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EVOLUTION OF VIRULENCE IN AN EXPERIMENTAL BACTERIOPHAGE SYSTEM

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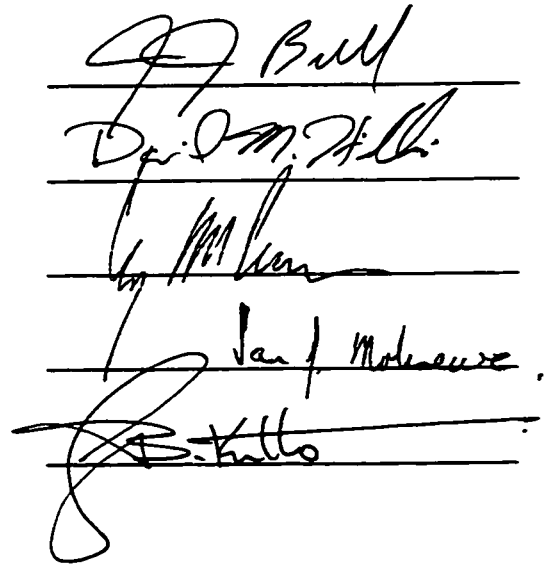

EVOLUTION OF VIRULENCE IN AN EXPERIMENTAL BACTERIOPHAGE SYSTEM

by

Sharon Lee Messenger, M.S.

Dissertation

Presented to the Faculty of the Graduate School of

the University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

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DEDICATION

To my parents, Miles and Valerie Messenger,

and my sister Lori,

for their support and their faith in me.

And to Jim, for his friendship, encouragement...

and for being there.

EPIGRAPH

"The very complexity of the natural systems with which the epidemiologist is faced often makes it difficult to tell which of the correlations that he observes are biologically significant. This difficulty is not lessened by the fact that it is quite easy to invent hypotheses that would, if they were true, fit attractively into our puzzle. It is much less easy to determine whether they are true or not; and this step has been omitted with a rather surprising frequency."

Topley, 1942.

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I especially thank Jim McGuire providing encouragement, support, and, most importantly, friendship. Jim has always been there for me whether it has been for scientific advice or for emotional support.

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EVOLUTION OF VIRULENCE IN AN EXPERIMENTAL BACTERIOPHAGE SYSTEM

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The University of Texas at Austin, 1997

Supervisors: James J. Bull and David M. Hillis

An experimental system using a bacteriophage virus and its bacterial host was used to test the hypothesis that increased virulence evolves in response to selection for increased transmission. This model assumes that virulence is correlated with traits that increase fecundity of the pathogen. Transmission rates to new susceptible hosts were experimentally manipulated, varying the relative intensity of selection against virulence. Phage with relatively high transmission rates benefit from increased phage production (fecundity) because their progeny represent a larger proportion of the population at the time of re-infection, while phage with low transmission rates increase their frequency in the population by allowing increased growth and cell division of the host. As predicted by the model, phage transmitted at high rates evolved higher virulence (indicated by a

slower host cell growth rate) and significantly higher fecundity (determined by phage titers) than phage subjected to low transmission rates. However, other assays of virulence, such as phage impact on host intrinsic growth rate, and long-term survival of the infected host, showed no significant difference whether infected with high or low transmission phage indicating that measures of virulence are sensitive to the method of assay. These results reveal the complexity of the dynamics of virulence and underscore the need for more experimental data to tease apart this complexity to gain a better understanding of the evolution of virulence.

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CHAPTER 1

REVIEW OF THE EVOLUTION OF VIRULENCE AND AVIRULENCE IN PARASITES AND PATHOGENS

INTRODUCTION

Only slightly more than a decade ago a previously unknown virus suddenly captured the attention of the medical community. Now, HIV has emerged as a full-blown epidemic. Afflicting over 29 million children and adults worldwide and resulting in a cumulative total of over 6 million deaths, HIV is now the leading killer for people between the ages of 15-44 (WHO, 1997). This disease has alerted us to the realization that epidemics with high death tolls are not just the subject of history books. There are still many unanswered questions about the exact timing of and evolutionary forces underlying the emergence of this virus in the human population, but there have been suggestions that changes in the virulence, or harmfulness, of HIV influenced the emergence and spread of the disease (Ewald, 1991a, 1994). In 1919, W. W. C. Topley suggested that changes in virulence of a pathogen were a contributory factor in the rise and fall of epidemics (See Lipsitch and Moxon, 1997). Since then changes in pathogen virulence have been proposed as the cause of epidemic spread in the influenza pandemics, including avian influenza epidemics (Webster, 1993; Taubenberger et al., 1997), plague epidemics (Lenski, 1988; Rosqvist et al., 1988), invasive group A *Streptococcus* (Stevens,

1995), and *E. coli* O157:H7 (Strockbine et al., 1986). Traditionally, the underlying causes of an epidemic (i.e., the occurrence of a disease at an unusual frequency) have been treated independently from those underlying virulence (i.e., the degree of harmfulness of a disease pathogen). But do changes in pathogen virulence have the power to spark epidemics?

A more current assessment of epidemics proposes that they are largely a population dynamic process whereby a pathogen can undergo rapid reproduction when the population density of susceptible hosts is high, but the epidemic will wane as the available host density decreases (dying out if the density drops below threshold density) (Anderson and May, 1991; Fine, 1993; Lipsitch and Moxon, 1997). Thus, in this view there is no hypothesized link between epidemics and virulence. But, are there differences in virulence between pathogen strains that predominate during epidemics versus endemic periods? Does a rapidly expanding epidemic favor a more virulent pathogen? If so, do these variants exist at low frequencies in the population ready to take over under the right conditions? From this perspective, the question is not 'do changes in virulence give rise to epidemics?', but 'do changes in population dynamics select for changes in parasite virulence?'.

Such proposals assume that there are general patterns of virulence evolution. If such patterns exist, it would clearly be of great interest to understand not only what factors influence the evolution of virulence, but also whether or not these patterns are general enough to apply across a diversity of parasites. The goal of this review is to evaluate the data on virulence evolution

and the prospects for identifying general rules of virulence evolution, and to consider research directions that might bring us closer to those aims.

What is Virulence?

It is worthwhile to discuss how virulence has been defined and how it will be used in the context of this review, because the definition can affect our perception of its evolution. Certain pathogens may be virulent by some definitions, but not by others. So, what is virulence? In theoretical population biology literature, virulence generally has been defined as the increase in host mortality caused by the parasite (Levin and Pimentel, 1981; Anderson and May, 1982, 1991; See Read, 1994 for a review). These models rarely include a specific term for morbidity resulting from pathogen virulence, although morbidity could play a important role in influencing how virulence evolves. Other definitions are more general encompassing any decrease in host fitness caused by the pathogen, which extends to host reproduction as well as survival. Virulence has even been used to refer to a pathogen that spreads particularly quickly, again intermingling the concepts of epidemics and virulence (See Fenner and Cairns, 1959).

For purposes of evolution, virulence is nearly always considered a property of the parasite. One difficulty in accurately assessing parasite virulence is that various host traits, such as resistance and health, can affect the harm caused by a parasite. The goal is to derive an understanding of how selection acts on virulence determinants (i.e., any trait of a pathogen whose loss decreases virulence). But identifying virulence determinants is not trivial. Virulence is

often determined by a complex set of traits, potentially encoded by several genes, thus an understanding of the evolution of virulence may require an understanding of the evolutionary forces acting on several virulence determinants and their interactions.

The focus on virulence determinants, however, can lead to a narrow view of the evolution of virulence. While virulence determinants by definition contribute to the virulence of a pathogen, other selective forces acting on the pathogen can also affect the virulence of the pathogen. For example, antibiotic resistance genes may not be categorized as virulence determinants, yet in the presence of antibiotics the acquisition of such genes can increase the pathogenicity of that bacterium by allowing colonization, propagation and transmission compared to the antibiotic sensitive variants. In the strict sense, one may not want to recognize antibiotic resistance as a virulence determinant because selection for resistance acts without regard to the virulence of that pathogen. Thus, understanding the evolution of antibiotic resistance does not address why parasites evolve to harm their hosts. The acquisition of antibiotic resistance genes, however, can enable a bacterial strain once held at bay to become a threat to public health. Temporal shifts in strains of cholera associated with epidemics in India appear to be influenced by resistance patterns to antimicrobial drugs (Mukhopadhyay et al., 1996; Waldor et al., 1996; Mooi and Bik, 1997). In 1992, a new serotype of cholera, O139, replaced the O1 El Tor cholera that had been present there for years. The O139 serotype was known to carry a conjugative transposon that encodes multiple resistance to antibiotics (Waldor et al., 1996). After a brief period, the O1 serotype resurged,

but upon further examination it was discovered that this O1 serotype differed from the one prevalent before 1992. The difference was that the new O1 strain was multidrug resistant (Mukhopadhyay et al., 1996). Although further testing would be required to rule out alternative factors such as changes in herd immunity of the hosts, these data suggest that resistance to antibiotics may have been an important selective agent in the shift of serotypes observed. This shift in serotypes associated with epidemics were perhaps erroneously hypothesized to be caused by changes in virulence of the pathogen (Ewald, 1991b, 1994; See below), but it may be the shift in serotypes in response to other selective pressures on the pathogen that caused the change in virulence, if indeed a change in virulence has occurred.

Similarly, overlooking non-genetic changes in virulence of the pathogen also may hinder studies of virulence evolution. There is a growing consensus that most emerging and re-emerging diseases result from ecological changes (e.g., human activities such as urbanization, agriculture, deforestation or global climate changes correlated with human impact) rather than evolutionary (i.e., genetic) changes in the pathogen (Morse, 1995; Schrag and Wiener, 1995). A related question is 'how important are ecological factors in affecting the virulence of pathogens?' Can such host and environmental influences of virulence tilt the balance in any given parasite-host interaction to favor different outcomes? Within the ecosystem of the host, a pathogen must contend with competitors, sometimes their own parasites, physical, chemical, and physiological niches, and temporal variability. A brief examination of some ecological factors and their effects on virulence is informative.

Host Factors, Ecological Factors and Virulence

Host genotype.— The effect of host genotype on susceptibility to infection has been documented for several infectious diseases (e.g., myxomatosis, cholera, malaria). In several cases, genetic differences in blood types are correlated to differences in susceptibility to disease. The mutation of hemoglobin associated with sickle-cell anemia is now well-known to be protective against malaria, as are glucose-6-phosphate dehydrogenase deficiency and other hemoglobinopathies (Allison, 1954, 1960, 1982; Livingstone, 1971; Miller, 1994). Individuals with blood group O exhibit more severe symptoms than those with other blood types when infected with cholera (Glass and Black, 1992; Richardson, 1994).

Other physiological factors can influence disease severity. For example, resistance to myxoma evolved in Australian rabbits after the introduction of the virus, and though the underlying mechanisms of resistance are not known, the evolution of resistance over a relatively short time period suggests genetic variation in host susceptibility in those rabbit populations (Fenner and Ratcliffe, 1965).

Hypochlorhydria (i.e., low stomach acid), which has both genetic and non-genetic causes, is known to be a risk factor for cholera infection and is associated with increased disease severity by effectively increasing the number of infecting bacteria that can survive to the intestine (Richardson, 1994). This condition could be associated with malnutrition, B_{12} deficiency, or gastritis.

Additionally, individuals regularly taking antacids are at particularly high risk for infection from cholera because the antacids slow down the production of stomach acids that normally kill bacteria (Richardson, 1994).

Dosage.— There is a linear effect of dosage on disease severity in humans infected with *Vibrio cholerae* (Glass and Black, 1992; Richardson, 1994) Dosage can be affected both by the medium by which *V. cholerae* is ingested (e.g., water or food) as well as by the physiological state of the individual ingesting the bacteria (e.g., Ingesting *V. cholerae* with a meal is shown to increase infective doses because some bacteria in a bolus of food may be physically insulated from the gastric juices in the stomach).

Age at infection.— Age at infection has been demonstrated to affect virulence in humans infected with poliovirus. Increased poliomyelitis attack rates (on the order of 20-fold higher) in Europeans living in Morocco in the 1950's were ultimately attributed not to differences in genetic makeup of the two ethnic groups, but to age at infection (Paul and Horstmann, 1955; Nathanson, et al., 1995). Moroccans often acquired polio infections while they were infants still protected by maternal antibody, but Europeans often were infected later in childhood. Ironically, the later age at first infection in the European population was attributed to increased purification of the water supply which reduced transmission of the virus– hence polio became a disease of improved hygiene. Similar results were found in studies that compared populations in Cairo, Egypt

with Miami, USA (Paul, 1971), and neither study found evidence of genetic variation in virulence of the polioviruses.

Acquired immunity.— Changes in acquired immunity in a host population impose strong selective pressure on pathogen populations. Acquired immunity to cholera has been suggested to cause cycling of different antigenic types (Mooi and Bik, 1997). Although there is cross-serotype immunity, immunity is more complete for the original infecting strain than for the cross strain. Sorting out changes in virulence of the pathogen from changes in acquired immunity in the host population could be especially problematic in regions such as Bangladesh where multiple biotypes (classical and El Tor) are present. Serotype conversion of El Tor cholera from the Ogawa to Inaba serotype within the course of a single infection has been documented and the switch was correlated with a relapse in the patient, suggesting that the Inaba serotype was able to temporarily evade the immune system (Gangarosa et al., 1967; Manning et al., 1994).

Presence of other microorganisms.— The presence of competitors or parasites (e.g., phage) have been discovered to influence the virulence of some pathogens. Toxins produced by *Vibrio cholerae* (Waldor and Mekalanos, 1996) *Corynebacterium diphtheriae* (Freeman, 1951), *E. coli* O157:H7 (Strockbine et al., 1986), *Staphylococcus aureus* toxic shock syndrome (Schultzer et al., 1983), and one of the toxins associated with group A *Streptococcus pyogenes* toxic-

shock like syndrome (Johnson and Schlievert, 1984; Musser, 1996) are some examples of bacteriophage-encoded virulence determinants.

The presence of other microorganisms within the host have also been linked to changes in virulence though the mechanisms are not always understood. For *Staphylococcus* toxic shock syndrome the presence of toxic shock syndrome toxin 1 (TSST-1) may act synergistically with other endotoxins from the normal bacterial flora to induce a dramatic immune response (Schlievert, 1982). Additionally, the presence of *E. coli* is suspected to affect disease severity of *Staphylococcus* toxic shock syndrome. Strains of *S. aureus* that produce toxic shock syndrome toxin (*tst*) are tryptophan-dependent (Trp^-) and evidence suggests that the *tst* determinant is situated within the Trp operon (Chu et al., 1988; Chu et al., 1989). Chu et al. (1989) showed that toxin producing (Trp^-) strains of *S. aureus* must obtain tryptophan exogenously, and are able to grow (and produce toxin) when they are co-cultured with *E. coli.*

Toxic-shock like symptoms associated with Group A *Streptococcus* are have been seen in individuals also infected with *Varicella* or *Influenza* and may have been a route of entry for the bacterium (Stevens, 1996). Dengue hemorrhagic fever (DHF), a particularly severe form of dengue, is associated with infections by more than one serotype of dengue virus (Monath, 1994). In these cases, disease severity is generally associated with over-reaction of the immune system.

Though experiments are still ongoing, *Helicobactor pylori* could be associated with increased risk of infection of cholera (Richardson, 1994). *H. pylori* is known to cause irreversible damage to the stomach lining resulting in

chronic hypochlorhydria. *H. pylori* is also transmitted under similar conditions as *V. cholerae.*

These descriptions cover just a sampling of the numerous cases in which non-parasite factors are associated with disease infection or severity. The question is how much do these factors impede our search for patterns of pathogen evolution, and are they influential enough to thwart us from accurately describing virulence evolution? The area where these factors have the greatest potential of obfuscating our understanding of pathogen evolution is in retrospective analyses, because many of these studies rely on interpretations of correlational data spanning long periods of time and which is often collected inconsistently.

