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Elisa Margolis	Date

Within–Host Ecology and Evolution of Nasal Colonizing Bacteria: *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*

By

Elisa Margolis
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Population Biology, Evolution or Ecology

Bruce R. Levin, Ph.D.
Advisor

Rustom Antia, Ph.D.
Committee Member

William M. Shafer, Ph.D.
Committee Member

Ifor R. Williams, M.D., Ph.D.
Committee Member

Accepted:

Lisa A. Tedesco, Ph.D.
Dean of the Graduate School

Date

Within–Host Ecology and Evolution of Nasal Colonizing Bacteria: *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*

By

Elisa Margolis
B.Sc., Brown University, 2000

Advisor: Bruce R. Levin, Ph.D.

An abstract of
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Abstract

Within–Host Ecology and Evolution of Nasal Colonizing Bacteria: *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*

By Elisa Margolis

Many bacteria, including *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*, colonize and reside harmlessly in the nasal passages of a substantial faction of the human population and are therefore considered commensal. However these same bacteria are also responsible for a great deal of morbidity and mortality. The goals of this dissertation are to understand the conditions under which these three species of bacteria can colonize the nasal passages, how they interact with each other and why they become invasive. Towards these ends, experiments in neonatal rats were performed on nasal colonization, intra- and inter-specific interactions and on the invasiveness of these bacteria. All three species readily colonized the nasal passages of neonatal rats and regardless of the inoculum size apparently reached a steady-state density. To ascertain how these three different species interacted, I introduced either the same or different species in rats already colonized with other strains or species. Established populations of *S. aureus* inhibit invasion of new *S. aureus* populations. And for both *S. pneumoniae* and *H. influenzae* the invading and established populations of the same species were able to co–exist. Previous reports had suggested that hydrogen peroxide (H_2O_2) production by *S. pneumoniae* reduces *S. aureus'* bacterial density, however in the neonatal rat model the density of *S. aureus* when colonized did not differ in the presence of a *S. pneumoniae* strain that was H_2O_2–secreting or non–H_2O_2 secreting. Surprisingly, the only multi-species interaction that we did observe was *H. influenzae* reaching higher densities when *S. aureus* or *S. pneumoniae* were present.One hypothesis for the rare invasiveness of these commensal bacteria is the evolution of invasive mutants in the population of bacteria colonizing the nasal passage or within–host evolution. When *H. influenzae* isolated from the blood and the nasal passages were re-inoculated, one out of the six blood isolates had an increased propensity to invade the bloodstream. These results provide support for within–host evolution as one but not the sole explanation for the occasional invasive disease of normally commensal bacteria.

Within–Host Ecology and Evolution of Nasal Colonizing Bacteria: *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*

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I can safely say that **Bruce Levin** is the best PhD advisor imaginable, however he would find me remiss if I didn't mention that "best" is defined over the set A_n defined as the number of professors who would put up with me and where n=1. You have not only been a great source of scientific inspiration but also extremely generous with your time, your patience, your kibitizing, your vast knowledge, those archaic words you insert to liven up manuscripts and your enthusiasm. I feel extremely honored to have had this opportunity to work with you.

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Introduction

Why when multiple people are exposed to an infectious disease does not everyone fall ill? Why will one person be suffering from a hacking cough, another one not at all visibly perturbed and others be dead? The large variation in outcomes after exposure to an infectious agent is one of the fundamental unknowns in the field of infectious disease and leads to far more questions than answers. Is this due to variation in the population of hosts or in the population of microbes? Can we link it to genetic variation in a particular operon or cytokine receptor family? Does the variation come from the presence of other microbes or differences in the amount of iron available to the microbe? Or is it simply due to chance alone, such that every once in a while when a pathogen and host encounter disease occurs? The investigations contained within this dissertation begin to address these issues for invasive bacterial diseases.

