued that commensalism is the ground state for most human-associated bacteria, which acquire their pathogenic habit via horizontal gene transfer (HGT) and genetic recombination. Similar-

ly, *Rhizobium* species can acquire by HGT genes that allow them to nodulate various host legumes and fix nitrogen within the nodule cells (Sullivan *et al.* 1995, Sullivan & Ronson 1998). However, sometimes the rhizobia are poorly effective or even ineffective after lateral transfer of a symbiotic island (Nandasena *et al.* 2006, 2007); these bacteria are cheaters. Because most studies evaluate symbiotic competence on the basis of whether nitrogen is fixed, strains defective in a single gene critical for nitrogen fixation could be considered symbiotically incompetent. Nevertheless, sanctions may not be imposed upon them if the plant is not starved for nitrogen.

Suffice it to say, as we learn more about the diversity of rhizobia-legume symbiosis, we will learn more about the altered states in which these organisms live. For example, additional similarities between mammalian pathogens and legume symbionts may become evident as studies proceed. The exploration of such convergences should provide a better understanding as to how this agriculturally and ecologically important mutualism between plants and bacteria evolved.

Evolution of virulence in attaching and effacing E. coli

Escherichia coli is a multi-faceted organism. It is an important member of the mammalian gastrointestinal microflora, and an essential tool for biochemical and genetic research. Although most strains are harmless commensals, pathogenic isolates exist, and cause a variety of diseases in human and animal hosts. Virulent *E. coli* strains can be divided into at least eight pathotypes based on clinical features, the epidemiology of infection, and virulence factors produced (Donnenberg & Whittam 2001). These include uropathogenic *E. coli*, an important cause of urinary tract infections, meningitis-associated *E. coli*, a cause of neonatal meningitis, and the diarrheagenic *E. coli* pathotypes which cause a diverse spectrum of gastrointestinal diseases (Nataro & Kaper 1998). Two diarrheal pathotypes, enterohemorrhagic *E. coli* (EHEC O157:H7) and enteropathogenic *E. coli* (EPEC) are members of a family of pathogens that share a common virulence mechanism, the formation of attaching and effacing (A/E) lesions. Recent studies have examined the similarities and differences between A/E family members, and have addressed the acquisition of virulence by these organisms.

EHEC and EPEC virulence determinants

The hallmark of infection by EHEC and EPEC is the formation of attaching and effacing lesions. Bacterial adhere intimately to intestinal epithelial cells, causing a dramatic rearrangement of the cytoskeleton, resulting in degeneration of the microvilli and the formation of actin-rich pedestal structures beneath the adhering bacteria (Knutton *et al.* 1989, Moon *et al.* 1983). Virulence is

mediated by a combination of common and pathotype-specific virulence factors. The major common virulence determinant is the chromosomal LEE (locus of enterocyte effacement) pathogenicity island. The G+C content is significantly lower than the region of the genome flanking the LEE, suggesting that it was acquired horizontally (McDaniel *et al.* 1995, Perna *et al.* 1998). The LEE contains genes required for A/E lesion formation, including a type III secretion system (TTSS), type III secreted effectors (EspS), and the bacterial ligand (intimin) and receptor (tir). EPEC and EHEC have evolved a remarkable mechanism for adherence and A/E lesion formation: They insert their own receptor, the bacterial protein Tir, into the plasma membrane using the TTSS. Tir then binds to the outer membrane protein intimin, resulting in A/E lesion formation (Deibel *et al.* 1998, DeVinney *et al.* 1999, Kenny *et al.*, 1997). This mechanism allows EHEC and EPEC to adhere tightly to the host in the absence of a specific host cell receptor. In EPEC but not EHEC, the LEE is sufficient to confer *in vitro* A/E lesion formation activity to non-pathogenic *E. coli* K-12 isolates (Elliott *et al.* 1999, McDaniel & Kaper 1997). Whether the LEE region alone is sufficient to convert K-12 from an avirulent strain to an EPEC-like pathogen is unknown, but highly unlikely due to the requirement for pathotype-specific virulence determinants.