Social Impact and Relevance

Virulence of a human or agricultural parasite is intimately linked to the impact of disease on society, but it is only part of the social equation. Disease impact must take into consideration not only virulence, but also the number of afflicted individuals. If the immediate goal of society is to reduce mortality and suffering from infectious diseases, one solution would be to find ways to avoid infection in the first place. This has been the traditional tack of medicine and public health with obvious merit. Despite this, emphasis is often placed squarely on virulence. Human deaths from the highly virulent rabies virus are a very rare occurrence in the United States (1-2 deaths/year on average; Rupprecht et al., 1995), yet rabies invokes greater fear than the much more prevalent influenza which in fact has a more considerable mortality.

An alternative solution, albeit less intuitive, is to select for less virulent pathogens. This has been the suggestion of Paul Ewald (1983, 1987, 1988, 1991a, 1991b, 1993, 1994) who has been the dominant figure investigating factors that contribute to the evolution of pathogen virulence. He has suggested that certain human behaviors, referred to as cultural vectors, may contribute to the evolution of more harmful pathogens by transporting pathogens more efficiently from an infected individual to susceptible hosts. If this is true, then we may be able to control the level of virulence of a given pathogen simply by changing relevant cultural practices. In other words, we may have the ability to force some pathogens to evolve to be less harmful.

These ideas are based on certain assumptions about how pathogens evolve that depend upon an association between transmissibility and virulence (See Models of Virulence Evolution). The idea that we may be able to manipulate parasites to our benefit (i.e., lower the transmission rates, reduce the virulence) is very enticing, not only because the solutions are within our current realm of technology, but also because they suggest that we can render parasites harmless without having to completely eradicate them. However, the feasibility of such an endeavor relies on a better understanding of the population biology and evolutionary ecology of disease pathogens as well as the existence of predictable responses of pathogens to given selective pressures. If we were to heed the message of Ewald's and select for decreased virulence in our pathogens, would we be better or worse off?

Many of Ewald's proposed solutions to select for less virulent pathogens (e.g., purified water systems, mosquito netting and screens for houses, more

rigorous sanitation procedures in hospitals), in fact, are the same solutions already proposed by the medical and public health community to avoid infections. Even if the long-term evolutionary responses of pathogens are unpredictable, the progress made by improved hygiene and water purification can attest to the power of prevention in fighting infectious pathogens. An important consideration to make, however, is whether or not the evolution of decreased virulence can also decrease the overall population mortality and morbidity attributed to that disease (i.e., disease impact). Despite a drop in deaths per infection, overall mortality could increase if the evolution of decreased virulence allows for an increase in the number of individuals infected as predicted by mathematical models (Lenski and May, 1994). Some have suggested that this is what has happened with the El Tor biotype of cholera (Mooi and Bik, 1997; See below) and must be considered as a possibility for other pathogens if we are looking at this from a public health standpoint.

ARE THERE GENERAL MODELS FOR THE EVOLUTION OF VIRULENCE?

A major issue in the study of virulence evolution is whether there are general rules that encompass the diversity of strategies used by infectious pathogens. If so, a better understanding of these rules could have tremendous impact for those fighting disease. If, however, every pathogen or parasite plays by its own rules and the complexity overwhelms any threads of generality, then we may have little hope of predicting or preventing infectious diseases via understanding the evolution of virulence. Evaluating the empirical support for

the models of virulence evolution can provide a context for where we currently stand.

Historical perspective

The Conventional Wisdom.— Historically, there was one general guiding principle of the evolution of virulence taught by medical, veterinary and parasitological texts which was believed to be largely true of all pathogens despite their known diversity in life history strategies, and this view is still commonly held. Parasite should evolve to be less harmful to their hosts because more virulent parasites could drive their hosts to extinction, dragging the parasites with them (Smith, 1934; Zinsser, 1935; Swellengrebel, 1940; Burnet and White, 1972; Hoeprich, 1989). An alternative form of this model is that the best adapted parasites inflict the least harm on their hosts. In this view, virulence is ultimately maladaptive for both the host and the parasite, thus selection should favor less and less virulent pathogens, and given enough time, pathogens should evolve toward a symbiotic (e.g., commensal) association. A corollary of this hypothesis is that more virulent parasites are indicative of a more recent association with a given host (Dubos, 1965; Burnet and White, 1972; Mims et al., 1995).

This hypothesis relished support from observational data. Many pathogens are observed to be less virulent in hosts of long association than they are in naive hosts. Mammalian diseases such as trypanosomiasis, malaria, myxomatosis, hemorrhagic fever and encephalitis, are known to be more

virulent in exotic hosts than in their native hosts (Fenner and Ratcliffe, 1965; Allison, 1982) Detractors from the traditional view focused on the exceptions to this rule (e.g., Rabies is believed to be an "old" disease of mammals, but is one of the most virulent mammal virus.), and further stated that the evidence to suggest that parasites inevitably evolve to become benign was insufficient (Ball, 1943; Andrewes, 1960; Coatney et al., 1971; Toft and Karter, 1990).

Despite the appearance of empirical support, this idea has been criticized on its reliance on group selection (Levin and Svanborg Eden, 1990; Lenski and May, 1994; Levin, 1996). Evolution of decreased virulence may be beneficial for the long-term survival of the lineage, but it would not necessarily be favored from the standpoint of the individual. Also, this theory was not easily rejected by data that seemed inconsistent, because there was no predictable time frame within which the expected changes in virulence were to occur. It is also the case that the data supporting "conventional wisdom" also support two alternative hypotheses: (1) highly virulent parasites select host resistance, decreasing the apparent virulence, and (2) selection on the parasite favors parasites that have lower virulence without compromising their reproductive capacity. The conventional wisdom met with more serious challenges in the early 1980's when theoretical population biologists derived mathematical models showing that individual selection could favor either increases or decreases in virulence depending on the specifics of the population dynamics of the parasite-host pair.

Current Framework.— An increasingly prevalent view of virulence evolution is that natural selection can favor high, low, or intermediate levels of virulence

(Levin and Pimentel, 1981; Anderson and May, 1982; Bremermann and Pickering, 1983; Levin, 1983; May and Anderson, 1983). Selection acts to increase pathogen fitness by maximizing the number of new infections derived from a single infection in a naive population (i.e., the pathogen's basic reproductive rate). Selection for increased virulence could be postulated if it were assumed that traits increasing virulence are positively correlated with traits that increase the reproductive potential of the parasite. Consequently, despite selective pressure to decrease virulence in general, selection against virulence may be weaker than selection to increase the reproductive potential and spread of the pathogen. Unlike the conventional wisdom, co-existence of pathogen and host can tolerate high virulence if the parasite regulates host density down to the point that a parasite has on average one successful infection per original infection (Lenski and May, 1994).

In this current view of the evolution of virulence natural selection provides the foundation upon which virulence can not only be maintained, but also can increase under some circumstances. Unlike the conventional wisdom, there is no universal prediction that lower virulence is expected to evolve for all parasites, rather the predicted magnitude and direction of virulence evolution relies on a more detailed understanding of additional parameters (e.g., transmission mode, within-host competition, etc.). From a "one model fits all" approach, this current framework has lead to more and more specific models of virulence evolution investigating the effects of host population structure (Lipsitch et al., 1995a, 1995b; Lipsitch et al., 1996), superinfection (i.e., hosts infected with multiple strains; Levin and Pimentel, 1981; Nowak and May,

1994; May and Nowak, 1995), or within-host dynamics (Nowak et al., 1991; Antia et al., 1994; Bonhoeffer and Nowak, 1994; Lipsitch and Nowak, 1995).

With this new generation of models of virulence evolution, do we now have a sufficient framework to understand pathogen evolution and still maintain enough generality to predict the "behavior" of a wide array of pathogens? If we find that these models are still too simple to accurately describe the dynamics underlying virulence without considering additional parameters, will we lose all hope of generality such that we will have to treat each pathogen-host pair individually?

Virulence: Adaptation or Coincidence?

Given that virulence can affect the fitness of the host as well as that of the pathogen, it is not unreasonable to suggest that traits determining virulence are acted upon by natural selection; however, it is important to recognize that virulence may not always be a major determinant of parasite fitness. In fact, it may be irrelevant to the pathogen if it occurs after the critical period of transmission has occurred, because at that point the progeny have already left the host (Levin and Svanborg Eden, 1990; Bull, 1994). Making the assumption that these traits are adaptations may confound more than clarify our understanding of pathogen evolution.

Some of the most sensational infectious diseases afflicting humans are, in fact, examples of pathogens that have invaded a non-traditional host (i.e., humans) (Craven, 1984; McKee et al., 1984; Peters, 1984; Morse, 1991; 1993). Ebola, hantaviruses, several hemorrhagic fevers, rabies, and viral encephalitis

are the diseases of headlines and best-selling novels, but the pathogens causing these diseases generally do not infect humans in their natural cycle, do not transmit well from human to human, and are clearly maladapted for utilizing humans as a host. Because they are maladapted to humans, these outbreaks generally do not travel far and tend to die out quickly. Examples of diseases of wildlife and domesticated animals (e.g., BSE, ebola in chimps, equine morbillivirus, Australian bat *Lyssavirus*) that spillover from a natural reservoir are also common, though for some diseases their occurrence may be a frequent enough to be considered a serious public health concern (Meslin, 1997).

Similarly, parasites may occasionally find themselves in new environments within a traditional host (e.g., tissue tropisms) to which they have not adapted. In these cases, genes encoding the virulence determinants evolve a function in a different tissue, but in this new environment virulence may result without benefit of increased transmission or reproduction when the pathogen colonizes. Levin and Svanborg Eden (1990) described this as coincidental selection for virulence. Examples include *E. coli* adhesins that appear to have a selective advantage by allowing the bacteria to adhere to and colonize the gastrointestinal tract where *E. coli* is not virulent, but cause infections upon entering the urinary tract where the adhesin does not confer a local advantage in colonization because the inflammation upon infection clears the bacteria (Levin and Svanborg Eden, 1990). Similarly, toxins produced by various soil bacteria (e.g., *Clostridia* spp.) may confer an advantage in that environment, but these virulent toxins do not appear to provide any advantage to the bacteria in colonization, proliferation, or transmission in human hosts.

It is important to identify cases in which adaptation of a pathogen in one environment manifests itself as virulence in a novel environment, because in these cases, a search to understand the underlying selective forces shaping virulence could be misleading. An additional consideration is that virulence may be selected for because it confers a local advantage, and yet it may lead to an evolutionary dead-end. That is, virulence evolves because it confers a local advantage within the microenvironment of the host, but winners of within-host selection may be poorer at transmission to new hosts. This short-sighted evolution model proposed by Levin and Bull (1994) provides an explanation for how certain pathogens persist despite known disadvantages in transmission. Assumptions of the short-sighted model have been clearly stated allowing a framework within which this model can be tested. First, the more virulent individuals are genetically distinct subpopulations that arise by mutation within that host. These genetically distinct subpopulations have a local advantage in colonization, proliferation or transmission that allows them to outcompete less virulent individuals locally. It is this local advantage within the host that translates into a disadvantage for transmission to new hosts, thus these "winners" are potentially evolutionary dead-ends.

How common is this phenomenon? HIV, polio, meningitis (*Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae*), and tuberculosis (*Mycobacterium tuberculosis*) have been cited as potential examples of short-sighted evolution of virulence (Levin and Bull, 1994). More importantly, however, this model is testable with the acquisition of the appropriate data and several of these examples have been examined more

critically within this framework. For example, meningitis (*Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*) may be caused by proliferation of the pathogen in a novel niche, the cerebrospinal fluid (CSF), which can cause central nervous system (CNS) damage when the hosts immune system reacts to the infection with inflammation. Virulence results from short-sighted exploitation of a novel tissue allowing that variant to achieve a local growth advantage and the over-reaction of the immune system by the host. By invading the CSF, however, these variants have no viable route of transmission to new hosts.

One interesting thought regarding this model is that despite selection against any long-term advantage for virulence, the mutation-selection balance could direct the evolution and maintenance of these more virulent pathogens.

MODELS AND THEIR SUPPORT

It is not our goal to exhaustively review all of the models of evolution of virulence proposed (for a comprehensive review see Frank, 1996), but rather to examine the evidence compiled in support of some of these models. Frank (1992), Bull (1994), and Levin and Bull (1994) proposed a general breakdown of models of virulence evolution in which successful reproduction for the pathogen is seen to occur in two stages (Table I). First, upon invasion of the host, the pathogen must achieve a high reproductive potential within the host while avoiding the host's immune system (within-host selection). Secondly, the pathogen must successfully transmit to a new host to begin the infection process again (between-host selection). Success at each of these stages is critical for the

survival of the pathogen, and different forces of selection at each stage contribute to the overall level of virulence that evolves within a pathogen. This breakdown of virulence models provides a useful framework for this review.

Historically, the focus has been on between-host models, not because within-host selection was viewed as less important, but because transmission imposes an obvious filter or bottleneck for parasite success (i.e., only a fraction of the pathogens in any given infected host will realize their reproductive potential by having their progeny successfully infect a new host). Thus, there are numerous models investigating factors that influence which variants get transmitted and how transmission modes alter these dynamics (Anderson and May, 1982; Ewald, 1983, 1994; May and Anderson, 1990; Anderson and May, 1991; Frank, 1992; Bull, 1994; Lenski and May, 1994; Levin and Bull, 1994; Lipsitch and Moxon, 1997). In some cases, the pathogens that achieve greater transmission are also the ones producing the most progeny within the host, but this is not always true (e.g., short-sighted evolution of virulence).

Because most of the empirical studies have focused on the link between transmission and virulence, especially the "trade-off" models, we will concentrate on the evidence in support of these models. Trade-off models suggest that there is a necessary coupling between traits that increase parasite fitness (e.g., increase fecundity) and virulence, and it is because of this coupling that virulence is maintained (Anderson and May, 1982; Bull, 1994; Ewald, 1994, 1996; Lenski and May, 1994; Levin, 1996; Lipsitch and Moxon, 1997; See also Fig. 1). This trade-off exists at the parasite's fitness boundary creating a constraint such that any increase in parasite transmission will cause an increase

in virulence (Bull, 1994). In this model, the prediction is that parasite transmission is maximized at some intermediate, or even high level of virulence that balances the forces of natural selection on the parasite to maximize fecundity and persist in the host long enough to transmit to a new host. If virulence is high, the host may die before transmission can occur, while if virulence is low, the host may clear the infection before transmission, or the parasite may lose out to another more rapidly reproducing strain of the same parasite within that host. From this perspective it has been suggested that selection to maximize transmission can favor increased parasite reproduction despite the concomitant decrease in host survival.

Testing Models of Virulence Evolution

In order to set up a framework for evaluating models, it is important to clarify what assumptions are made. Theoretical models often base their predictions on what is expected under some equilibrium (Non-equilibrium dynamics may be too complex to describe with simple models; Frank, 1996). We assume that virulence, in and of itself, generally does not benefit the pathogen (and by definition never benefits the host). Additionally, the following are specific assumptions of the trade-off models that should be tested:

(1) Virulence is due to genetic changes within the parasite.

(2) High virulence yields high transmission rates (when the host is alive).

(3) High rates of transmission and accompanying high virulence are beneficial and evolve only under certain conditions.

(4) Within-host variation in virulence is minor relative to between-host variation.

In evaluating the evidence in support of trade-off models of virulence (using Table 1 as a framework) we want to consider and to be able to reject alternative hypotheses:

Virulence is due to genetic changes within the parasite.— Host (e.g., resistance) and environmental factors (e.g., infective dose) can influence our measures of virulence even if no genetic change has occurred in the population of the pathogen (See *Ecological Factors and Their Effect on Virulence*). Thus, non-genetic changes in the parasite, as well as genetic and non-genetic changes in the host, must be excluded to accept assumption 1. Given that there are sufficient data to reject the null model of no evolution, alternative models (Table I) should be rejected.

High virulence yields high transmission rates.— We assume a positive association between reproduction within the host and probability of infecting a contacted host (transmission), and that the association between reproduction and virulence, the trade-off, is fixed (Bull, 1994; Pease and Bull, 1988). Virulence is viewed as a "side-effect" of these other traits that contribute to increased fecundity and transmission of the pathogen. The data should be able to demonstrate that increased transmission rates and increased virulence are coupled. For example, experimental data can demonstrate this association by altering transmission rates and measuring virulence.