There are many examples of bacterial species (e.g. *Staphylococcus aureus*, *Escherichia coli*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*) which colonize and reside for extended periods of time in substantial fractions of human populations without causing symptomatic disease and are therefore considered commensal. Here I will consider three of these species: *S. aureus*, *S. pneumoniae* and *H. influenzae*. The selection of these three particular species is due not only to experimental tractability but also to their relevance to human disease. In particular these normally commensal bacteria species are responsible for considerable morbidity and mortality associated with meningitis, pneumonia, otitis media, soft tissue infections, bacteremia and sepsis[88, 4, 93].

1.1 Ecology of Nasal Colonizing Bacteria

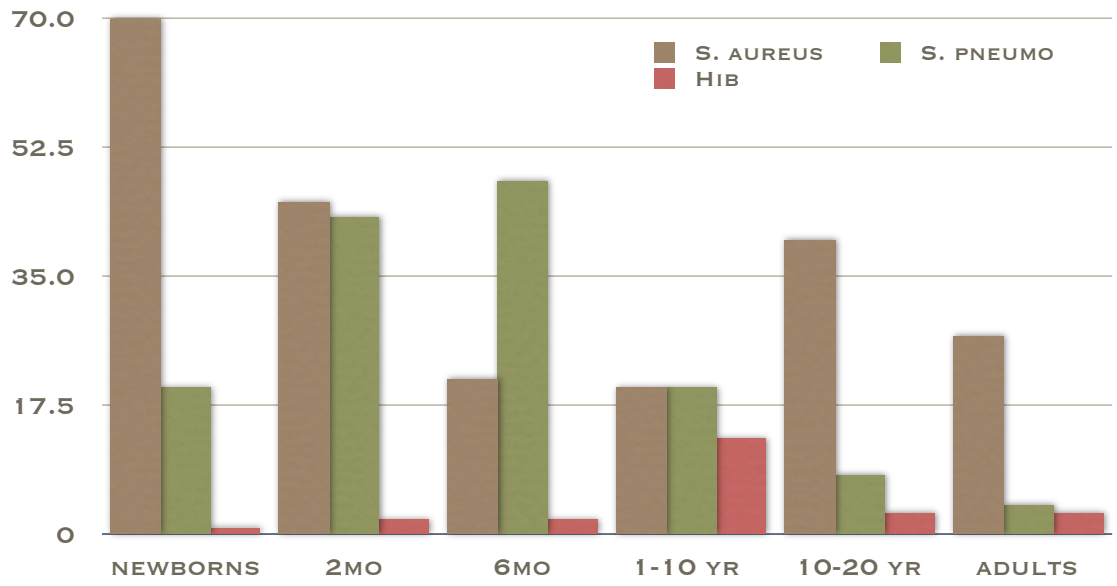
The human epithelium, including the gut, genitals and nose, provides a habitat for an almost inconceivable number of bacteria. In the adult human intestine alone there are up to 100 trillion microbes[73]. The nasal mucosa, which harbors a diverse prokaryotic microflora, is no exception. *S. aureus*, *S. pneumoniae* and *H. influenzae* together with *Moraxella cattarrhalis*, *N. meningitidis* and various other staphylococci and streptococci make up the commensal flora of the upper respiratory tract. The majority of these species that colonize the nose are either entirely harmless or commensals that on very rare occasions cause invasive disease (i.e. meningitis, bacteremia or endocarditis) or disease in adjacent tissues (otitis media or pneumonia). However, very little is known about the ecology of these human microbial flora. Beginning with early portraits of gut habitats[16] through recent investigations into the impact of obesity[44], much of the work on microbial ecology of habitats in the human body is descriptive— describing the number of species, number of habitats/niches or more recently the number of discernible genomes. A more dynamical approach is necessary in order to understand what governs the number of species, the number of niches available or the number of microbes that can reside in these habitats.