In addition to the LEE, both EPEC and EHEC express pathotype-specific virulence determinants. EPEC contains a large plasmid (pEAF) that contains genes encoding a type IV bundle-forming pilus (BFP) (Sohel *et al.* 1996). The BFP is involved in both the formation of bacterial microcolonies and non-intimate adherence to the intestinal epithelia, and is essential for full EPEC virulence (Bieber *et al.* 1998). EHEC isolates are defined by the production of shiga toxins and the absence of the BFP (Nataro & Kaper 1998). The two shiga toxin subtypes (STX1 and STX2) are encoded on lysogenic phages, and inhibit host cell protein synthesis, resulting in severe gastroenteritis and systemic disease. EHEC and EPEC are also distinguished by their host specificity and particular niche within the gastrointestinal tract. EPEC is predominantly a pediatric pathogen, and colonizes the small intestine (Nataro & Kaper 1998). In contrast, EHEC colonizes the large intestine of humans of all ages and ruminants. In humans, EHEC is a pathogen, whereas it can asymptotically colonize the lower GI tract. Whether this interaction is truly mutualistic is controversial. Although colonization does not result in disease, the interaction is not benign, as EHEC can cause A/E lesions in adult cattle and animals and mount an immune response to STX.

Acquisition of virulence determinants by EPEC and EHEC

An intriguing question is how did virulence arise in *E. coli*? Although numerous possibilities present themselves,

experimental data suggests two different mechanisms. Work from Reid and colleagues suggest a stepwise acquisition of virulence, based on multi-locus sequence analysis and the distribution of virulence determinants within 21 different isolates (Reid *et al.* 2000). On the basis of a rate of d_s of 4.7×10^9 /site/year, the authors suggest the radiation of clones began 9 million years ago, and that EHEC O157:H7 and *E. coli* K12 separated from a common ancestor 4.5 million years ago. The authors suggest a stepwise and additive acquisition of virulence factors, with the repeated gain and loss of genes over time. This process began with the insertion of the LEE pathogenicity island in the EPEC and EHEC chromosomes. Acquisition of the LEE is thought to have occurred several times in parallel, with insertion occurring in different chromosomal locations (predominantly pheU and selC tRNA genes) generating distinct clonal lineages. Comparison of the genes encoded by the EHEC O157:H7 and EPEC LEE identified regions with both high sequence similarity and variability (Perna *et al.* 1998). The genes encoding structural components of the TTSS showed low rates of both d_s and d_N substitution, whereas genes encoding Tir, intimin and the type III secreted effectors were highly variable, with more gaps and an increase in both d_s and d_N . This is consistent with observations that genes encoding the secretory apparatus are well conserved across the various bacterial species that express TTSS, but that the secreted effectors are highly variable (Hueck 1998). This also suggests that recombination and mutation occurred within the Tir/intimin and effector genes, (allowing fine tuning for differences in host/lifestyle). Both the EPEC and EHEC lineages subsequently acquired pathotype specific virulence factors, including the genes encoding Shiga toxins and the pO157 plasmid by EHEC, and the plasmid encoding the BFP by EPEC. These factors most likely contribute to differences in host specificity, and the enhanced virulence observed with STX-producing EHEC strains.

A second model suggests that the LEE may have been acquired in multiple steps, rather than by the horizontal transfer of one large region of DNA (Sandner *et al.* 2001). The authors examined the prevalence of LEE-encoded genes for Tir and its chaperone CstT, the type III secreted protein EspB, and the operons encoding the structural subunits of the TTSS, in *E. coli* strains isolated from a variety of species of wild mammals. Markers for the LEE were found in 40% of the strains tested, but surprisingly were not always found together in a given strain. These data suggest that the LEE is a dynamic region, and that the LEE-encoded genes may have other functions when expressed individually in nonpathogenic bacteria. Pathogenic *E. coli* may have acquired LEE-encoded genes by horizontal gene transfer, which were then assembled into a pathogenicity island. A second explanation for these data suggests a loss of virulence by commensal *E. coli*. The LEE may have been initially acquired in its entirety,

and some LEE-encoded genes lost over time through rearrangement and deletion. A compelling question is whether the isolated LEE genes found in wild *E. coli* strains are still functional, and if so, do they play a role in a more mutualistic lifestyle? These questions might be further addressed through additional comparisons of genomes from a compliment of both mutualistic and pathogenic *E. coli* strains. Future research comparing a wider array of *E. coli* genomes may provide further insight into how virulence and pathogenicity islands radiated throughout *E. coli* phylotypes.