High rates of transmission and accompanying high virulence are beneficial.— Distinguishing among alternative between-host models requires that virulence be demonstrated to be specifically detrimental to the fitness of the parasite. The direct benefit model suggests that virulence benefits transmission of the pathogen (e.g., sneezing associated with cold viruses), while the neutral model suggests that virulence has no impact on pathogen fitness (e.g., HIV?). Since virulence, if severe enough, could negatively impact any host to the point where transmission is reduced, there is not always a clear distinction between virulence that is beneficial to transmission and virulence that is detrimental. It is also important to consider that cases in which virulence appears to be irrelevant to transmission (e.g., neutral) may in fact fall into the category of coincidental evolution of virulence. The distinction in this case seems to be in determining whether or not the pathogen is observed within the environment under which it evolved (i.e., neutral) or within a novel environment such as a novel host or novel tissue tropism (i.e., coincidental evolution) to which it is not adapted.

Within-host, relative to between-host, variation in virulence is minor.— Increased virulence may be favored within the host regardless of whether or not virulence ultimately benefits transmission. In most multicellular hosts, within-host dynamics are quite likely and have been demonstrated to influence the evolution of virulence (Levin and Pimentel, 1981; Bremermann and Pickering, 1983; Knolle, 1989; Anderson and May, 1991; Antia et al., 1994; Garnett and Antia, 1994; van Baalen and Sabelis, 1995a). Testing among these alternatives

is not trivial given the complex nature of virulence, especially if the plausible scenario exists that increased transmission rates are positively correlated with increased multiple infections and within-host competition. The framework of Bull (1994) offers four alternative models for the evolution of virulence resulting from selection at the within-host level. Within-host models can be tested independently to detect any influence of these factors in determining virulence. Although there is currently less empirical evidence to support any given within-host model, data gathered in support of the trade-off model has correspondingly provided evidence of the role that within-host factors play in the evolution of virulence (Diffley et al., 1987; Dearsly et al., 1990; Herre, 1993, 1995; Ebert, 1994). If both within- and between-host factors are discovered to impact virulence, it is not obvious how the relative contributions of within- and between-host factors can be qualified. Data to test the trade-off models have come from direct and indirect measures of transmission rates and virulence as well as experimental studies that alter transmission rates and measure the resulting levels of virulence that evolve.

Retrospective Epidemiological Data

There is a vast literature focusing on the epidemiology of disease pathogens, consisting largely of correlational data, and these data could be exceedingly useful for formulating hypotheses of pathogen evolution, such as the evolution of virulence.

A large body of evidence gathered in support of a trade-off between transmission and virulence comes from correlational retrospective studies

(Ewald, 1983, 1987, 1988, 1991a, 1991b, 1993, 1994). The premise for the trade-off is that host mobility is critical for transmission: If increased virulence decreases host mobility, resulting in fewer host-host contacts and reduced transmission, then lower virulence should be selected. If, however, pathogens can bypass their reliance on host mobility and utilize alternative strategies (e.g., vectors) for transmission, the selective pressure to decrease virulence would be weakened. Ewald has loosely defined vector to include such vehicles as water and human cultural practices that influence timing and mode of pathogen transmission and promote transfer of pathogens from infected to susceptible hosts. Ewald's "cost-benefit theory of virulence" makes several predictions about the levels of virulence that should evolve in a diversity of pathogens (applied largely to human pathogens) depending upon their mode of transmission. For example, arthropod-borne parasites are predicted to evolve higher levels of virulence than parasites directly transmitted from host to host: Not only does the arthropod vector bypass the problems of host immobility, but with increased reproduction by the parasite and a consequent increased immobility of the host, transmission by the vector may be improved (Ewald, 1983, 1994). Like arthropod-borne parasites, water-borne parasites and parasites exploiting other vectors (e.g., hospital attendants) should gain relatively small fitness costs and large fitness benefits from extensive reproduction inside hosts (Ewald, 1991a, 1991b, 1993, 1994, 1996). Additionally, Ewald (1991a, 1993, 1994, 1996) suggested that changes in human behaviors that increased the rate of horizontal transmission of pathogens

led to the evolution of increased virulence in HIV, influenza, and several bacterial pathogens associated with nosocomial infections.

Ewald has suggested that HIV had existed in the human population before it evolved increased virulence, and that the increased virulence evolved in areas with higher rates of unprotected sexual contact and greater numbers of partners (Ewald, 1991, 1994). That is, increased sexual promiscuity weakened the selective pressure to be less virulent because the virus did not have to persist in these hosts as long.

Additionally, Ewald (1991, 1994) suggested that the 1918 influenza pandemic that killed over 20 million people worldwide was caused by a virus of increased virulence that arose in the trenches during World War I. The trenches presented extremely crowded conditions in which sick soldiers, though immobilized could still transfer infections to their neighbors. Also, the personnel responsible for transporting sick individuals from the trenches to the hospitals and replacement soldiers to the trenches could act as vectors for the virus further providing an appropriate environment for the selection of a strain of increased virulence.

Nosocomial, or hospital-acquired, infections have been demonstrated to increase with increasing contact via hospital attendants (Ewald, 1993, 1994). Ewald (1993, 1994) documented increases in virulence in bacterial pathogens (e.g., *Escherichia coli*) in neonatal wards by showing that the length of outbreaks of pathogenic *E. coli* in hospitals was correlated to increased contact with hospital attendants and decreased stringency of hygiene.

Evidence gathered to support Ewald's predictions come largely from epidemiological data. There is a danger, however, in relying solely on these epidemiological studies to test hypotheses of pathogen evolution, because these data often are consistent with several hypotheses and rejecting alternative hypotheses using such correlational data often prove to be difficult. By using cholera as a case study, we can demonstrate the limitations of retrospective analyses and show that epidemiological data often cannot reject alternative models of the evolution of virulence.

Limitations of Retrospective Analyses: Cholera, a case study

The model: Water-borne pathogens are more virulent than directly transmitted pathogens.— Ewald (1991a, 1991b, 1993, 1994) presented cholera and several other diarrheal diseases as evidence for the "cultural vector hypothesis". Cultural vectors are characteristics associated with human culture that can behave similarly to an arthropod vector by transmitting pathogens from infected, immobilized hosts to susceptibles, and their predicted impact on virulence evolution parallels that predicted for arthropod-borne diseases. He concluded that virulence of water-borne pathogens should be positively associated with their tendencies for water-borne transmission. That is, the more these pathogens rely on water for transmission, the greater virulence they are predicted to evolve (i.e., water-borne transmission selects an increase in virulence). The rationale for this model is that the less reliant a pathogen is on the mobility of the host for transmission, the less cost that pathogen incurs if the host is incapacitated. The

reduced cost of higher virulence frees up the pathogen to increase the rate of reproduction.

Data in support of the model.— Ewald (1991a, 1991b, 1994) surveyed the literature on pathogens causing diarrheal diseases (e.g., *Vibrio cholerae*, *Shigella*, *E. coli*) and upon finding a positive correlation between degree of water-borne transmission and virulence, suggested that variation in virulence could be explained by the degree of water-borne transmission. Furthermore, the advantage to the rapidly reproducing, virulent pathogens would be lost if water supplies were purified, and a shift from severe to benign species after water purification would be predicted. In the 1950's and 60's as water supplies were purified throughout the world, a less virulent El Tor biotype of cholera replaced the more virulent classical cholera biotype. Water purification occurred in stages and those countries that successfully cleaned up water supplies earlier, observed an earlier shift from the more virulent to the less virulent cholera biotype. In Bangladesh, where water purification was hindered by burgeoning populations, the classical biotype persisted. Similar trends with *Shigella* and *Salmonella* were cited. Additional evidence came from the recent Latin American cholera epidemic that began in 1991 in Peru where it was observed that mortality in urban populations where the epidemic emerged was lower than that seen in rural inland areas where the epidemic spread. Correlated with this was increased access to purified water in the cities compared to rural areas.

Factors that affect virulence.— Though the data collected by Ewald in support of the cultural vector hypothesis are consistent with this model and compelling, these data also should be capable of rejecting alternative models. First, the data should be able to reject the hypothesis that the virulence of the pathogen has not evolved. It is worth mentioning briefly here that the El Tor and classical biotypes of cholera are not variants within a population but in fact are independent (possibly separated a long time ago) lineages and are known to coexist in some regions (e.g., Bangladesh). Ewald (1994) recognized this and suggested that closely related species (e.g., within the same genus) could act as proxies for variants within a population in this model, but that interspecies competition would be expected to be weaker than that encountered within species. Whether or not interspecies competition is qualitatively similar to intraspecies competition is debatable, but for the purposes of this review we will make the assumption that we can extrapolate from inter- to intraspecies levels.

Host Genetic differences: Since the epidemiological data are correlational, the possibility exists that hidden variables not considered in the model could be causing the observed trend. One obvious confounding factor in measuring virulence is how to separate changes in the pathogen from variation in host susceptibility. Host susceptibility can be affected by physiological and physical properties of the individual, both genetic and non-genetic. Infectious diseases have been suggested to cause genetic polymorphisms in humans (Haldane, 1949; Clark, 1975, 1976; Duncan et al., 1980). Similarly, genetic differences in host susceptibility have been hypothesized to explain the differences in severity of symptoms observed for a number of diseases. For

example, people with blood group O, though not more susceptible to infection by cholera, are known to develop severe symptoms to infection more often than people of other blood groups (Glass and Black, 1992; Richardson, 1994). Amerindians are known to have predominantly blood group O, thus it is possible that differences in the genetic make-up between rural and urban populations of Latin America could account for a difference in mortality (Mata, 1994).

Treatments: Ewald (1994) cited an increase in mortality as cholera spread from urban to rural areas of Peru as potential confirmation of his hypothesis, but stated that additional data were needed to rule out the possibility that better treatments in urban areas contributed to the trend. Rehydration therapy is an extremely effective and simple treatment for cholera and can reduce mortality of grave infections from ~50% to <1% (Gangarosa and Tauxe, 1992; Bennish, 1994). Rehydration therapy can be administered by family members with household ingredients found commonly even in impoverished areas. Dissemination of information about the treatment, however, generally lags in rural areas. Differences in treatment regimes remain a viable alternative hypothesis for the observed higher mortality rates in rural areas.

Dosage: Levin and Svanborg Eden (1990) suggested that the increased virulence of the water-borne pathogens may result from higher infective doses. A linear relationship between dosage and severity of cholera infections has been verified experimentally in humans (Glass and Black, 1992; Richardson, 1994). Mothers caring for sick children can become infected by their children if they receive large innocula of *V. cholerae*, despite having antibodies that generally indicate acquired immunity to cholera. The more virulent classical cholera is

more dependent upon water transmission, while the El Tor biotype is often food-borne (Mata, 1994). Whether or not the route of transmission (water versus food) has an effect on the dosage of infecting bacteria has yet to be tested.

Acquired immunity: Acquired immunity is a trait of the host population that clearly influences the evolution of disease pathogens. Ewald (1994) recognized the importance of acquired immunity to cholera and incorporated this factor into his cultural vector hypothesis as a way to explain the cycling of El Tor and classical strains in Bangladesh. He further stated that if water systems were less pure than those encountered in Bangladesh, cholera would be predicted to become more virulent, citing as an example the outbreak of 1992 and 1993 in India and Bangladesh. This outbreak was the first known case of a cholera epidemic caused by a non-O1 serotype, called O139 (Mukhopadhyay, 1996; Mooi and Bik, 1997). Further investigation suggested that O139 was derived from the insertion of a new O antigen into an otherwise characteristic O1 type, and evidence is accumulating that this transfer was phage-mediated (Waldor and Mekalanos, 1996). In populations where O139 emerged, there was no acquired immunity to this antigenically-different strain, and it spread quickly. O139 also differed from the characteristic O1 type in possessing a multiple-drug resistance plasmid (Waldor et al., 1996). Despite his earlier acknowledgment that acquired immunity can affect pathogen evolution, Ewald (1994) has suggested that the emergence of this strain occurred in response to lower water quality and consequent increases in transmission rates. The data, however, cannot reject the possibility that the strain emerged because of a competitive advantage offered by either the absence of herd immunity in the host population

or by multiple drug resistance. Ewald (1994) suggested that acquired immunity to cholera was a factor consistent with the cultural vector hypothesis. However, a strain for which its host population has no acquired immunity will have an advantage regardless of its level of virulence, and thus does not shed light on why parasites evolve to harm their hosts.

Additionally, it was noted early on that when cholera returned to areas that had previously experienced outbreaks, severity and mortality rates were lower (Svennerholm et al., 1994). This was attributed to acquired immunity that may be retained in some individuals for much longer periods than in others. If an epidemic is recurring in a given geographic region (whether we recognize this fact or not), this could be interpreted dubiously as a decrease in virulence even if the pathogen has not changed.

Environmental factors: Environmental factors influence not only the evolution of virulence, but also our ability to accurately measure virulence. Ewald (1991a, 1991b, 1993, 1994) has assumed that pathogenic *Vibrio cholerae* rely heavily on the human gastrointestinal tract for reproduction and transmission and do not persist for long periods of time in water (i.e., water is the conduit for transmission, but *V. cholerae* can only survive for a few days to a few weeks), while the non-pathogenic forms of *V. cholerae* are almost strictly aquatic and only occasionally invade humans whereby they incidentally cause mild disease. This, however, does not fit with more recent data that suggest that pathogenic strains of *V. cholerae* 01 can exist in aquatic environments for long periods of time in a nonculturable but viable state that can revert to a potentially pathogenic state upon reentry into an amenable environment (Colwell and Huq,

1994a, 1994b). If *V. cholerae* can remain viable for long periods of time outside of the human gut, then there is not a strong reliance on host mobility for transmission for either the El Tor or classical biotype. That is, there should be no greater cost of increased virulence for strains that are less dependent on water-borne transmission (El Tor) than for strains that are more water-borne (classical) if both strains survive well in the environment.

Data suggest that *V. cholerae* 01 are found in association with plankton species (Colwell and Huq, 1994a, 1994b). In some areas where cholera is endemic there is a seasonal component to outbreaks, suggesting that some environmental cues might somehow trigger either the plankton (planktonic blooms) upon which *V. cholerae* is attached or the *V. cholerae* directly. Furthermore, outbreaks caused by different strains appear in divergent localities within days of each other, providing additional support for an environmental component contributing to outbreaks (Glass and Black, 1992).

Alternative Models of Virulence Evolution: Within-host competition.— Coincident with the phenomenon of seasonal outbreaks in certain endemic regions, it is common for people in these regions (e.g., Bangladesh) to become infected with more than one strain. Members of the same family can contract different strains indicating that multiple strains occur sympatrically, and cases are not uncommon in which more than one strain infects a single individual (Glass et al., 1982; Glass and Black, 1992). These regions of high endemism are also categorized by Ewald (1994) as regions of high virulence where a recurrence of the more virulent classical biotype has been observed. If in these

regions hosts are more often plagued by multiple infections of strains that are not closely related, one cannot rule out within-host competition to explain the increased virulence in that area if indeed it is an evolved increase. Similarly, in the Latin American epidemic two El Tor serotypes, Ogawa and Inaba, originally detected in different countries, are now found sympatrically in some regions. (Glass et al., 1982).

Change in virulence or change in reporting?— As surveillance and detection techniques improve, detection of mild or asymptomatic cases are more feasible. Accurate interpretations of long-term retrospective studies, especially those spanning over decades, rely upon consistent measures of parameters. Increased sensitivity in detecting asymptomatic cases could be interpreted as a reduction in virulence (defined as % mortality/infection) even if virulence did not change. It is not clear how such problems can affect accurate estimates of the relative virulence of different contemporaneous strains in comparative studies.

In cholera it is suggested that a large number of asymptomatic infections may play an important role in transmission; however, the number of asymptomatic infections have been very poorly documented (Glass and Black, 1992). Ewald (1991a, 1991b, 1994) defines virulence as deaths per infection, thus any increased sensitivity to detecting asymptomatic cases would be reflected as a decrease in virulence. This may not be problematic unless there are systematic biases in reporting in different geographic regions.