1.1.1 Epidemiology of Nasal Colonizing Bacteria

Within seconds after birth (whether cesarean section or vaginal delivery) a human infant encounters bacteria[3], and during the first months of life the nasopharyngeal flora become established[20]. In fact, humans are constantly exposed to new bacteria throughout our lives. Therefore it is no surprise that at some point in our lives we will encounter *S. aureus*, *S. pneumoniae* or *H. influenzae*. Whether that encounter leads to carriage (a persistent detectable bacterial population) or rapid clearance depends on whether the encountered bacteria successfully colonize our nasal passages. Every human is likely to be carriers of one or all of these bacteria during a lifetime. In the case of *H. influenzae*carriage is most likely when we

are young children; as carriage rates are low in the first 6 months of life, peak between 3 and 5 years and decline in adulthood (Figure 1.1.1)[2]. For *S. aureus* a slightly different trend emerges with over 70% of newborn babies having at least one positive nasal culture, reducing to less than 50% during the first 8 weeks to 20% in a healthy adult population[93]. While *S. pneumoniae* carriage varies considerably from population to population and study to study it is clear that the peak incidence of pneumococcal colonization is in 6 months – 3 year-olds (55%) with a steady decline until 10 years of age (8%) and a further decline in adulthood[5].

Figure 1.1: Percentage Carriers by Age Group. Prevalence of *S. aureus*, *S. pneumoniae* and *H. influenzae* carriage based on epidemiological surveys[93, 69, 51].



Nasopharyngeal colonization is a dynamic process with turnover of species, strains or serotypes. The degree of turnover appears to vary and three pattern of carriage can be distinguished: persistent carriage, intermittent carriage and non-carriage. In the case of staphylococcal carriage, 20% (range 12–30) are persistent, 30% (range 16–70) are intermittent and 50% (range 16–69) are non-carriers[93].

While the definitions vary from study to study[86], some general trends emerge. Persistent carriers are often colonized by a single strain of a particular species over long time periods and generally have higher densities of bacteria[59]. Intermittent carriers often carry different strains over time. And for all three species, children are more likely to be persistent carriers than adults[13, 28].

What determines who becomes a persistent or intermittent carrier or non-carrier? For this (and most of the questions in this dissertation) there are three broad answers, none of which are mutually exclusive. The first factor could be the host. For various reasons (e.g. age, genetic background, smoking habit, obesity, sex, etc.) there is variation in the host's physiological and immunological makeup allowing for bacteria to not only colonize but persist. The second factor could be variation in the colonizing population of bacteria- either genetic or purely phenotypic; such that differences in physiological responses and/or gene expression allow that specific population of bacteria to persist. Lastly there are inter–species (or intra–species) interactions which could determine who becomes a carrier. There is modest evidence for all three of these factors occurring. Host characteristics play a substantial role in determining who will be a carrier or non-carrier. (Presumably because exposure is almost universal– the relevant host characteristics are due to heterogenity in host susceptibility.) In a recent study where both non-carriers and persistent carriers were challenged with a mixture of *S. aureus* strains, the non-carriers eliminated the challenged *S. aureus* [58]. Intriguingly in the same study, the resident *S. aureus* strain they had had originally was selected for in the persistent carriers. This suggests that not only does variation in bacteria matter but that there is strong selection on this variation in each individual host. While there are numerous suggestions of inter–species interactions in the literature, perhaps the most striking evidence is the use of introducing one bacterial species to usurp another (probiotics). For example, introduction of a *Corneybacterium* species has been demonstrated to eradicate methicillin-resistant *Staphylococcus aureus* [84].

Epidemiological studies reveal two fundamental features about these nasal commensals:

1. Carriers are the most important source of transmission within the community [38]

2. Invasive disease is preceded by nasopharyngeal colonization (with the same strain) [29, 20, 35]

The first can be inferred from probability: there are vast numbers of carriers of these bacteria and only a tiny fraction of the population have disease; so carriers' contribution to the transmission cycle is much more extensive. The second point was first reported by Danbolt in 1931[75] and widely validated for *S. aureus* (including demonstrating that the nasal colonizing and infecting strain share the same phage type or genotype[85, 89]). However it has been slower to find acceptance in the streptococcal scientific community, as the point was diluted by the emphasis on defining particular serotypes as 'invasive' and others as 'colonizing' [8] .