Conjugation in *Bacteroides* species: A paradigm of efficiency

Bacteria have evolved sophisticated mechanisms to survive in their environment, and the ability to acquire DNA from the environment confers a selective advantage to many bacteria (Moore & Holdeman 1974, Wang *et al.* 2003). The most efficient method of DNA acquisition is by conjugation, where DNA is transferred from a donor to a recipient bacterium that are in close physical contact (horizontal gene transfer). A large size range of DNA can be transferred by conjugation, including small genes, plasmids and transposons, and even pathogenicity islands. Inter-generic transfer is common, and conjugation-proficient bacteria can acquire genetic material from diverse and frequently, unrelated donors. Conjugation is thus considered a fast and efficient pathway for bacterial evolution.

Members of the genus *Bacteroides* are part of normal human gut flora, and are one of the major anaerobic genera in the colon. *Bacteroides* organisms can grow to high cell density, and are often present at 10^{10} - 10^{12} colony forming units per gram fecal matter (approximately 30% by weight of fecal matter). *Bacteroides* are important human symbionts. Among their many functions, one important contribution is aiding in host digestion by degrading plant polysaccharides and other compounds. Polysaccharide breakdown products are further used as energy sources by the bacteria. The extent and importance of this symbiosis is only now being fully appreciated—the recently released genome sequence of *Bacteroides thetaiotaomicron* reveals the presence of an astonishing number of genes whose predicted products are involved in polysaccharide metabolism (Comstock & Coyne 2003). Although *Bacteroides* are normal human commensal flora, events that lead to their spillage from the colon can have serious clinical consequences, including abscess formation and life-threatening infections (Brook 1989, Elliott *et al.* 2000, Hecht *et al.* 1999). The clinical picture may be further compounded by the fact that many *Bacteroides* organisms are resistant to one or multiple antibiotics (Hecht *et al.* 1999). Resistance is mediated by a plethora of genetic elements, many of which are mobile, and can be efficiently transferred within and from the genus.

For this reason, *Bacteroides* have been referred to as “reservoirs” of antibiotic resistance (Salyers 1999, Salyers & Amabile-Cuevas 1997, Shoemaker *et al.* 2001).

Mobile genetic elements are responsible for gene transfer

Mobile genetic elements harbored by *Bacteroides* may be either plasmids or transposons (Salyers *et al.* 1995a, b, Smith *et al.* 1998). For efficient DNA transfer, two independent sets of biochemical processes are required—(a) processing of the DNA molecule destined for transfer, and (b) assembly of a mating bridge or conjugation pore across the donor and recipient cell envelopes to allow passage of the transferred DNA. Mobile elements that encode both sets of functions are referred to as conjugative transfer factors, whereas those that encode only the processing functions are referred to as mobilizable transfer factors. Mobilizable elements physically transfer from donor to recipient bacteria only when they are co-resident in the donor cell with a conjugative transfer factor, whose mating bridge they can utilize. In *E. coli* and other aerobic bacteria, mating bridge functions are encoded by large conjugative plasmids (drug-resistance or “R” factors), whereas in *Bacteroides*, conjugation functions appear to be encoded primarily by conjugative transposons. Since the latter are chromosomally localized, stably inherited, and mobile, there is efficient and high-frequency dissemination of antibiotic-resistance and other DNA within and from the *Bacteroides*. More than 80% of clinical *Bacteroides* isolates are now resistant to tetracycline, due to the dissemination of resistance genes mediated by conjugative transposons (Shoemaker *et al.* 2001).

In order to understand how DNA is transferred with high efficiency during conjugation within and from the *Bacteroides*, research has primarily focused on analyses of the initial processing events that result in transfer-ready DNA molecules (Sitailo *et al.* 1998). In the case of both plasmids and transposons, these processing events occur within a specific region of the transferred DNA called the origin of transfer (*oriT*), and are catalyzed by proteins called mobilization (Mob) proteins. A major difference between the Mob proteins encoded by transfer factors from different bacterial genera is exemplified by anaerobic mobilizable transfer factors, many of which require fewer Mob proteins to complete initial DNA processing reactions. Multiple small *Bacteroides* and *Clostridium* mobilizable plasmids and transposons encode only one or two Mob proteins (Bass & Hecht 2002, Crellin & Rood 1998, Li *et al.* 1995, Smith & Parker 1998, Vedantam *et al.* 1999) whereas those harbored by *E. coli* and other aerobic bacteria encode 4, 5 or more Mob proteins for DNA processing (Howard *et al.* 1995, Pansegrau *et al.* 1988). Thus, one can argue that Mob proteins derived from anaerobic DNA transfer factors have evolved to be more efficient in the DNA processing reactions. In addition, it has been observed that Mob proteins encoded by mobile