Cholera as a Case Study for Social Impact.— Ewald (1994) has suggested that replacement of more virulent parasites by selection for less virulent strains will reduce mortality. For example, if in Bangladesh classical cholera, with a per infection mortality of 15%, is supplanted by El Tor cholera, with a per infection mortality of 1.4%, mortality rates could be reduced ten fold. This assumes that the number of infections is unaffected. If, however, the El Tor biotype can outcompete the more virulent classical biotype by exploiting more hosts, then it should spread to more individuals, and potentially contribute to greater morbidity and mortality population wide, or at least offset the per-case reduction in mortality (Lenski and May, 1994).

Retrospective studies can be problematic not only because of lack of data, but also because these data often cannot be interpreted unambiguously, and alternative hypotheses cannot be rejected (van Baalen and Sabelis. 1995b). In the cholera case study, data were consistent with the cultural vector hypothesis, but could not reject the null hypothesis of no evolution of virulence (i.e., variation in virulence may result from differences in host susceptibility, treatments, or dosage). Similarly, an alternative model suggesting that increased within-host competition contributed to increased virulence in endemic regions of high virulence (i.e., Bangladesh) cannot be rejected by the data.

Prospective Epidemiological Data

Observational data in which specific parameters could be measured either directly or indirectly offer additional support for the trade-off model.

Such studies afford more rigor and, in some cases, provide the necessary data to reject alternative models of virulence evolution.

Microsporidian parasites of Daphnia.— Ebert (1994, 1995) studied the population dynamics of *P. intestinalis*, a microsporidian gut parasite of *Daphnia* (a planktonic crustacean). This parasite is transmitted by the fecal-oral route and has strictly horizontal transfer. The gut parasite produces vesicles containing spores within the intestinal epithelium of *Daphnia* and this sporeload (i.e., the number of sporophorous vesicles found within a host's gut epithelium) increases over the course of the infection. Ebert used sporeload to estimate the replication rate of the parasite and measured virulence by three separate assays (i.e., impact on fecundity, host mortality, and growth of a clonal host). He found not only a positive correlation between probability of transmission and sporeload, but also between sporeload and virulence (defined as host mortality). In addition to confirming a link between transmission and virulence, Ebert also found that all three virulence measures decreased significantly with increasing geographic distance (i.e., estimate of genetic distance) between the parasite and host pools. Parasites were more virulent in hosts that were geographically closer, which he equated with genetic closeness, to the host in which the parasite evolved. Parasite infectivity (i.e., the ability to infect) was also significantly lower when comparing novel to host clones, but variance in the virulence measures of novel hosts was large, and geographic distance explained only part of that variation. Despite this, decreased virulence is still apparent with increased geographic distance, even when controlled for infectivity .

The increase in virulence observed with increased transmission rates is consistent with the predictions of the trade-off model, and the positive correlation of sporeload with both transmission and mortality rule out that this virulence factor is either neutral or beneficial. The data, however, cannot rule out within-host competition as the predominant factor for the increased virulence observed in the geographically closer lineages. If the parasite produces a higher sporeload in sympatric host clones, then those clones may also receive higher doses of parasites in their water environment. Higher doses may lead to higher mixed infections which could increase within-host competition among the parasites within a single gut.

Nematode Parasites of Fig Wasps.— Herre (1993, 1995) used a comparative approach to study the evolution of virulence in a group of closely-related Panamanian fig wasps and their species-specific nematode parasites. In these studies Herre found population structure to be an important factor in resolving the relationships between nematode reproduction, transmission and the long-term survival of its wasp host. The population structure in this system dictated the relative amount of vertical (i.e., from parent to offspring) versus horizontal (i.e., among unrelated individuals) transmission. Female fig wasps lay their eggs in the inflorescences of fig trees. Different species of fig wasp establish different degrees of vertical and horizontal transmission depending upon how many females lay their eggs in a single inflorescence . Since the nematode parasites are species specific and their life cycles are tightly coordinated to those of their host, their transmission opportunities are tied to the degree of population

structure of the host, allowing a system to test the impact of population structure on the evolution of virulence. The trade-off model would predict that the virulence of a parasite relying strictly on vertical transmission would evolve to be lower than that of a parasite that also transmits horizontally (Bull et al., 1991; Bull, 1994). Herre was able to make comparisons between single foundress wasps in which the nematode relies on strict vertical transmission and multiple foundress wasps in which horizontal transmission is also available to the nematode. Herre directly measured virulence (i.e., impact on the lifetime reproductive success of the wasps) by estimating the ratio of offspring produced by infected versus uninfected foundresses in single foundress figs. He found that the species with the greatest opportunity for horizontal transfer also exhibited the highest virulence. Herre acknowledged, however, that parasites within a fig fruit of single foundress wasps are likely to be more closely related to each other than parasites from multifoundress wasps, and that within-host competition in multifoundress broods therefore would be expected to be greater. Simulation models confirm that increased within-host competition can select for increased virulence in the multifoundress broods and even suggest that it is the predominant force in selecting for increased virulence (Herre, 1993, 1995; Frank, 1996). Thus, the data cannot discriminate between the trade-off model and within host evolution of virulence.

Experimental Data

Even with detailed and careful analyses, correlational studies of virulence evolution cannot control for all variables that can potentially influence

the evolution of virulence to the degree that well-designed experiments can. So, have experimental data contributed to a greater understanding of the evolution of virulence than correlational studies ? At this point, the number of experimental studies of virulence evolution has lagged behind both theoretical and other empirical studies. The experimental work that has been carried out has provided ambiguous support for the trade-off model.

Early Experiments.— In the early part of this century Topley and others (Topley, 1919; Greenwood et al., 1936; Topley, 1942) tested many epidemiological models by infecting mice with a naturally encountered pathogen, the bacterium *Pasturella muris*. Topley (1942) injected *Pasturella* directly into the tissues and assayed virulence by measuring mortality rates and epidemicity by measuring transmission rates. Epidemicity, or the potential for epidemic spread, was measured as the number of mice that become infected upon contact with an infectious individual, while virulence was determined by the size of the dose required to kill half of a standardized stock of mice (i.e., LD_{50}). Two strains of *Pasturella* were found to have high epidemicity and high virulence, two strains had low epidemicity and high virulence, and one strain had low epidemicity and low virulence. Thus, Topley did not find a positive correlation between transmission (i.e., epidemicity) and virulence. It is difficult to assess the results, however, because the method of pathogen infection (e.g., injection of bacteria into tissues) was unnatural. It is possible that the measures of virulence would have been different if animals were assessed after obtaining infections via the natural route of entry.

These studies exemplify a general problem for interpreting virulence evolution. When changing the conditions by which infection normally occurs in wild populations, there is the risk that the adaptation observed in the laboratory does not resemble what would have occurred in nature (Bull, 1994; Lipsitch and Moxon, 1997). For example, when injecting pathogens into the host, a natural filter is bypassed. If variants that normally could not invade the host via natural routes of transmission obtain access to the host, unnaturally greater within-host variation could result, potentially leading to selection for greater virulence.

Myxomatosis.— Fenner's classic studies on the virulence of myxoma virus are often cited as the most convincing studies to date supporting a trade-off between virulence and transmission (Fenner and Cairns, 1959; Fenner and Ratcliffe, 1965; Fenner and Myers, 1978; Fenner, 1995). The myxoma virus was introduced into the European rabbit (*Oryctolagus cuniculus*) population in Australia in 1950 to reduce the impact of this drastically over-populated, exotic species. Although myxoma was known to be relatively benign in its native host, the South American tropical forest rabbit (*Sylvilagus brasiliensis*), it was discovered in the late nineteenth century to be highly virulent in the European rabbit and was subsequently developed in the laboratory as a pest control agent Sanarelli, 1898; Fenner and Ratcliffe, 1965; Fenner and Myers, 1978). Within a decade of the release of myxoma in Australia, progressively less virulent strains of the virus (when measured in standard laboratory strain rabbits) evolved and replaced the more virulent strains.

It was believed that selection to maximize net transmission reduced the virulence of the myxoma virus, a vector-borne pathogen, after its initial introduction into Australia (Fenner et al., 1956, Fenner and Ratcliffe, 1965). It seemed that the more virulent strain killed rabbits too quickly to be transmitted efficiently by the mosquito vector, favoring strains of lower virulence. This idea was further supported by the observation that the virulence of myxoma was lower in the winter when mosquitoes were not available for transmission and increased in the summer as populations of mosquitoes increased.

There was still the question of whether or not a trade-off between transmission and virulence existed; however, alternative hypotheses can explain these data. The virulence (e.g., percentage host mortality, mean survival time) of different strains of myxomatosis was used to define six grades (I-II, IIIA & B, IV-V) with grade I being the most virulent and grade V the least virulent. Assays of these different strains of myxoma virus measured in standardized laboratory rabbits showed that the strains of highest virulence, strains I, II, and IIIA & B, did not exhibit lower titers, suggesting that a trade-off between parasite fecundity and long-term persistence may not exist (Fenner et al., 1956; Fenner and Cairns, 1959; Fenner and Ratcliffe, 1965). In fact, titers of virus in the skin were not apparently different among grades I-IV (only grade V was significantly lower in titer). Additionally, grades I-IV did not show significant differences in transmissibility (again, only grade V showed significantly lower transmissibility). The trade-off model would predict that the level of virulence that evolves should depend on certain ecological parameters, such as host density. That is, under conditions of high host densities, more virulent

pathogens would have an advantage despite the fact that they may be more harmful, because they will benefit from greater early transmission. In the case of myxomatosis, even in populations of high host density, the more virulent virus does not have an advantage during early reproduction because it does not exhibit a higher titer or greater transmissibility than grades II-IV (Bull, 1994). In this respect, myxomatosis does not support the trade-off model.

The difference in transmission among grades I-IV was in the number of days over which the virus could transmit. Grades IIIA & B and IV provided a significantly longer period of time during which the lesions remained open and virus could be transmitted. Rabbits infected with grades I or II died too quickly, while rabbits infected with grade V recovered too quickly. These results suggest that the virus that persists is the one with the greatest net transmission (i.e., net transmission equals the titer times the number of days over which transmission could occur), and in this case that is the virus with intermediate virulence.

Using a simple model, Anderson and May (1982) approximated the level of virulence that would be predicted to evolve to maximize the intrinsic reproductive rate (R_0) if there were no trade-off between virulence and rate of transmission. Transmission rate was treated as a constant, but hosts lived longer and thus transmitted longer when carrying the less virulent viruses. They predicted that the virulence level that should evolve would be intermediate (grades IIIA-IV). Although the calculations were rough, there was a close fit to the observed data, again suggesting that a trade-off may not exist.

Evidence from flea-borne myxoma virus used in Europe does hint at a trade-off between transmission and virulence (Mead-Briggs and Vaughn, 1975;

Anderson and May, 1982). These studies found an inverse relationship between the percentage of rabbit fleas becoming infective (i.e., transmission) and the survival time of the rabbit (i.e., virulence) for all but the most virulent (grade I) strains of the virus. Thus, there is the possibility of a trade-off, but the fact that the most virulent grade of myxoma has one of the lowest transmission rates provides ambiguous support.

Serial Transfer Experiments.— Serial transfer experiments have been undertaken with a diversity of pathogens and hosts and have identified a link between virulence and transmission (Orrego et al., 1982; Diffley et al., 1987; Dearsly et al., 1990; Bull and Molineux, 1992), but, as discussed by Lipsitch and Moxon (1995), these experiments bypass the normal route of infection and can select for individuals which may not necessarily be favored under natural conditions.

Bacteriophage f1.— Experimental studies using a nonlethal bacteriophage virus (f1) and *Escherichia coli* as the model parasite and host have tested whether or not virulence evolves as a correlate of selection for increased transmission by varying the relative degree of vertical versus horizontal transmission (Bull and Rice, 1991; Bull et al., 1991; Bull and Molineux, 1992; Messenger et al., submitted). The model would predict that those pathogens most restricted in their transmission opportunities should evolve decreased virulence. Early experiments (Bull et al., 1991; Bull and Molineux, 1992) compared strictly vertical transmission (from parent to daughter cells) to a regime in which

horizontal (infectious transfer) was allowed. Those viruses restricted to vertical transmission evolved lower virulence and reduced progeny production suggesting a trade-off between virulence and transmission; however, this design not only selected for decreased virulence in the vertical transmission line, but also relaxed selection to maintain the ability to transmit infectiously.

More recent f1 experiments (Chapter 2; Messenger et al., submitted) varied the relative intensity of selection for transmission while maintaining selection for transmissibility and observed changes in virulence in response to this selection. In the f1-*E. coli* model within-host selection can be discounted because only a single f1 virus can infect each host cell. In all experimental lines, selection invariably opposed virulence; however, one line applied relatively greater selection for transmission (relative to selection against virulence) than the other. The prediction was that the greatest levels of virulence would evolve in the line subjected to the most intense selection for transmission. Virulence was measured as the negative impact on infected host cell growth; transmission was estimated from the phage titer. A significant trade-off was observed between phage fecundity (i.e., titer) and virulence. While the qualitative predictions of the trade-off model were confirmed by these results, the magnitude of the response to selection was weak relative to day to day heterogeneity in assay measurements. Additionally, when virulence was measured in other ways (e.g., impact on host growth, long-term survival of infected hosts, and impact on intrinsic rate of reproduction), the null hypothesis of no trade-off could not be rejected. The inconsistent assays indicate that

additional parameters not considered in the design have an important influence on virulence.

Summary of Between-Host Models

Despite the suggestion that transmission modes do play a role in determining the level of virulence that evolves, we are far from the point of predicting the direction or magnitude of virulence that evolves or applying general rules to a diversity of pathogens. Some tests of between-host models (Herre, 1993; Ebert, 1994) also have hinted that within-host factors may be predominant in determining the level of virulence that evolves, though dynamics occurring within the host have remained a black box. We currently are seeing a shift of emphasis toward within-host models that has been spurred on by advances in immunology and improved molecular techniques, allowing detailed analyses of pathogen-immune system interactions. These theoretical studies have begun to divulge the intricacies of pathogen evolution occurring within the host to avoid succumbing to the immune response of the host. When it comes to evading or manipulating the immune system, the diversity of strategies utilized by pathogens increases, and many of the traits acquired to do so are considered virulence determinants. Thus, there is still much to be discovered about the factors affecting virulence, and within-host studies lead to a promising direction for progress.

Within-Host Models

Though within-host models have not been tested to the degree as between-host models, there are data consistent with the predictions of these models, and formal testing of them could greatly expand our current level of knowledge of virulence evolution. In addition to competition between variants within the host, competition between pathogens and the immune system is gaining support as an important determinant of virulence (Marrack, and Kappler, 1994; Smith, 1994; Bangham, 1995; Bothamley, 1996; Graham, 1996; Daenke, 1997).

Antia et al. (1994) modeled the within-host population dynamics of a parasite and the immune system and concluded results similar to those found for the trade-off model. Selection should favor parasites of intermediate virulence, because less virulent parasites are cleared quickly by the immune system, while more virulent parasites kill their hosts too quickly. The model can also accommodate a number of different strategies utilized by pathogens to evade the immune system (e.g., latency, resistance to immune attack, and suppression of immune responses). This theoretical model provides a framework in which to experimentally test the assumptions and predictions, and promises to greatly expand our understanding of virulence evolution.

Nowak et al.'s (1991) antigenic diversity model was developed to explain the observed patterns of HIV infections within a given host. Over the course of infection within a single host there is an increase in the diversity of genetically distinct strains of HIV thought to result from selection to evade the rapidly changing population of $CD4^+$ T-lymphocytes. The model predicts that

the increase in diversity of HIV variants will at some point overwhelm the population of T-cells precipitating a collapse of the immune system. This is what has been observed by molecular biologists. Thus, antigenic diversity is thought to be the virulence factor that causes disease. Other models have reported similar results (McLean, 1993; Mittler, et al., 1995).