1.1.2 Ecology of Bacterial Colonization

The dynamics of bacterial colonization and the bacterial load of a given habitat is determined by three ecological levels: resources, bacteria–bacteria interactions and predators (i.e. immune system and bacteriophage), Figure 1.2. As the nasal mucosa is rarely an unoccupied habitat, most colonizing bacteria must confront a plethora of resident bacteria that can affect all three ecological levels. Two scenarios are predicted by ecological theory for a new bacteria population colonizing a habitat with resident bacteria: coexistence or competitive exclusion. Competitive exclusion is predicted in four situations, when the residents:

1. grow better on the available resources (exploitation competition; limiting resource)

2. produce bacterocins or harmful substances (interference competition; allelopathy) [9, 71]

3. induces an immune response to which the invading population is more sensitive than the resident (apparent competition; immune mediated)[26, 62].

While it was once thought that stable coexistence of ecologically similar strains or species was unlikely [30], a large body of theoretical and experimental work suggests that stable coexistence of multiple strains of the same and different species is not only possible but may even be prevalent. Scenarios that allow for this outcome include differential utilization of multiple resources (including metabolic byproducts) [61, 79, 39, 31, 81, 6], spatial or temporal variability in a habitat [10] and the presence of predators (i.e. bacteriophage or the immune response) [43].

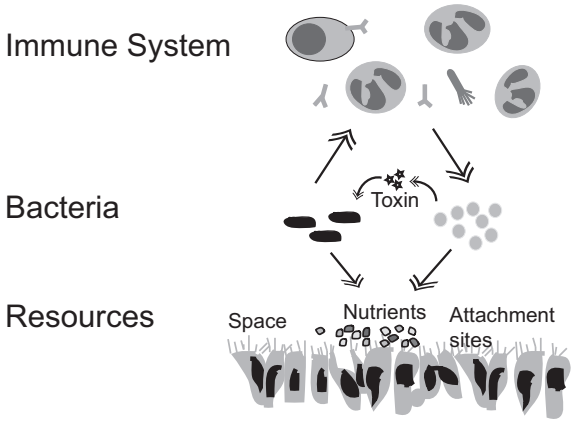


Figure 1.2: Whether colonization can occur depends on three ecological levels: 1) Resources- how bacteria populations utilize the resources (energy sources, nutrients, attachment sites, etc.); 2) Bacteria- how bacteria interact with each other (including toxin production) and 3) Immune Response- how susceptible bacteria are to a particular immune response (phagocytosis, complement, antibodies, etc.).

There are numerous reports of residential bacteria affecting the colonization of an invading species. In the case of same species, Wickman has shown for *S. aureus* that the organism arriving first will outcompete the burn wound[94]. Lipsitch and colleagues have demonstrated that some strains of *S. pneumoniae* (serotype 6B) can limit the nasal colonization of another *S. pneumoniae* (serotype 23F) [45]. There are notable investigations into competitive interactions in complex bacteria

communities- for instance host inflammation leads to gut microflora dominated by *Enterobacteriaceae* [47] and *Bordetella pertussis* increases attachment of *S. aureus* , *H. influenzae* and *S. pneumoniae* to cultured respiratory cilia[80]. In the nasal passages, α -haemolytic streptococci and viridans streptococci have been found to inhibit colonization by *S pneumoniae*, *H influenzae*, *S aureus* and *M catarrhalis*[83, 78]. Below is a more thorough consideration of the ecological conditions for allelopathy, specifically H_2O_2 affect on nasal colonization, and immune–mediated competition.

H_2O_2 Allelopathy

An attractive hypothesis to explain why *S. aureus –S. pneumoniae* co-colonization is rare than expected[4, 69, 50, 90, 49], is that *S. aureus* is killed by H_2O_2 produced by *S. pneumoniae* [70]. H_2O_2 is produced by *S. pneumoniae* as a by-product of pyruvate oxidase synthesis of acetyl phosphate. This enzyme has a role in metabolism, signaling and oxidative stress[67]. The possibility of H_2O_2 mediated allelopathy has wide-spread implications for nasal colonizers as some produce H_2O_2 (including *S. pneumoniae* [64] and the viridans streptococci [82]) while many more are particularly sensitive to H_2O_2 (including *S. aureus* [70], *H. influenzae* , *N. meningitidis* and *M. catarrhalis*[63]).