elements of anaerobic bacteria retain activity in *aerobic* bacteria as well, where mobilization levels similar to, or higher than those observed in the anaerobic background can be obtained. (Novicki & Hecht 1995, Vedantam *et al.* 1999). It is not completely understood how these proteins are expressed and function in diverse backgrounds. Preliminary analyses reveal that the Mob genes of Gram-negative *anaerobic* bacteria have a % G+C content midway between those of *aerobic* Gram-positive and Gram-negative origin. Such data may provide clues to why anaerobic Mob proteins are functional in diverse backgrounds (Vedantam & Hecht unpublished), and hence, might have evolved from different organisms.

Evolution of mobile cassettes

The plethora of *Bacteroides* transfer factors, their ability to be disseminated within and from the genus, and their stability and further transfer from unrelated genera all raise important questions about the evolution of mobile DNA cassettes. A close analysis of Mob genes from *Bacteroides* reveals that there may be a shuffling of important functional modules that has the potential to generate great diversity of mobile elements. This concept of functional module permutation and combination is now being recognized as an important means of generating gene diversity (Burrus *et al.* 2002, Roberts *et al.* 2001, Rowe-Magnus *et al.* 2002, 2003).

Irrespective of the type of mobile element being transferred in *Bacteroides*, it is likely that the same type of conjugation apparatus will be required to physically transport the DNA. Since the apparatus itself is non-discriminatory in terms of the type of DNA transferred, it then becomes all the more important to gain a deeper appreciation of its nature and structure, so that effective blocking agents can be developed to counter the widespread dissemination of DNA carrying antibiotic-resistance, and pathogenesis-related genes.

In summary, *Bacteroides* harbor a wide variety of mobile DNA transfer elements, many of which carry antibiotic resistance genes, and are capable of inter-genus transfer, survival and expression. In many systems studied to date, the transfer of these elements within the *Bacteroides* is sensitive to, and up-regulated by, sub-inhibitory concentrations of a common, widely used antibiotic, tetracycline. Further, in many cases, transfer factors appear to be composed of functional modules that can mutate, mix and match to generate a great diversity of mobile elements. Thus these normal human commensal organisms have evolved to become "reservoirs of resistance" (Salyers 1999), and infections involving such bacteria can have serious clinical consequences. Since they are part of the normal colonic flora, future research will need to incorporate ingenious approaches to eliminate only antibiotic-resistant variants of these organisms.

Microbial toxins promote biodiversity in a real-life game of rock-paper-scissors

There has been increased interest in the exploration of the role of spatial scale in explaining the maintenance of biological diversity (Chesson 2000, Tilman & Pacala 1993). Ecological theory suggests that local interactions and dispersal promote diversity (Durrett & Levin 1994). Further, multiple species can co-exist when they have non-hierarchical, non-transitive relationships (Durrett & Levin 1997). The children's game of rock-paper-scissors illustrates the concept of non-transitive relationships, with rock crushing scissors, scissors cutting paper and paper covering rock. In this game, with all three phenotypes present, no one wins, the game cycles and thus diversity is maintained.

Evolution of colicin gene clusters

Recent work has focused on developing both *in vitro* and *in vivo* models with which to test the impact of non-hierarchical relationships on microbial diversity (Durrett & Levin 1997, Kerr *et al.* 2002). Some of the more successful of these models are based upon one member (the colicins) of the diverse and abundant family of bacterial toxins known as bacteriocins (Chesson 2000, Kerr *et al.* 2002). The bacteriocin family includes a diversity of proteins in terms of size, microbial targets, modes of action and immunity mechanisms. The most extensively studied, the colicins produced by *Escherichia coli*, share certain key characteristics (James *et al.* 1996). Colicin gene clusters are encoded on plasmids and are comprised of a colicin gene, which encodes the toxin, an immunity gene, which encodes a protein conferring specific immunity to the producer cell, and a lysis gene, which encodes a protein involved in colicin release from the cell. Colicin production is mediated by the SOS regulon and is therefore principally produced under times of stress. Toxin production is lethal for the producing cell and any neighboring cells recognized by that colicin. A receptor domain in the colicin protein that binds a specific cell surface receptor determines target recognition. This mode of targeting results in the relatively narrow phylogenetic killing range often cited for bacteriocins. The killing functions range from pore formation in the cell membrane to nuclease activity against DNA, rRNA, and tRNA targets.