Despite evidence of between-host selection affecting evolution of virulence in some systems (e.g., f1 bacteriophage), within-host selection could be the predominant force in others (e.g., HIV?), and a balance between within- and between-host selection, in which between-host factors predominate under some conditions and within-host factors under others, is likely. We have focused on within- or between-host factors in isolation, but it is not clear how much that will tell us about what happens when the two levels are operating in concert, but it is a necessary step.

CONCLUSIONS

Tremendous advances in detection, surveillance, and treatment of infectious diseases (including advances in modern medicine and improved sanitation and hygiene) have occurred within this century, yet we are currently losing ground. Today there is an explosive literature on emerging and re-emerging diseases, resistance of microbes to once-effective treatments, and speculation about factors contributing to disease emergence and evolution. A potential contribution of evolutionary biology to the fight against infectious diseases is to understand how pathogens evolve, to determine whether or not we can predict the evolutionary responses of pathogens, and if so, to use this

knowledge to counter certain pathogen strategies. An understanding of pathogen virulence has been viewed as pivotal in this endeavor.

So, what contributions has evolutionary biology made in understanding pathogen virulence? Have we been able to identify general patterns of virulence evolution or does the complexity overwhelm the signal? The greatest amount of work to date has been in the theoretical realm, and theoretical modeling has created a solid framework in which to further test models of virulence evolution. Though the empirical data so far has not provided unambiguous or conclusive information about patterns of virulence evolution, studies have demonstrated a link between virulence and transmission and that increased virulence is associated with increased transmission rates. What the data cannot do at this point is support the notion that models of virulence evolution are predictive and general. This should not be surprising given that virulence is but one "characteristic" of a pathogen upon which selection acts, and that the selective forces acting upon virulence itself are inextricably linked to selection acting on the host.

Where should we go from here? The area of study that has lagged behind is also the area that provides us with the best hope of untangling some of the complexity underlying virulence evolution, namely experimental studies. The prospects for the future of model testing are great. Advances in molecular techniques, and a rapidly increasing database of knowledge of immunology, epidemiology, and pathogen biology allow for more sophisticated experimental designs. There is a mutually beneficial relationship between molecular biologists, epidemiologists and evolutionary biologists that produces a far more

comprehensive picture of disease evolution than any of these fields could produce in isolation creating a realistic possibility of improved understanding of pathogen evolution.

However, in addition to expanding experimental research, the current body of evidence requires that we develop an appreciation for the complexity of virulence and further suggests that we may not find many unifying general principles. Greater progress in countering infectious diseases may be realized by avoiding sweeping generalizations and embracing the details that, though they may not extend beyond the specific case, at least have proven utility in that specific case.

CHAPTER 2

EXPERIMENTAL EVIDENCE FOR THE EVOLUTION OF VIRULENCE

INTRODUCTION

A triumph of modern medicine has been the prevention and treatment of infectious diseases. Historically and into the first part of this century, bacterial diseases were mankind's biggest killers; many viral diseases were also scourges, although the tolls from viruses never evidently reached the numbers taken by TB, pneumonia, and plague. At least for those of us lucky enough to have lived in Western Civilization in the last 50 years, the medical advances of antibiotics, vaccines and good hygiene diminished these threats so much that the major sources of mortality shifted to heart disease and cancer.

These achievements of modern medicine are now challenged by microbial evolution. Drug resistance has either neutralized antibiotics or is threatening to do so in virtually all major bacterial pathogens. Most viral vaccines have retained their efficacy, but antiviral drugs have historically been few, and those typically succumb to viral resistance even faster than antibiotics do. One hope for overcoming these problems lies in technology — continually developing new drugs, as has been done with antibiotics. Yet, despite the promise of new technology, there is concern that the microbes will win.

New technology isn't necessarily the only alternative. Paul Ewald (1983, 1991, 1993, 1994, 1996) proposed that humans can use principles of population biology to coerce virulent microbes to evolve into benign ones. That

is, we can be the beneficiaries of microbial evolution and turn pathogens into non-killers. Whereas medical technology may be failing to withstand the tide of microbial evolution, this population biological approach uses microbial evolution as an ally to beat microbes at their own game.

Ewald's arguments predict that virulence levels in a parasite evolve in response to the population dynamics of the parasite, and consequently, that manipulation of parasite dynamics should select higher or lower levels of virulence. Social practices, whether through the provision of hygienic drinking water, sewers, providing access to sterile needles for drug users, or the mere specification of ventilation systems on public transport, all influence the spread of infectious organisms. According to Ewald, therefore, these social practices thereby influence the evolution of virulence in our infectious microorganisms. Whether by legislation or other means of influencing cultural practices, societies have the ability, not only to influence the number of people infected by harmful microbes, but societies can also cause future changes in how deadly those infections become.

If Ewald is correct, it would mean that evolutionary biology — a discipline viewed by the public as arcane and objectionable — could provide a social benefit of unprecedented magnitude. But are Ewald's proposals sufficiently well-founded to warrant such claims? The models underlying Ewald's proposals have been tested only minimally, chiefly through tabulation of epidemiological data from the literature. Many of the experimental tests to date are serial passage experiments in which virulence has been observed to increase upon selection for increased transmissibility (e.g., Diffley et al., 1987;

Dearsly et al., 1990), but these experimental procedures often bypass the natural routes of infection and transmission, thus the link between transmission and virulence warrants further investigation. Interpretation of results of other experiments (Bull et al., 1991; Bull and Molineux, 1992) are hindered by the fact that selection acted on more than one trait. Although the design selected for reduced virulence in f1, strict vertical transmission of the virus also reduced selection to maintain transmissibility. The vertically transmitted phage lost their ability to infectiously transfer. This, however, is an outcome that likely would not be favored under natural conditions. Additionally, some of the best observations of evolutionary changes in virulence (Fenner et al., 1956; Fenner and Ratcliffe, 1965; Herre, 1993; Ebert, 1994) even fail to address the crux of Ewald's arguments. It thus may be premature to advocate such views. The study described here was undertaken to begin filling this void. We offer the most thorough experimental test yet of models for the evolution of virulence.

The Trade-off Model

Parasites, by definition, harm their hosts. Although this harm (virulence) is measured as the parasite's deleterious effect on its host, it also is generally believed that virulence feeds back negatively on parasite fitness: high virulence kills the host too quickly for the parasite to spread, and lower levels of virulence are at least mildly deleterious to parasite fitness because a healthy host is needed for transmission. The maintenance of virulence has thus been explained as a necessary evil of parasite growth and metabolism in the host. Furthermore, it has been argued as plausible that the level of virulence increases with parasite

load and reproduction, generating a coupling between virulence and parasite reproduction, or a "trade-off" between avirulence and parasite reproduction (Levin and Pimentel, 1981; Anderson and May, 1982; May and Anderson, 1983). Under this model selection will favor an intermediate level of virulence, balanced between selection for high parasite reproduction within the host and selection against high host mortality (fig. 1).

An optimum balance between parasite reproduction and host mortality should thus be sensitive to environmental factors affecting rates of transmission between hosts. The prediction from this trade-off is that lower virulence will evolve when periods of transmission are infrequent (relative to duration within a single host), whereas higher virulence will evolve as transmission becomes more frequent. Ewald (1983, 1991, 1993, 1994, 1996) has applied this model to predict the impact of various cultural practices on virulence in human pathogens.

The Basic Design

Here, we tested this model of virulence evolution experimentally, using a nonlethal bacteriophage as the model parasite and *Escherichia coli* as the model host. To determine if evolutionary changes in transmission rates affect the evolution of parasite virulence, we serially propagated lineages of infected hosts and then varied the rate of infection of new hosts. Thus, hosts were infected with single parasites and propagated for n days (in this design n = 1 or 8 days) after which parasite progeny were recovered from the infected hosts and used to re-infect a new batch of hosts for the next cycle (fig. 2). In this particular

experimental system, only one parasite could exist in each host, thereby minimizing within-host competition, and no susceptible hosts were available during the propagation phase of the cycle, allowing full control over the rate of infectious transfer.

f1, The Model Parasite

Along with phage fd and M13, f1 is known as a filamentous phage because of the long, filament-like structure of the virion (Model and Russell, 1988). Several properties of filamentous phages are suited to studies of the adaptive significance of virulence: they do not kill their hosts outright but merely retard host growth rates; they prevent superinfection and so allow tight control of within-host competition; and their genomes tolerate inserts of foreign DNA that facilitate monitoring their frequencies and genotypes. The lack of strict lethality of an f1 infection means that changes in virulence can be selected up or down gradually, in small increments. Host lethality, however, is not impossible with this phage. Clear plaque formers of M13 (Salivar et al., 1964) and fd (Hoffmann-Berling et al., 1963) are known to occur at relatively high frequencies (10^{-4}), which likely have high virulence, and gene expression of the eleven phage genes is tightly coupled so that amber mutations in any of 9 of those genes is lethal to the cell.

MATERIALS AND METHODS

The Experimental System

The selection experiments were carried out using an F-piliated, kanamycin-sensitive strain of *E. coli* (IJ338: *E. coli* K12 Δ(*pro-lac*) *sup*D Tn*10* *hsdS*/F' *traD*36 *proA*$^+$*B*$^+$ *lacZ*ΔM15; C. Lark, University of Utah. IJ338 similar to UT481 of Bull and Molineux, 1992) as the host. Some assays were carried out using an *E. coli* strain (called UT481-lac$^+$) that was isogenic to IJ338 with the exception that the F′ is *traD*$^+$ and *lac*$^+$ (F128).

The model parasite was a filamentous bacteriophage (f1) genetically engineered to be resistant to kanamycin [JB5: similar to R386 (described in Terwilliger et al., 1988; Bull and Molineux, 1992) but with the Pst I fragment of plasmid pUC4K containing a kanamycin resistance gene (aminoglycoside 3′-phosphotransferase) cloned into CGF3]. The presence of the kanamycin (Kn) resistance gene in the phage allows infected hosts to grow in antibiotic-containing media, while uninfected cells die. We constructed a second phage by PCR amplification of a fragment containing a Kn-resistant gene coding for the 6′-aminoglycoside acetyltransferase [AAC(6′)] 2′′-aminoglycoside phosphotransferase [APH(2′′)] enzyme from an *E. coli* plasmid JM109 (J. Ferretti, University of Oklahoma Health Sciences Center; See Ferretti et al. 1986). Nsi I restriction of the PCR product produced the same overhangs as Pst I permitting ligation into CGF3. This second phage was called JB17.

The resistance gene placed in JB5 confers resistance to both kanamycin and neomycin, while the gene placed in JB17 confers resistance to kanamycin

but not neomycin. The differential antibiotic sensitivities in these phage allowed for testing of superinfection by f1.

Bacteriological and molecular methods followed Bull et al. (1991) and Bull and Molineux (1992), although methods specific to this study are described here. All incubations were carried out at 37°C.

Selection Experiments

Long-term Selection (24 Days).— We generated evolved phage lines by infecting *E. coli* IJ338 with JB5 and then dividing the phage/bacteria mixture into two lines, henceforth referred to as the high- and low-selection lines. A cycle of selection consisted of the following steps: (1) infection of new hosts (infection phase) (2) growth of infected hosts for n days in the absence of susceptible hosts (growth phase) (3) recovery of phage produced in the last hour of growth. In our design, n = 1 day in the high-selection line and n = 8 days in the low-selection line. Both lines were selected over a 24 day period, during which the high-selection line infected new hosts 24 times (24 cycles), while the low-selection line infected new hosts only three times (3 cycles). These 24-day (i.e., long-term) selection experiments were replicated three times (selections I, II, and III).

To initiate the selection, 2 ml log phase cells of IJ338 were infected for 20 minutes with JB5 at an MOI of approximately unity. 50µl of the cell/phage mixture were transferred to each of two tubes (the high- and low-selection lines) containing 2 ml of LB broth with 50µg/ml kanamycin (LBKn). Uninfected host

cells cannot survive the antibiotic media, and progeny JB5 cannot infect already-infected hosts because of resistance to superinfection; therefore, reinfection could not occur in the antibiotic-containing tube. During the *n*-day growth phase, phage could increase their representation in the population only through enhanced host survival and reproduction, thus selecting in favor of less virulent phage. Every 24 hours, cells were washed twice with LBKn and diluted 1000-fold into a new 2 ml culture of LBKn for the next 24 hour period of growth.

On the terminal day of a cycle, cells were washed as above, but were then resuspended in fresh medium at the same cell density and grown for 1 hour. Phage produced during that one hour growth were retrieved. A new cycle was initiated by infecting a 2 ml of a fresh population of log phase cells with 1 µl of the retrieved phage. The two washes ensured that most of the phage used for reinfection were produced by infected cells during the preceding hour of growth.

Short-term Selection (8 Days).— Eight-day selection experiments were performed in which the starting population of phage was comprised of a mix of both the evolved high- and low-selection lines from selection I. By starting with a mix of two types of phage, the response to selection should have been accelerated over the 24-day selections. Furthermore, it was important to see if both increases and decreases in virulence could be selected. Mixtures were set up with (i) 99% high-selection phage and 1% low-selection phage, or (ii) 99% low-selection phage and 1% high-selection phage, hence both lines contained a population with a rare genotype and a common genotype. The selection regime

over the following eight days matched that from which the minority phage had been taken, attempting to favor the rare phage type over the common type.

Assays

We assessed phage virulence in infected hosts in three ways, (i) cell density at 24 hours, (ii) growth rates at low density, and (iii) long-term survival. Reproductive capacity of the phage was assessed by measuring phage titers produced during a brief interval. Assays were carried out in the presence of kanamycin unless stated otherwise. The phage used in all assays were collected from the final day of the selection experiments and were either samples of the entire supernatant ("whole cultures") or were single phage isolates obtained from single colonies of infected cells ("isolates"). Assays were carried out with phage collected from each of the three selection experiments (More measurements were taken on phage from Selection I than either Selection II or III, and all assays for the short-term selection were carried out on phage from Selection I only.). All host cells used in the assays were taken from a frozen stock which were assumed not to have been infected previously with f1, thereby eliminating host evolution.

Virulence Assays.—

Infected Cell Density: We estimated cell densities after 24 hours of infected cell growth, this interval representing the duration of the daily growth phase in the selection experiment. Log phase cells were exposed to phage for 20 minutes to allow infection, after which 10 µl of the mixture were transferred to 2

ml LBKn and grown for 24 hours at 37°C. Cell densities were determined by plating a known dilution on LBKn plates. Colonies were counted after 18-24 hours of incubation at 37°C.

Growth Rate (r): This assay estimated the intrinsic rate of increase (r) of infected host cells during 6 hours of exponential growth. Log phase cells were exposed to phage for 20 minutes to allow infection. Infected cells were added to LBKn to an initial cell density of less than 1×10^4 cells/ml (measured as colony-forming units, CFUs) to ensure that the culture would not become saturated within the 6-hour assay period. CFUs were determined immediately after the 20 minute infection and again after 6 hours. The intrinsic growth rate was calculated as $r = (\ln [CFU_6] - \ln [CFU_0])/6$; N_0e^{rt} represents the expected number of infected cells at time t, starting with an initial number of N_0.

Long-term Survival: This assay measures the mortality of infected cells over a 96 hour interval in the absence of fresh media. After log phase cells were exposed to phage for 20 minutes to allow infection, 100 µl of cells were transferred to 25 ml LBKn in a 125-ml sidearm flask and grown for 96 hours at 37°C. During the 96-hour assay period, no new nutrients were added. Cell densities were assessed by plating on LBKn plates every 24 hours, and the cell density measured at 24 hours post infection was used as the maximum density against which survival was measured.

Phage Infection Assays.—

Phage Titers: Because filamentous phages do not kill their host cells, but rather retard host growth, plaques formed by filamentous phages are moderately

turbid. Host cells infected with f1 selected for low virulence grow nearly at the same rate as uninfected cells; thus, these selected phage may no longer form visible plaques. An alternate method for assaying phage titers, in which phage were measured as colony-forming units on antibiotic-containing media, was developed. 10µl of a known dilution of phage supernatant was spotted on a 2.5 ml layer of top agar embedded with .2 ml log phase *E. coli* cells (overlaying 25 ml bottom agar). Soon after the spot of phage had dried in the incubator, a second 2.5 ml layer of top agar was added. After 2.5 hours of incubation at 37°C, a third 2.5 ml layer of top agar containing 65 µl of Kn was added. CFUs were counted after 24-48 hours of incubation. Phage titers per cell (i.e., phage output per cell) were calculated as the ratio of infected cell density to phage titer in the supernatant.