Ecological conditions can effect whether a toxin producing bacteria will have a competitive advantage in a particular habitat. For instance Chao and Levin, demonstrated that a toxin producer growing on agar surfaces was able to overcome its fitness disadvantage as they had an increase access to resources in the zone of inhibition around colonies[9]. In broth culture this was not the case and toxin producing bacteria had an advantage only when they were common enough to produce sufficient toxins (frequency-dependence). In theoretical studies, when there is a relative cost to toxin production, toxin producing strains persist in rich habitats where resource competition is lower.[22]. However, a patchy distribution of resources can allow for coexistence of toxin producers and toxin sensitive bacteria[17]. Interestingly empirical studies have shown that large differences in

growth rate, lysis rate and amount of toxin produced per bacterium translate into relatively small differences in colonization dynamics[71].

In the case of H_2O_2 mediated allelopathy in the nasopharyngeal habitat, there are three ecological conditions that are important to consider:

1. the half-life of the H_2O_2 ,
2. the amount of H_2O_2 produced per bacterium, and
3. the relative growth rates of the H_2O_2 –producing and sensitive bacteria.

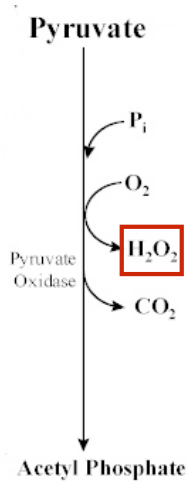


Figure 1.3: *S. pneumoniae* H_2O_2 production

H_2O_2 can freely diffuse across bacterial membranes and cause single-strand nicks in DNA as well as oxidation of critical respiratory chain components[32]. However, bacteria and the host epithelial cells (and all aerobic organisms) possess enzymatic to neutralize (i.e. catalases) and scavenge (i.e. reduced glutathione) H_2O_2 [12]. The bacterial defenses alone are sufficient to lower the half-life of the H_2O_2 , such that H_2O_2 –sensitive bacteria can survive and multiply in the presence of H_2O_2 –producing competitors[48]. The second factor, the amount of toxin produced per bacterium, is important because in broth when *S. pneumoniae* is at low densities relative to *S. aureus* there are insufficient amounts of H_2O_2 to inhibit the growth of *S. aureus* [69]. And lastly the rate of replication of the toxin sensitive strain may make up for the killing by a toxin, as demonstrated by Cornejo and colleagues[11].

Immune–Mediated Competition

Immune-mediated competition requires two conditions:

1. an immune response must be elicited during colonization or be constitutively present

2. the colonizing population must be more sensitive to that immune response than the resident (or vice versa)

Previously it had been assumed that commensal flora did not elicit immune responses as these were reserved for pathogens. However, it was recently found that microbial commensals are recognized by Toll-like receptors (TLR) under normal conditions and this recognition is necessary to maintain epithelial homeostasis[66]. Colonization by *S. aureus*, *S. pneumoniae* or *H. influenzae*likely elicits an immune response. For example, *S. aureus* colonization density has been shown to be increased in TLR2-deficient mice (which are also highly susceptible to *S. aureus* sepsis[77])[24]. And both *S. pneumoniae* and *H. influenzae* elicit the production of the chemokines MIP-2 (during mouse colonization) and IL-8 (when cultured with human respiratory epithelial cells)[68]. Together this suggests that epithelial cells respond to *S. aureus*, *S. pneumoniae* or *H. influenzae*bacteria by generating the signals, such as cytokines, necessary to trigger inflammation. As to the second condition, I am not aware of any studies directly comparing how different species/strains differ in their sensitivities to specific immune responses that might be elicited by commensal bacteria.

1.2 Evolution of Virulence

Why do infectious diseases cause harm? Or from the microbes perspective, why be virulent? It is easier to understand why microbes are infectious, as transmission from one host to another increases a pathogens populations' long–term fitness. It is much more difficult to explain why these pathogens harm their host. In fact conventional wisdom suggests that pathogens that are dependent on their hosts for survival- most bacteria, viruses and parasites- should eventually evolve to not harm or kill their hosts. Yet if that is the case how can we explain why there are so many infectious diseases.