Diversity among colicin phenotypes in natural populations

Various mathematical and experimental models have been used to explore the relationship between the three colicin-related phenotypes found in all natural populations of *E. coli*; sensitive cells (which can be killed by colicin toxins), resistant cells (which have altered receptor and translocation systems and are thus resistant to

Strain below	Wins against	Loses against
Killer	Sensitive	Resistant
Sensitive	Resistant	Killer
Resistant	Killer	Sensitive

Table II. - Chemical warfare among microbes as a non-transitive, three-way game similar to the “rock-scissors-paper” game.

colicins) and producer cells (which carry colicin plasmids and produce toxin when induced) (Durrett & Levin 1994, 1997, Kerr *et al.* 2002). Pair-wise interactions among the strains have the non-transitive structure of the childhood game of rock-scissors-paper (Table II). The colicin producer strain beats the sensitive strain, owing to the toxin's effects on the latter. The sensitive strain beats the resistant strain, because only the latter suffers the cost of resistance. And the resistant strain wins against the producer, because the latter bears the higher cost of toxin production and release, while the former pays only the cost of resistance.

Kerr and coworkers (Kerr *et al.* 2002) employed *in vitro* experimental methods and mathematical modeling to illustrate that the maintenance of diversity in this system requires spatial structure; in the well-mixed environment of liquid nutrients in a flask, diversity is rapidly lost - with producer cells killing sensitive cells and then producer cells replaced with more rapidly growing resistant cells. When spatial structure is introduced, in this case by plating cells onto an agar plate, the three different cell types “chase” each other across the plate - with producer cells chasing, and killing, sensitive cells, sensitive cells chasing resistance cells (as they outgrow them) and resistant cells chasing producer cells (as they outgrow them).

Kirkup & Riley (pers comm) have recently shown that a similar cyclical pattern is observed when the same three cell lines are introduced into a mouse colon model. This *in vivo* model employs streptomycin to rid the mouse colon of its Gram-negative bacterial flora. Streptomycin resistant strains can then be introduced into the system, through the mouse's water source, and the establishment and interaction between strains observed. Kirkup and Riley have shown that, just as was observed when spatial structure was introduced in the *in vitro* model, all three strains (sensitive, producer and resistant) cycle through the mouse colon with the same relationships as predicted by theory, i.e. the sensitive cells are only transiently retained when present with producer cells and resistant cells outgrow producer strains. If migration into this close system of caged mice is allowed, then the predicted cycling between strains ensues.

Understanding the interactions between these three microbial phenotypes does more than simply aid in our understanding of how microbial diversity is maintained in natural communities. Such information can directly

inform applied studies as well. For example, one can envision a similar sort of dynamic involved in the evolution of antibiotic resistance. When antibiotics are applied in an environment, it is analogous to introducing a toxin producing strain. The antibiotic rapidly kills sensitive cells and inadvertently selects for resistance. Resistance then dominates until the current antibiotic is no longer useful. Once the selection pressure is removed (antibiotics are no longer applied), then the sensitive population will reinvade the population or community simply because it grows faster than the resistant strains. Viewing the evolution of antibiotic resistance from this perspective allows us to incorporate evolution and ecological theory into drug design. Resistance is usually considered to be an undesirable, but unavoidable consequence of microbial evolution. However, armed with evolutionary theory we can make rational decisions about how to design drugs that are more difficult to resist (thus slowing the transition from sensitive to resistant) or more costly to resist (thus speeding up the transition from resistant to sensitive).

HIV evolution and virulence

A practical implication of the evolution of infection and virulence is adaptive microbial evolution to therapeutic interventions. One good example is the evolution of drug resistance by human immunodeficiency virus type 1 (HIV-1). Drug therapy to HIV infection is typically done with combination therapy has dramatically reduced the rate of HIV-1 and AIDS-related morbidity and mortality. The lack of compliance to drug administration may result in suboptimal therapy, which can lead to drug resistance. Drug resistance limits the clinical benefit of drug treatment and can select for new variant viruses with altered virulence and tropism.

The HIV-1 mutation rate is high (i.e., 4×10^{-5} mutations per target bp per replication cycle, which correlates to about one mutation in every 3 new genomes produced) and likely aids in the rapid development of drug resistance during suboptimal therapy (Mansky & Temin 1995). Transmission of HIV-1 with reduced susceptibility to antiretroviral drugs may compromise the efficacy of drug therapy (Garcia-Lerma *et al.* 2001).