Defective Interfering Particles

Passaging f1 phage at high MOI selects for defective interfering particles (DIPs) (Horiuchi, 1983; Model and Russell, 1988) which are deletion mutants (about 1/3 the length of wildtype) that compete with wildtype phage for the cells resources. DIPs can cause significant declines in f1 titers, potentially altering the selective environment and the evolution of virulence. We assayed for the presence of DIPs in our evolved phage lines by isolating the double-stranded replicative form of f1 from 10 isolates from each of the evolved phage lines. High titers of DIPs are readily detected by agarose (0.8%) gel electrophoresis because they contain a single large deletion of ~4900 bp (Horiuchi 1983).

Superinfection

f1 is said to resist superinfection (Model and Russell, 1988), but our study required that this assumption hold strongly. To test for superinfection, we mixed cultures in which cells and phages each carried distinguishable markers and assayed for heterotypic phage-host combinations after mixed growth. *E. coli* UT481 (lac^-) were infected with JB5 (Kn^R, Nm^R) and *E. coli* UT481-lac^+ infected with phage JB17 (Kn^R, Nm^S). After a 20 minute infection period, 10µl of each infected cell type were mixed into a single tube with 2 ml LBKn and grown for 24 hours. The infected cell mixture was plated on LBKn plates with IPTG and X-gal at zero time and after 24 hour incubation. White (Lac^-) and blue (Lac^+) colonies were tested by stabbing them onto a Kn and an Nm plate, in this order. If no superinfection has occurred, white colonies, infected with Kn^R/Nm^R JB5, should remain resistant to both kanamycin and neomycin. Blue colonies, infected with Kn^R/Nm^S JB17, should remain kanamycin resistant but neomycin sensitive. Reciprocal infections were also carried out.

DNA Sequencing

To determine how many and what types of molecular genetic changes are found in lines that evolved changes in virulence, the entire phage genome (6425 bases) of a single isolate of each of the evolved lines (high and low lines) was sequenced and compared to the published f1 sequence (Beck and Zink, 1981; Hill and Peterson, 1982). The PstI insert, containing the antibiotic resistance gene, was not sequenced. When differences were found between either of the evolved lineages and the published sequence, those regions were

also sequenced in the ancestral (JB5) lineage. Log phase UT481 cells were exposed for 45 minutes to either of two isolates (high line-isolate c and low line-isolate a) from the 24th day of the first selection experiment to allow infection. 100 μl of infected cells were transferred to 25 ml of LBKn and grown for 36 hours. Single-stranded DNA from these two isolates was extracted from supernatant using the PEG protocol described in Sambrook et al. (1989), and double-stranded DNA was prepared from cells using the Promega Wizard Miniprep DNA purification protocol. Primers, spaced approximately every 450 bases, were designed from the published sequence of f1.

RESULTS

Evolution of Virulence in 24-day Selections

24-hour Infected Host Density.— A total of 147 cell density assays, conducted on both whole cultures and phage isolates, were spread over 26 separate days. We observed significant heterogeneity in the results of assays carried out on different days ($p < 0.0001$, ANOVA) so comparisons were restricted to assays from the same day. Virulence was invariably higher for 1-day (high-selection lines) evolved phage than for 8-day (low lines) evolved phage ($p < 0.0001$, nested ANOVA; fig. 3a). This conclusion applies both to comparisons between whole cultures and to isolates from these cultures, although the figure presents data for whole cultures only. Because isolates behaved similarly as the whole cultures, unless otherwise stated, we present only the whole culture results. Whole cultures are less prone to sampling error. Cells infected with either high-

or low-selected phage achieved a greater 24-hour cell density than cells infected with the ancestral phage JB5. These assays suggest that both the high- and low-selection phage evolved lower virulence from their common ancestor, and that the low-selection line evolved lower phage virulence than the high-selection line.

Growth Rate (r).— Assays of the impact of phage on host cell growth rate during log phase growth (r values) were inconsistent across three independent trials, each trial using the same four low- and four high-selection phage isolates (fig. 4). Although in trials 1 and 2 the high- and low-selection lines exhibited significant differences in growth rates ($p = 0.008$ and 0.025, respectively; unpaired t-test), the growth rates for the low-selection lines were greater than the high-selection lines in trial 1, but less than the high-selection lines in trial 2. In trial 3, there was no significant difference in growth rates between the selected lines ($p = 0.053$, unpaired t-test). The data obviously do not reject the null model of no difference in virulence between high- and low-selected lines, nor do they reject the trade-off model .

Long-term Survival.— Another measure of virulence is the impact of the phage on long-term host survival. Less virulent phage should allow greater long-term survival. This assay consisted of growing infected cells to 24 hours and then monitoring their survival during the subsequent 72 hours at 37°C with aeration but no replacement of media. Cell densities were measured every 24 hours and virulence was assessed as the survival of infected cells from 24 to 96 hours

(plotted as ln[survival]); for one isolate, survival was measured only to 72 hours. The same four phage isolates from each of the high- and low-selected lines were assayed in two independent trials. Three of the four low-selection isolates conferred higher survival than did the high-selection phage (fig. 5). Analysis of variance indicated that the four isolates from each selection line were not identical, requiring a non-parametric test of survivals. The survival of cells infected with low-selection phage isolates was not significantly different from that of cells infected with high-selection phage by a binomial test ($p = 0.071$). So again, neither the null model nor the trade-off model is rejected.

Phage Particle Production (Fecundity)

Phage Titers.— 240 phage titers were determined, spread over 23 different days. As was seen in measures of infected cell density, assays of the same isolates between days are significantly heterogeneous ($p < 0.0001$, ANOVA), so comparisons are restricted to assays done on the same day. In all cases, the high-selection phage exhibited higher titers than low-selection phage measured on the same day ($p < 0.0001$, nested ANOVA; fig. 3b). These titers are for the entire culture, so given the observations in figure 3a (i.e., the assay for infected cell density) the per cell phage productivity of the high-selection line is even greater than indicated by culture titer. Low-selection phage also produced fewer progeny (either per ml or per cell) than the ancestral phage (JB5); however, high-selection phage exhibited similar titers to or, in some cases, higher titers

than the ancestral phage JB5, suggesting that the ancestral phage may have been poorly adapted to growth under the conditions of the selection.

The Trade-off

We looked for evidence of a trade-off between virulence and phage reproduction by plotting ln [infected cell density] (i.e., virulence) against ln [phage titer] (i.e., fecundity) for evolved whole culture lineages selected under high versus low selection (fig. 6). Assays were carried out on six different days, and on all assay days we observed a negative correlation between ln [phage titers] and ln [cell density]. The null model of no trade-off is therefore rejected by a non-parametric test ($p < 0.01$). Although there is significant heterogeneity between measures taken on different days (See *24-hour Infected Host Density* and *Phage Titers*), the correlation across all days between phage titers and infected cell density are still significantly negative ($r^2 = 0.1$, $p = 0.01$).

Short-term Selection

Virulence and phage fecundity were selected during four short-term experiments. Two lines were selected for low virulence but were started with 99% of phage from a high-selection line; two lines were selected for high virulence but were started with 99% of phage from the low-selection line (fig. 7a).

If there is a trade-off, we would expect lines selected for low virulence to reduce their impact on host growth (i.e., exhibit increased ln [host cell density]), and to decrease phage productivity (i.e., exhibit decreased ln [phage titer]).

Lines selected for increased virulence should exhibit increased phage titers and decreased cell densities (fig. 7b). In trial 1 the whole cultures behaved as predicted in both selections. The phage isolates for both selections, however, exhibited changes in phage titer that were completely reversed not only from the whole culture results, but also from the prediction of the model (i.e., Selection for low virulence is predicted to decrease phage titers, while selection for high virulence should increase phage titers.). In trial 2, the results were as predicted from the model, except that the whole culture line selected for low virulence exhibited a slight decrease in cell density, whereas the model would predict an increase.

If we assume that the selected lines are independent, each line has by chance a 0.25 probability of ending up in the correct quadrant (i.e., the quadrant described by the criteria predicted in the model) in figure 7b. However, 9 out of the 15 lines fell into the correct quadrant, thus rejecting the null hypothesis ($p = 0.005$) of no trade-off; these data thus lend support for the trade-off model.

Defective Interfering Particles

10 isolates from each of the evolved phage lines were examined for the presence of DIP's using agarose gel electrophoresis. We found none and do not dwell on this possibility further.

Superinfection

Two superinfection assays (including reciprocal infections) were carried out as described in the Materials and Methods using JB5 and JB17.

Superinfection should be detected by observing changes in neomycin (Nm) sensitivity over the course of 24 hours of growth of cells infected with a mixed culture of JB5 and JB17. We tested 50-100 colonies of each infected cell type per assay and found that Lac$^-$-JB5 remained entirely Kn^R/Nm^R and Lac$^+$-JB17 remained entirely Kn^R/Nm^S, indicative of no superinfection. If superinfection had occurred then JB5 would have infected Lac$^+$ cells and JB17 would have infected Lac$^-$ cells. Reciprocal infections were also tested for superinfection and none was detected.

DNA Sequence of Evolved Phage

Complete phage genomes (6425 nucleotides) were sequenced for a single isolate from each evolved line. Of the 6425 bases sequenced only two point mutations were found in the high-selection line and only one point mutation was discovered in the low-selection line (Table 1). The single change found in the low-selection line was the same as one of the two changes in the high-selection line. An additional two point mutations found in both the high and low-selection lines were found also to be present in the ancestor (JB5).

DISCUSSION

Support for the Trade-off Model

The trade-off model assumes that pathogen virulence evolves as a pleiotropic effect of increased transmission. Thus, increasing the within-host reproductive rate of the pathogen increases the probability of transmission but also increases virulence. In this study, the existence of a trade-off would be

demonstrated if the more virulent phage have higher fecundity than the less virulent phage. Plotting phage titers against 24-hour host cell density shows that a trade-off does exist: a comparison of high- and low-selection lines shows that virulence is higher with higher fecundity, but that the trade-off is weak. The pattern is made much clearer by restricting the comparison to within assay days. Data from the short-term selection experiments lend additional support for the model by demonstrating that selection regimes differing only in rates of transmission can effect increases or decreases in virulence. Although these data support the trade-off model, the magnitude of the response to selection relative to the day-to-day variation in assays was weak.

Indeterminate Support for the Trade-off Model

It is not surprising that some assays failed to yield the expected patterns even if the trade-off model is correct. Assays with a large measurement error will simply not have the power to reject the null model with these sample sizes. Further, if the trade-off is embedded in a suite of phage reproductive traits, assaying of a subset of those traits need not reflect the true trade-off (Pease and Bull 1988). What does appear from these assays is that the magnitude of any such trade-off is not great. Although this study suggests a trade-off between transmission and one measure of virulence, results of other assays do not support the trade-off model over a model of no trade-off.

No Resolution.— When virulence was measured as a decrease in long-term survival of infected hosts, we could not reject the model of no trade-off. The

results in two independent trials were consistent, and in both cases the variation between isolates within a treatment overwhelmed the variation between treatments. Given larger sample sizes the differences between treatments may have been significant, but these results emphasize the small magnitude of the response to selection in the evolved phage.

Host Evolution?— Although these experiments were designed to minimize host evolution, the host population may have been responding to the infection. During the selection experiments, low-selection phage remained with a given host for eight days before reinfection, and during this time hosts could have evolved in response to the infection, thus altering the "environment" in which the phage were evolving. After 4 and 5 days of the competition, some long-term competition experiments between marked cells carrying different phage isolates showed dramatic reversals of the trend up to that time. This sudden change in frequency of two cell types (after days in culture) is the hallmark of newly-arisen adaptive mutations, although we did not investigate whether those changes occurred in the phage or bacterial genome. Such host evolution would obscure viral evolution and may explain the lack of a stronger result in the selections. Nonetheless, if genetic variation for major differences in virus virulence existed, the design allowed abundant time to select those variants even in the presence of any potential host evolution.

Inconsistent Assays.— Assays of the impact on growth rate of infected hosts during log phase growth (r) were inconsistent across three independent trials,

despite using the same isolates in each trial. Thus, these results do not allow us to reject the model of no trade-off. The lack of difference in growth rates is consistent with findings of Merriam (1977), who demonstrated that infections by M13 have no major impact on the generation times of *E. coli* cells growing at low density during the log phase.

We performed additional assays to assess differences between the high- and low-selection lines by directly measuring changes in relative fitnesses of infected cells when grown together in competition. These assays involved three steps; (i) infecting cells with either the high- or low-selection phage, (ii) growing in a mixed culture for either 24 hours or eight days, and (iii) measuring changes in the relative frequencies of each infected cell line over the duration of the assay. The almost isogenic cells differed by being either Lac^- or Lac^+. These competition assays usually revealed results similar to those obtained from 24-hour infected cell densities. Cells infected with low-selection phage achieved a higher density after 24 hours than cells infected with high-selection phage, and these same infected cells grew faster in mixed culture than cells infected with high-selection phage. Yet we observed serious inconsistencies in the results of the competition assays that have eluded explanation despite numerous experiments to identify the cause. After carrying out seven replicate competition assays using phage from Selection I, we observed a complete reversal of relative fitnesses of these phage lines during an additional seven assays, after which the relative fitnesses reversed back. Despite attempts to identify experimental conditions that could have affected the assay results (e.g., changes in composition of LB broth, temperature, host cell stocks, etc.), we

found the assays to be robust to any of these perturbations. One factor identified was that relative changes in frequencies of each of the evolved phage during growth are dependent upon the initial frequency of each competitor. Control experiments in which the relative frequencies of each infected host were manipulated demonstrated that the relative fitness of the infected host was negatively correlated with the initial frequency of that host. We have no definitive explanation for the inconsistencies, but appears to be, at least in part, a reflection of the weak response obtained from the selection. Whatever variables contribute to reversals in relative virulence, errors in measurement should be been inconsequential if the response to selection were larger. In our view, therefore, the inconsistency of these assays highlight the lack of strong response to selection.

Is the Trade-off Model a General Model for Virulence Evolution?

It is not clear what the magnitude of the response of virulence would be to variations in the transmission rate, and thus how virulence evolution can be influenced by other selective forces (e.g., within-host competition). It is important to note that this f1-*E. coli* model system was chosen because it is among the simplest of parasite-host pairs (i.e., a clonal unicellular host with no immune system and virtually no within-host competition and one of the simplest of all parasites), and we expected to see a strong response to selection in this system. Despite this apparent simplicity, factors such as host evolution, frequency-dependent fitnesses, and other unmeasured variables may have influenced the evolution of virulence. In light of these considerations, one has

to expect difficulty in extrapolating these data to virulence evolution in a more complex system. Conversely, one also has to expect that more complex systems (e.g., viral infection of humans) will not be amenable to analysis by simplistic models and in the absence of extensive data obtained under controlled experimental conditions.

Should we take Ewald's advice and put efforts into reducing transmission rates of a diverse array of pathogens to try to force these pathogens to evolve lower virulence? We suggest the evolution of virulence is not a sufficient motivation for this course of action at present. First, our results suggest that even if transmission rates influence patterns of virulence evolution, virulence may not change greatly in response to reduced transmission. Secondly, there are more complex models that suggest that even if a trade-off does exist, virulence evolution in any specific case may not be predictable without knowledge of additional parameters (e.g., sexual transmission, within-host competition, population structure of host; see Bremermann and Pickering, 1983; Nowak and May, 1994; Lipsitch and Nowak, 1995; Lipsitch et al., 1995). Thirdly and more importantly, there are alternative models that do not rely on a trade-off (Levin and Svanborg-Eden, 1990; Antia et al., 1994; Bull, 1994; Levin and Bull, 1994; Levin, 1996), and they too appear to have some empirical support in specific cases. Several of these alternative models do not treat virulence as an adaptation to enhance transmission: virulence may exist because of a maladaptive response either by the host (e.g., over-reaction of the immune response) or the parasite (e.g., invasion of a novel tissue that increases pathogenesis but which may lead progeny parasites to dead-end with respect to

transmission). Several examples of "emerging" diseases (e.g., hantavirus, Ebola, Machupo and other hemorrhagic fevers) result from horizontal transfers to new species (e.g., humans) to which these pathogens are clearly maladapted, and virulence cannot be construed to be an adaptation in these novel hosts.