There are a number of evolutionary explanations for why pathogens are virulent, or cause damage to the host due to their presence. The simplest (the con-

ventional wisdom mentioned above) is that although pathogens will eventually evolve to be benign parasites (purely commensal) those that cause disease haven't been in contact long enough (or their have been impediments) to their evolving more gentle life styles. While this may be true it does not provide a mechanism to explain how evolution evolves. The epidemiological explanation for virulence is that the damage done to the hosts is an adaptation for the persistence of a pathogen in the host population[1, 19]. In this case virulence induced by bacteria will be maintained by natural selection if it aids in the pathogen's ability to transmit. Alternatively parasite traits responsible for virulence may have evolved for other functions[41]. For example, Shiga toxin may not have evolved to cause hemolytic uremic syndrome but to protect *E. coli* from grazing protozoa[76]. For invasive diseases there is an additional hypothesis: short-sighted within–host evolution[40]. In this case, colonizing populations include or generate variants that have a short-term advantage because they are able to replicate in sites where virulence occurs. In the past, within–host evolution has always been characterized as short–sighted because for invasive disease the site where virulence occurs is usually not where transmission occurs. However if the site of virulence is also the sight of transmission, within–host evolution becomes a more explicit mechanism for the epidemiological explanation. In theoretical studies, within–host selection among competing strains can lead to higher levels of virulence[42, 60]. This has been demonstrated for rodent malaria; more virulent parasites outcompeted less virulent parasites and obtained higher transmission to the mosquito host[14]. This illustrates how for a given pathogen these hypotheses may not be mutually exclusive.

While there has been extensive theoretical and some experimental work on the evolution of virulence, the majority has ignored two aspects of infectious disease. The first is that even though infectious disease can be responsible for serious illness and death, not all encounters with pathogens cause such extensive damage. This is especially true for the set of organisms that I have been considering, commensal bacteria (such as *S. aureus*, *S. pneumoniae* and *H. influenzae*) that only on rare occasions cause invasive disease. The second aspect is that most of the work

on evolution of virulence has seen virulence as a pathogen trait and has ignored the hosts contribution to the damage.

1.2.1 Within–host Evolution of Invasive Bacterial Disease

Evolution is simply the process whereby variation for a trait like antibiotic susceptibility in a natural population can lead to individuals with the beneficial variants for that habitat to be more likely to propagate. Evolutionary processes while typically thought to occur over a long time scale can occur over the course of a single bacteria infection, for instance the emergence of antibiotic resistance (Figure 1.4). The microbial characteristics that allow evolution to occur over short periods of times (during a single infection) includes their large population sizes, high mutation rates (for some) and short generation times. In fact, there are some viruses that are so extreme in these characteristics, such as HIV where viruses isolated from a single patient over six years yields as much variation as influenza viruses collected from sites all over the world[37]. This within–host evolution process can lead to selection of a variety of traits that are beneficial for the pathogens including antibiotic resistance, immune escape [33, 21], cellular tropism[91, 92] (or preference for a particular habitat) and in the case of polio virus [87] and coxsackie virus [23].

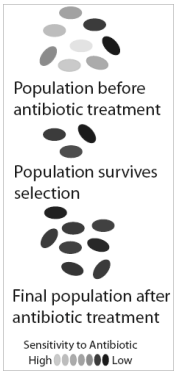


Figure 1.4: Within–Host Evolution. Variation in antibiotic susceptibility that exists in natural populations can be selected by antibiotic treatment resulting in a population having mostly individuals that have a low sensitivity to the antibiotic.