Drugs, drug resistance, increased HIV mutation rates

The impact of drugs and drug-resistant virus on HIV-1 mutation rates was done (Mansky & Bernard 2000) in light of earlier observations made with other retroviruses (Julias *et al.* 1997, Julias & Pathak 1998, Pathak & Temin 1992). Both drugs and drug resistant virus were capable of increasing the virus mutation rate (Mansky & Bernard, 2000). Recent studies with other drugs indicate that drug treatment may generally lead to increased virus mutant frequencies during HIV-1 replication (R Chen & LM Mansky unpublished observations) (Mansky 2003, Mansky *et al.* 2003). These observations suggest that when virus replication occurs in the presence of suboptimal concentrations of drug, drug-resistant virus is selected for and that replication of drug-resistant virus in the presence of drug could further increase the virus mutation rate. This has been shown to be the case (Mansky *et al.* 2002). Interestingly, different drugs used in conjunction with a drug-resistant virus can cause the same affect (Mansky *et al.* 2002). This indicates that when new drugs are added in drug therapy regimens they could also act with the drug-resistant virus to further increase virus mutant frequencies even though the drug-resistance phenotype is associated with another drug.

Although perhaps counterintuitive, an intentional increase in mutation rate has been speculated as a rational approach for antiviral treatment of RNA virus infections (Drake & Holland 1999). RNA viruses have high mutation rates and are particularly vulnerable to increases in mutation rate that could extinguish virus replication, by error catastrophe. The inhibition of RNA virus replication with ribavirin, a ribonucleoside analog, has been associated with error catastrophe for some RNA viruses (Contreras *et al.* 2002, Crotty *et al.* 2000, Severson *et al.* 2003), but not others (LCMV) (Ruiz-Jarabo *et al.* 2003). Promutagenic nucleoside analogs, which are incorporated into the viral genome during nucleic acid replication and result in a progressive accumulation of mutations that would ultimately lead to a drastic reduction in virus replication and fitness, have also been used to extinguish HIV-1 replication (Loeb *et al.* 1999). Given that the majority of mutations are deleterious, selection against such variants would reduce virus yield within a single cycle of replication and allow the maintenance of some significant level of virus fitness within the population. The success of eliminating HIV-1 replication by error catastrophe (also called lethal mutagenesis), has yet to be tested outside of cell culture systems.

Vertebrate host cells have evolved powerful strategies to eliminate retroviral infections by lethal mutagenesis. The apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC 3) proteins are cytosine deaminases that provide intrinsic antiviral immunity to HIV-1 infection (Harris & Liddament 2004). In the case of HIV-1, the APOBEC 3G and APOBEC 3F proteins have

been shown in particular to attack and destroy infectious virus by C-to-U hypermutation of the viral genome during minus-strand DNA synthesis (Hache *et al.* 2005, Harris *et al.* 2003, Liddament *et al.* 2004, Sheehy *et al.* 2002). These APOBEC 3 proteins attack the viral nucleic acid after being recruited into virus particles. HIV-1 normally evades such attacks by deflecting the APOBEC 3 proteins from particles with the HIV-1 Vif protein, which targets the Vif-APOBEC 3 protein complexes for degradation by the proteasome (Sheehy *et al.* 2003). The development of small molecule inhibitors of the Vif-APOBEC 3 protein interaction will be important for the application of lethal mutagenesis of HIV-1 by the APOBEC 3 proteins.

Antimicrobial drug resistance and increased pathogen mutation rates

There is a growing body of literature indicating that mutator alleles are selected for in microbial populations, particularly in response to environmental stress (Bjedov *et al.* 2003, Sniegowski *et al.* 1997). For instance, the emergence of antimicrobial resistance during drug therapy can increase the likelihood of selection for mutator alleles, as well as increase the probability of failure of subsequent drug therapies (Martinez & Baquero 2000, O'Neill & Chopra 2001) (Table III). The generation of drug resistance depends on the rate of emergence of resistant mutants which is defined by the mutation rate. In bacteria, there are many examples indicating that antibiotic treatment not only selects for antibiotic-resistant bacteria, but also selects for mutator alleles which confer a higher mutation rate (Giraud *et al.* 2001, 2002, Kohler *et al.* 1997, Mamber *et al.* 1993, Negri *et al.* 2002, Oliver *et al.* 2000, Ren *et al.* 1999, Tenaillon *et al.* 2001). Correlations between mutation rate and the efficacy of antimicrobial drug treatment have recently been observed (Gerrish & Garcia-Lerma 2003). Error-prone polymerases and mutations of the mismatch repair system, along with mutations of enzymes that protect DNA from DNA damaging agents and enzymes that degrade modified nucleotides, have been implicated as the ultimate mechanisms