By altering cultural practices (e.g., purification of water systems, increased hygiene among hospital attendants) to evolve less virulent pathogens, Ewald (1983, 1991, 1993, 1994, 1996) is suggesting that we could see large scale changes in pathogen virulence over relatively very short periods of time. This might be reasonable to predict if there were a strong correlation between transmission and virulence. Given the weak response of the evolved phage to what we considered dramatic changes in transmission opportunities, significant changes in virulence would likely require much longer time scales that would be largely irrelevant to discussions of human intervention strategies.

These examples just begin to explore the levels of complexity that influence how virulence evolves. Does it seem reasonable then to extrapolate from our experimental results with bacteriophage to other more complex systems? In most multicellular eukaryotic systems, additional parameters influencing virulence evolution, such as within-host competition, are inevitable. How additional factors, such as these, could alter the population dynamics of the evolution of virulence has been addressed in mathematical models, but has not yet been tested in an experimental framework. In order to estimate the impact of each parameter (within-host vs. between-host) on the evolution of virulence, the affects of each parameter should be adequately quantified through appropriate

experimentation. Without this level of detailed study, we may be hard pressed to justify any attempts to predict the course of the evolution of virulence.

Molecular Changes Associated with Virulence

Little is known to date about the molecular basis of virulence, and there are many questions surrounding the numbers and types of molecular changes required to cause observable differences in virulence. The difficulty in assessing virulence here offers little insight into the complexity of these changes. The fact that experiments were carried out over 24 days allows time for many changes to accumulate if selection is strong. However, major changes in virulence can arise from few base substitutions. Comparative studies of the plague bacterium, *Yersinia pestis* and the closely related *Y. pseudotuberculosis* (which vary in their virulence), have identified a non-sense mutation in the *yopA* gene of *Y. pestis* that contributes to the virulence of the strain (Lenski, 1988; Rosqvist et al., 1988). Additionally, f1 and fd clear plaque mutants are known to occur with rather high frequency (10^{-4}) and can arise from a single wild type isolate (Hoffmann-Berling et al. 1963; Salivar et al. 1964), suggestive of small numbers of changes.

Sequencing of evolved and ancestral lines of f1 provide an opportunity to qualify and quantify the molecular changes correlated with changes in virulence. The entire phage genome of 6425 bases was sequenced and, like the case of *Yersinia pestis*, very few changes were found. A total of two point mutations were identified in the high-selection line while only a single change was found in the low-selection line.

The mutation occurring in both evolved lines (position 957) falls within gene V and causes an amino acid change (Asn --> Asp). This gene produces a single-stranded DNA binding protein that binds to newly-synthesized (+) strand DNA and positions it for encapsidation. By binding to the ssDNA, gp V prevents the (+) strand DNA from forming a dsDNA RF, helping to maintain a balance between the rate of phage protein synthesis from the dsDNA replicative form (RF), and the rate of virion formation from the ssDNA (Model and Russell, 1988; Russell, 1995). An imbalance in the relative amounts of RF and ssDNA can kill the infected host cell. This protein also regulates the rate at which phage virions are produced by how well it binds to the ssDNA. If the observed mutation decreases binding efficiency of the protein V, reduced virion formation could result. A decrease in phage production (phage titer) was observed in both lines relative to the ancestor.

The mutation found only in the high-selection line (position 5692) falls within the intergenic region, specifically within the (-) strand origin which is important for complementary strand synthesis to form the dsDNA RF. Kim et al. (1981) demonstrated that deletion mutants in the (-) strand origin exhibited delayed formation of RF DNA and progeny as seen by turbid plaques. Thus, there is evidence of a link between mutations in this region and reduced progeny production.

Given that the low-selection line evolved a greater difference in virulence from the common ancestor than the high-selection line, it is puzzling that more changes occurred in the high-selection line, and that the low-selection line did not evolve any unique changes. Without further analysis to identify the

roles of each of the changes in phage reproduction; however, it is not clear whether or not the unique change in the high-selection line increases or decreases (or has no effect on) virulence. At this point, there is only an observed correlation between molecular genetic changes and differences in virulence. Additional investigation (e.g., site-directed mutagenesis) is required to establish that these mutations are the cause of the observed variation in virulence. Also, further investigation is necessary to determine if these mutations are found in all isolates of an evolved line, or if there is more than one way to evolve decreased virulence. The changes do occur in regions associated with phage DNA replication, however, making the causal link between genetic changes and virulence a plausible one.

TABLE 1. Models of Virulence Evolution[1]

LEVEL OF SELECTION	MODEL
Between host:	
Selection acts on rate of parasite spread. Virulence evolves as correlated character.	*Direct benefit* – Virulence factor directly benefits parasite transmission.
	Neutral – Virulence has no impact. Occurs after the selectively relevant period of transmission
	Trade-off – Virulence is detrimental to parasite. Maintained as a side effect of parasite fecundity.
Within host:	
Virulence correlated with traits conferring a competitive advantage within a single host.	*Associated* – Faster growing strains are more virulent.
	Reversed – Faster growing strains are less virulent.
	Tissue tropism – Virulence results from infection and replication in particular tissues not invaded by avirulent variants.
	Diversity – Virulence results from attack on the immune system by a diversifying, population of parasites in a single host.
Combined:	
Virulence evolves as a balance between within-host and between-host selection.	*Far-sighted* – Winners of within-host selection transmit at least as well as losers.
	Short-sighted – Winners of within-host selection transmit more poorly than losers.

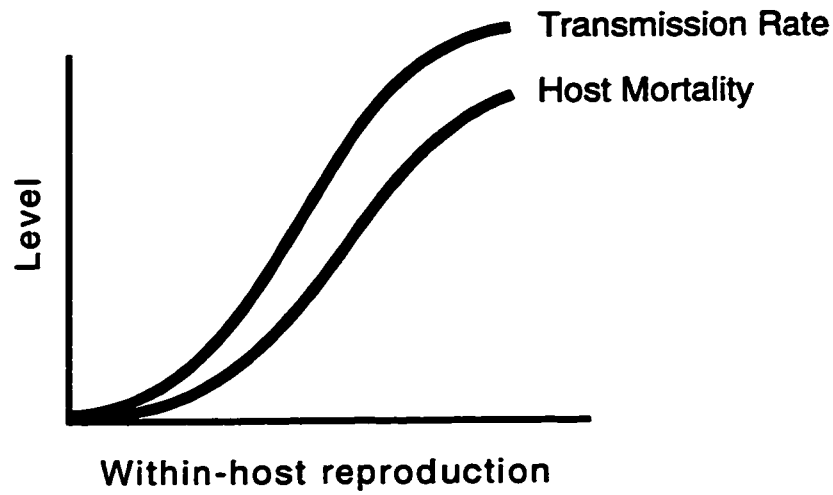
[1](adapted from Bull, 1994)

TABLE 2. Molecular Changes Observed in Evolved Phage.

Position	Gene	Mutation	AA Change	Lines changed
957	V	A ---> G	Asn-->Asp	High, Low
2439	III	G ---> C	Gly-->Gly	JB5, High, Low
5692	intergenic	T ---> G	N/A	High
6196	II	C ---> T	Ser-->Phe	JB5, High, Low

Figure 1. Predictions of the trade-off model. (a) The trade-off model assumes that increases in within-host pathogen reproduction unavoidably cause increases in the probability of transmission as well as increases in pathogen virulence. (b) If increased pathogen reproduction within the host also leads to an increased probability of transmission of that pathogen then the trade-off model predicts that maximum net transmission occurs at intermediate levels of virulence.

(a)


Transmission Rate
Host Mortality
Level
Within-host reproduction

(b)

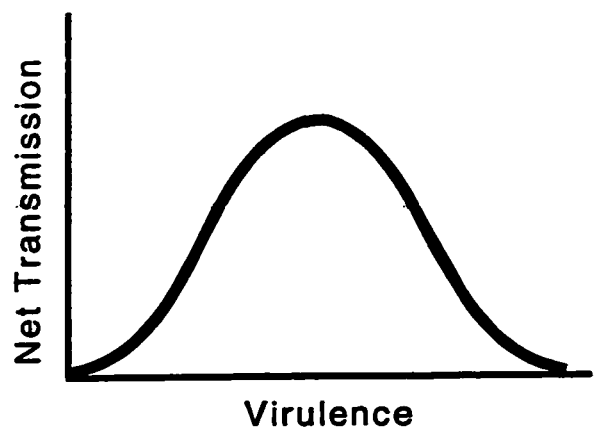

Net Transmission
Virulence

Figure 2. Experimental design for the selection experiments. The fundamental unit of selection of the f1 bacteriophage is a cycle of propagation over n days. Starting a cycle, phage infect *E. coli* cells. They are then grown for n (n = 1 or 8) days with no opportunity for infectious transmission during growth. At the end of n days, phage are recovered and used to initiate the next cycle. The number of days of a given cycle determines the transmission rate.

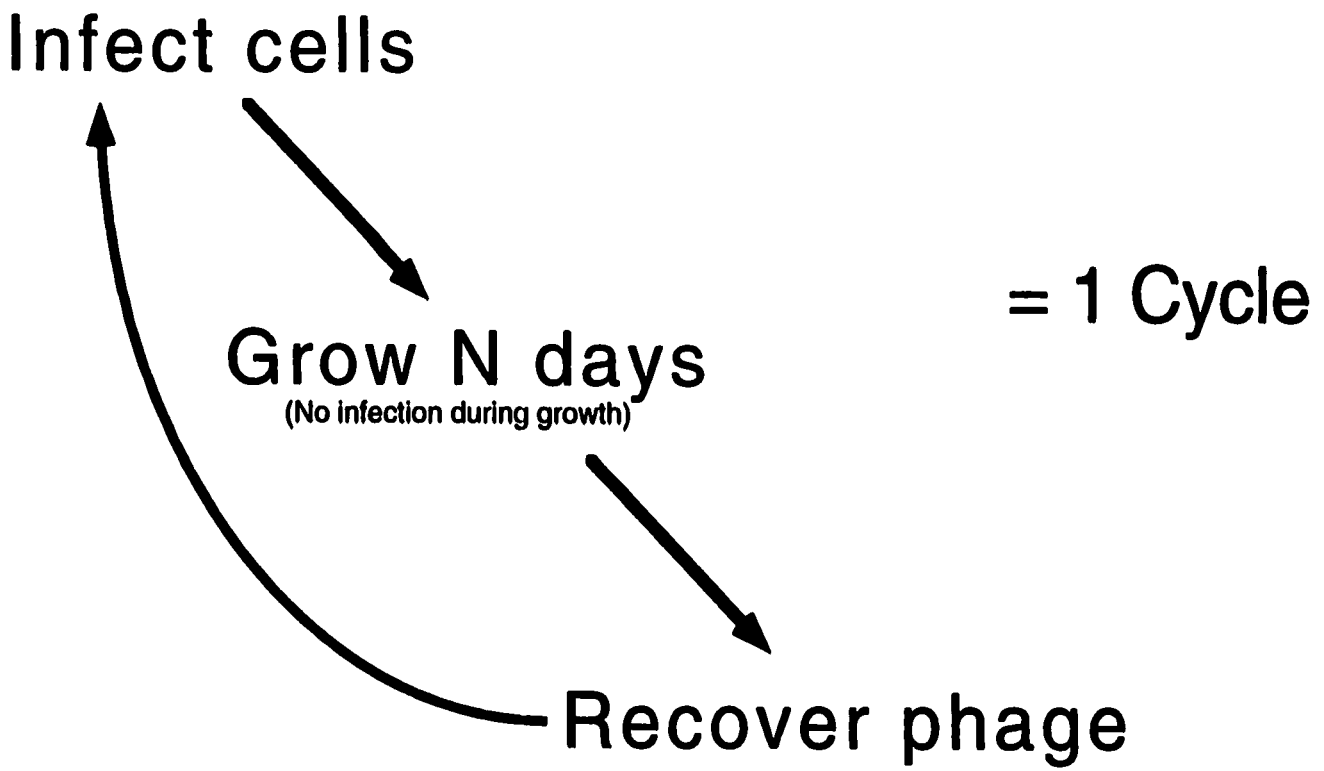

Infect cells
Grow N days
(No infection during growth)
Recover phage
= 1 Cycle

Figure 3. Avirulence and fecundity of evolved phage. Natural logarithms of (a) infected cell density (avirulence) and (b) phage titers (fecundity) for whole cultures of the evolved high-selection phage are plotted against those for the low-selection phage. Each point represents a different day on which the high- and low-selection lineages were assayed. The diagonal line represents points at which the high- and low-selection cultures have identical measures, thus the further away the points lie from the diagonal the greater the difference between them. Data points in the shaded area of the graph would be inconsistent with the predictions of the trade-off model. Though some data points lie close to the diagonal, none lie in the shaded regions. Lineages from all three selection experiments were assayed (open squares = Selection I, closed squares = Selection II, shaded squares = Selection III). If more than one assay was performed on the same day for the same selection, values were averaged and the number of assays performed placed inside the square. Each grouping of squares enclosed by a circle represents cultures from Selections I, II, and III that were assayed on the same day.

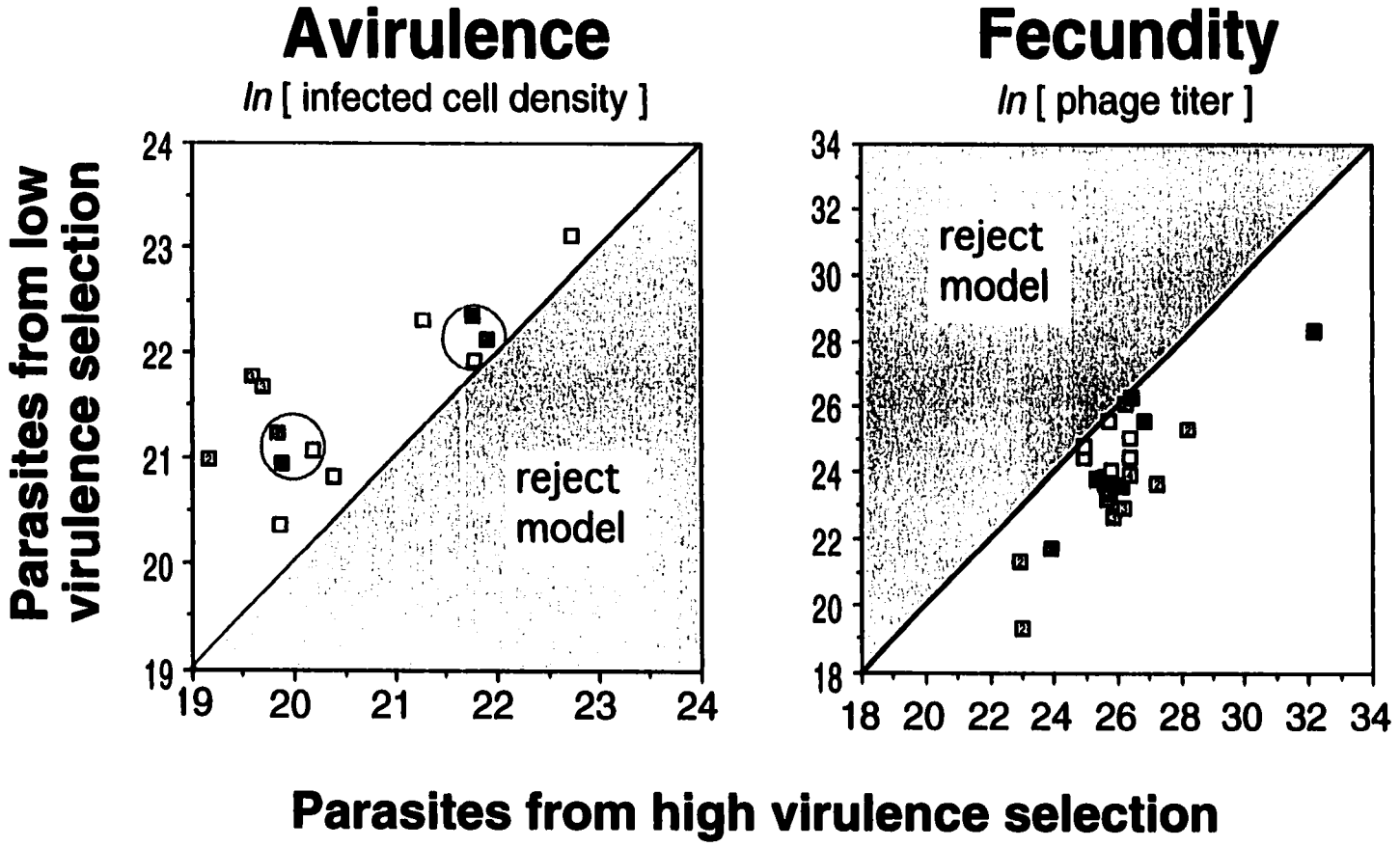

Avirulence
ln [infected cell density]
Fecundity
ln [phage titer]
Parasites from low virulence selection
reject model
reject model
24
23
22
21
20
19
19 20 21 22 23 24
34
32
30
28
26
24
22
20
18
18 20 22 24 26 28 30 32 34
Parasites from high virulence selection

Figure 4. The intrinsic rate of increase (r) per hour for host cells infected with either evolved high- or low-selection phage isolates. Data were obtained by growing infected host cells over a 6-hour period of exponential growth at low density and measuring cell densities. The assay detects no difference in phage impact on cell growth between the evolved phage lineages. Calculations for r are based on the following equation: $r = \Delta \ln [\text{cell density}]/\Delta t$. The assay was replicated three times, measuring four phage isolates (connected by dashed lines) in each replicate. Not only is there between-day heterogeneity in growth rates (especially in the high-selection lineage), but growth rates of the host cells infected with low-selection phage also overlap with those of host cells infected with the high-selection phage.

r (per hour)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
1
2
3
replicate
□ = high line
○ = low line

Figure 5. Long-term survival of host cells infected with evolved high- versus low-selection phage. Host cells infected with either high- or low-selection phage were grown in LB broth for 96 hours without replacement of media to identify differences in long-term survival related to type of phage carried by the host. The points represent averages (based on two replicate assays) of ln survival of hosts infected with different phage isolates (4 high-selection and 4 low-selection isolates). Although the means of survival in high- versus low-selection phage are significantly different, this difference may not be significant given larger sample sizes.

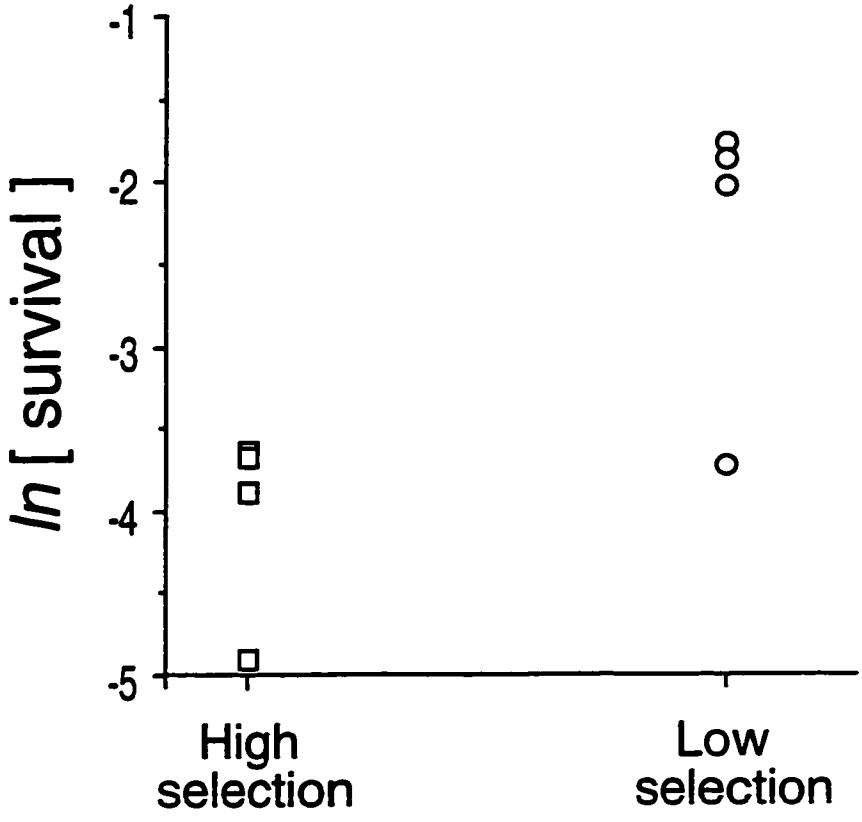

ln [survival]
-1
-2
-3
-4
-5
High selection
Low selection

Figure 6. The relationship between ln [phage titer] and ln [infected cell density] for evolved whole culture lineages selected under high versus low selection. Large symbols on each graph represent the lineages measured that replicate day, but all points are plotted on each graph to illustrate the between-day heterogeneity in measurements, as well as to provide orientation for where the points on any given day lie with respect to the entire distribution. In a-e, the highlighted points represent assays of lineages from the first selection experiment, whereas in f, the large symbols include assays from each of the three selection experiments, illustrating that between-day heterogeneity is greater than heterogeneity between the three selection experiments.

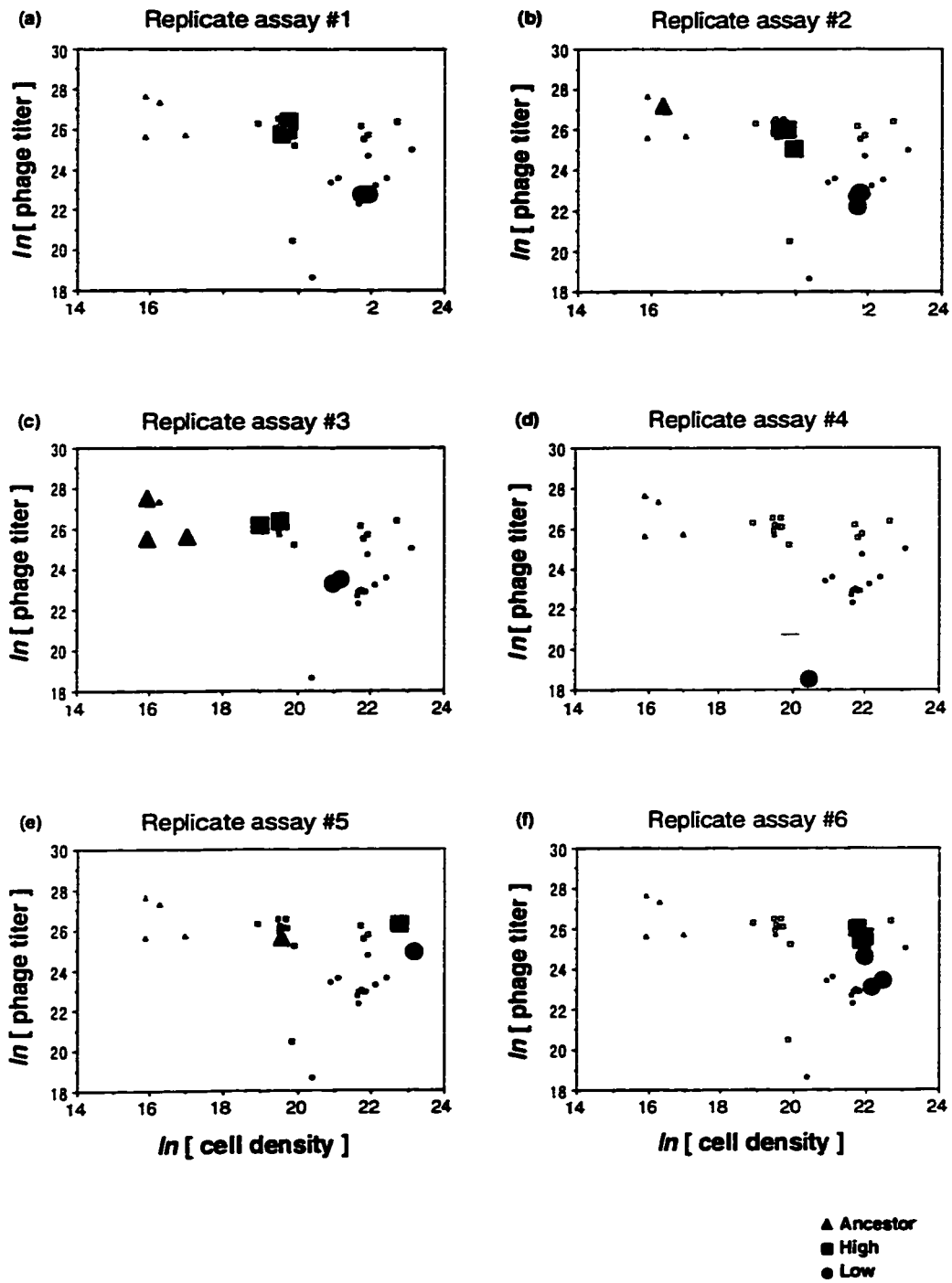
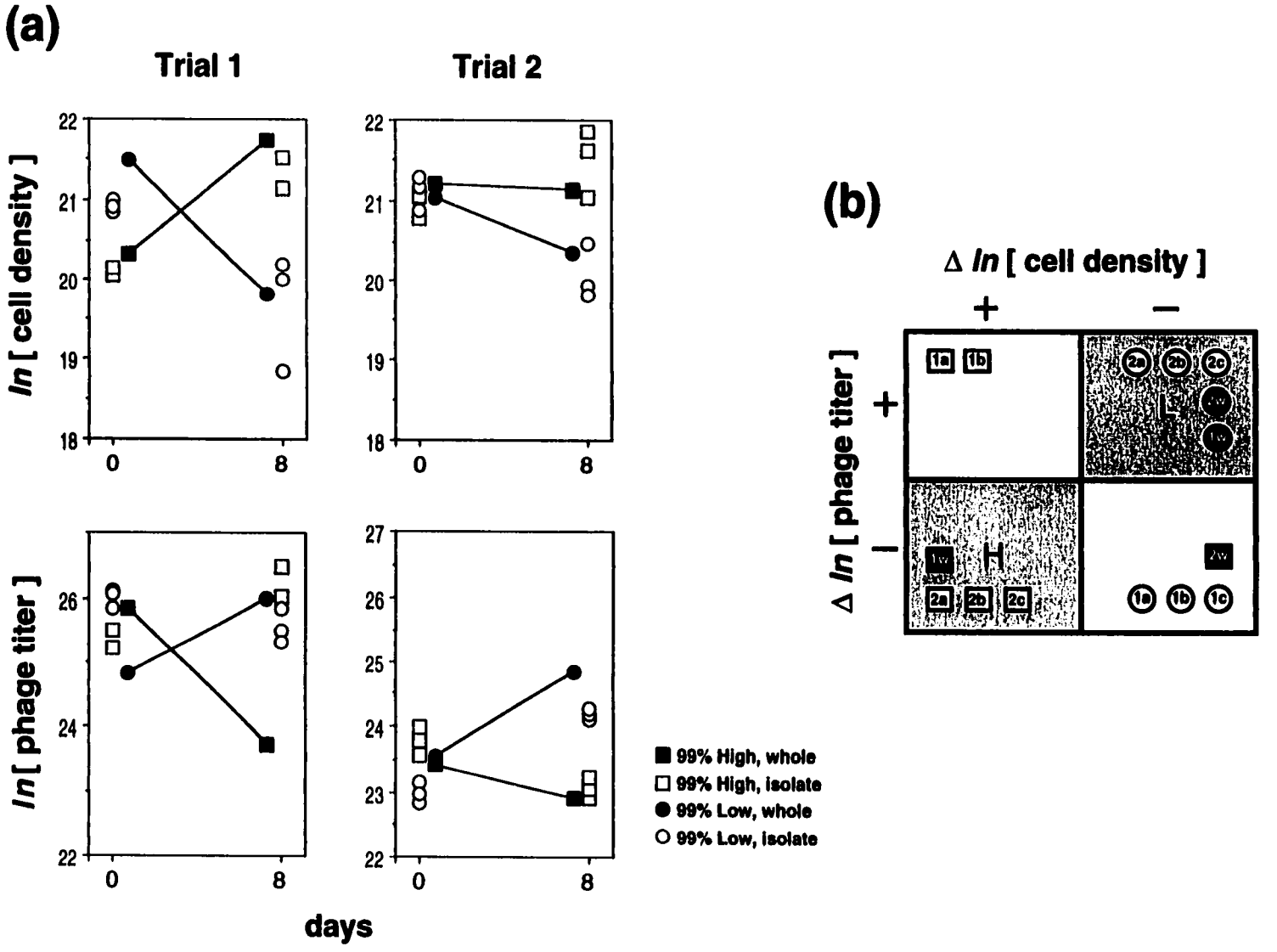

(a) Replicate assay #1
(b) Replicate assay #2
(c) Replicate assay #3
(d) Replicate assay #4
(e) Replicate assay #5
(f) Replicate assay #6
ln [phage titer]
ln [cell density]
▲ Ancestor
■ High
● Low

Figure 7. Two short-term selection experiments in which the initial culture contained a mixture of evolved 99% high:1% low-selection phage (= squares) or 99% low:1% high-selection phage (= circles). Solid symbols represent whole cultures, while open symbols represent individual phage isolates taken from the whole culture. In (b) numbers within symbols indicate the trial, and letters indicate isolates a, b, or c, or whole culture (w). (a) Over the course of eight days, the transmission regime for the mixture was the protocol used for the 1% phage in the original 24-day selection. For each trial, the ln [cell density] and ln [phage titers] are plotted on day 0 and 8 to show the change in response to selection. (b) The trade-off model predicts that the 99% high-selection phage would evolve decreased phage output and impact on cell growth (shaded quadrant labeled "H"), while the 99% low-selection phage would increase phage output and impact on cell growth (shaded quadrant labeled "L").


(a)
Trial 1
Trial 2
ln [cell density]
ln [phage titer]
days
99% High, whole
99% High, isolate
99% Low, whole
99% Low, isolate
(b)
Δ ln [cell density]
Δ ln [phage titer]
+
−
1a
1b
2a
2b
2c

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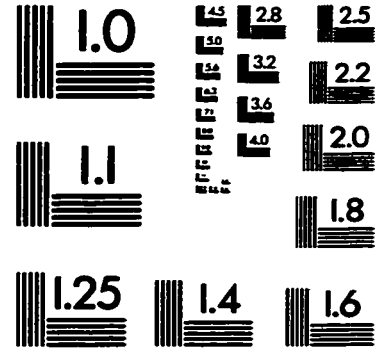

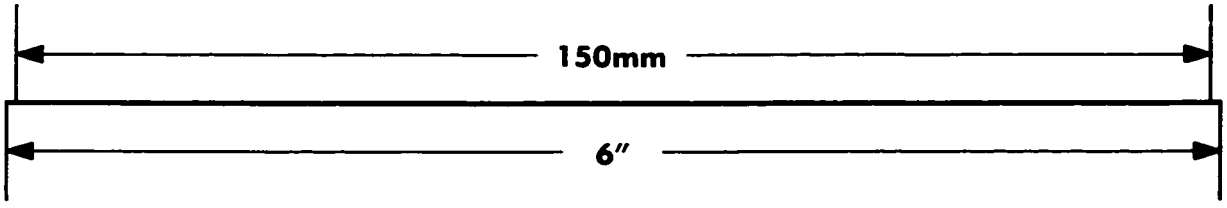

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