Table III. - Examples of antimicrobial resistance associated with increased pathogen mutation rates

Example	Reference
<i>E.coli</i> /rifampin resistance	(2)
<i>E. coli</i> /streptomycin resistance	(5)
<i>S. aureus</i> /vancomycin resistance	(6)
<i>S. pneumoniae</i> /cefotaxime resistance	(3)
<i>P.aeruginosa</i> /rifampicin resistance	(4)
<i>H. pylori</i> / rifampicin resistance	(1)

responsible for these mutator phenotypes (Table III) (Boshoff *et al.* 2003, Denamur *et al.* 2000, LeClerc *et al.* 1996, Oliver *et al.* 2000, Radman 1999).

In summary, increased HIV-1 mutation rates can be associated with the evolution of drug resistance, and this observation correlates with observations made in bacterial systems with antimicrobial drug resistance. The transmission of drug-resistant HIV-1 along with the development of drug-resistant virus raises concerns about the efficacy of drug regimens due to the presence of mutator phenotypes. Future studies should be directed at determining the risk of these mutator phenotypes in HIV-1 drug resistance. In addition, the unintentional increase in HIV mutagenesis by drugs could be used for improving the efficacy of drug therapy by the rational selection drug combinations that either minimize the potential for HIV mutagenesis or intentionally increase HIV mutagenesis to induce (perhaps along with a mutagen) error catastrophe. Drug resistance may select for new variant viruses with altered virulence.

CONCLUSIONS

The direct impact of “evolved” virulence is subject to many interactions at a variety of levels. Previous thought on the evolution of both pathogenic and mutualistic symbioses considered different roles for interactions between hosts and their microbial partners. Given the variety of examples in this article, and the mechanisms that determine whether they are benign or virulent, raises many questions on the evolution of similar processes of infection and colonization. Understanding, monitoring, and predicting the evolution and spread of infectious disease may depend upon our knowledge and capabilities of anticipating the behavior of symbiotic systems such as those discussed in this paper. The ability to modify or interfere with these infection processes can be approached by first understanding the conditions under which interventions fail. Subsequently, designing protocols to prevent these failures requires the application of evolutionary theory and deciphering complex molecular interactions (i.e. antibiotic resistance). Although there has been a growing interest in understanding the evolution of infectious diseases and anticipating their emergence, the integration of evolutionary biology with the study of pathogen interactions will significantly contribute to the development of predicting disease resistance in such systems. Considering that similar mechanisms exist between mutualistic and pathogenic associations are remarkable yet can be misleading. For example, many model systems have been adapted for use in laboratory experimentation, but do not represent naturally occurring populations in the wild. Comparing both laboratory and field based experiments will help further our understanding of the nature of symbiotic relationships, and their overall evolution as novel “species”.

ACKNOWLEDGEMENTS. - MKN is supported by NSF (DEB-0316516 and IOS-0744498) and NIH (S06-GM08136-26 and NIAID 1SC1AI081659-01). AMH's research is supported by NSF (EF-0626896 and IOS-0747516), US-Mexus (200708551) and the Shanbrom Family Foundation. RD is supported by Canadian Institutes for Health Research, the National Sciences and Engineering Council of Canada and the Canadian Bacterial Diseases Network, and is an Alberta Heritage Foundation for Medical Research Scholar. MAR is supported by NIH (GM58433). LMM is supported by the American 797 Cancer Society (RPG0027801), the Campbell Foundation, and the NIH (GM56615 and 798 AI053155).

This mini-review was the outcome of a symposium held at the Society for the Study of Evolution in Chico, CA in June 2003 entitled “Understanding the evolutionary patterns and processes of virulence”. SSE partially supported the speakers for travel and registration to attend the symposium.

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Zipfel C 2008. Pattern-recognition receptors in plant innate immunity. *Curr Opin Immunol* 20.

Received August 1, 2007

Accepted May 28, 2008

Associate Editor: S v Boletzky