One explanation for the rare invasiveness of commensal bacteria is the evolution of invasive mutants in the population of bacteria colonizing the nasal passage or within-host evolution for invasive dis-

ease. Some of the most appealing lines of evidence for this explanation can be found in experiments by Meynell and Stocker (1957) (and repeated by others) investigating whether invasion is an independent event for each bacterium crossing or orchestrated cooperatively by the colonizing bacteria[53]. In these experiments, equal frequencies of two genetically marked but otherwise isogenic strains were inoculated into rodents. While both strains were present in the site of colonization, in most cases only one or the other marked strain was recovered from the blood. If in fact invasion involved many bacteria crossing into and proliferating in the bloodstream, then both marked strains would be expected to be recovered. This experiment was first done with oral inoculation of *Salmonella enterica* serovar typhimurium of mice [53, 52] and repeated with intranasal inoculation of *Haemophilus influenzae* [56], oral inoculation of *Escherichia coli* K1 [65] and intraperitoneal inoculation of *Streptococcus agalactiae* [72] in neonatal rats. An alternative explanation for these experiments' results (that does not assume that the invading population of bacteria is genetically different from those colonizing) is that by chance alone one or a few bacteria pass through the host defenses and establishes a blood population. Of note the Meynell and Stocker original experiment has been extended with greater resolution (13 flourescence marked *Salmonella enterica*) in order to determine the intracellular demography of the bacteria.[7].

1.2.2 Contribution of Host's Immune System to Virulence

In all of the current hypotheses for the evolution of virulence (i.e. epidemiological, coincidental or within–host evolution) the onerous is on the pathogen and explaining why this pathogen ends up causing harm. The host is completely passive in these interpretations. Virulence is assumed to be due to greedy parasites which are damaging their hosts in order to exploit their host resources. Its certainly true that if not for the pathogen than the host would not be sick. But when one looks at the immediate cause of the symptoms of most infectious diseases it is often due to the host's immune defenses screwing up[27]. This immune screw-up has previously been referred to as immunopathology but I'll refer to it as an over-

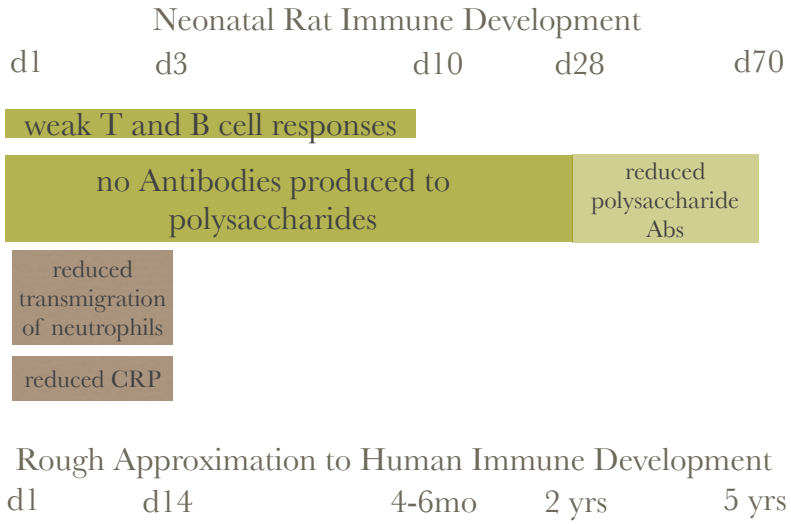
response. In these cases, virulence is due to microbes interacting with the host in such a manner that leads to an inappropriate or excessive immune response. While numerous bacterial examples are presented in **Chapter 4** I'll illustrate this concept with West Nile Virus (WNV) infection. The damage to mammalian hosts due to WNV occurs when viruses cross into the central nervous system (CNS) resulting in inflammation. What allows WNV to cross into the CNS is an excessive immune response in the peripheral tissue that involves Toll-like Receptor (TLR) 3 recognition of WNV and the subsequent release of cytokines (IL-6, IFN and TNF-α) which increase the permeability of the blood brain barrier[15] . Therefore the virulence of WNV can be thought of as being primarily due to inflammation in peripheral tissue leading to inflammation of the CNS.

1.3 Neonatal Rat model

The neonatal rat model for colonization of the nasal passages and investigating the natural course of invasive disease, used extensively throughout this dissertation, was developed by Richard Moxon to study *H. influenzae* meningitis[57]. Nasal colonization (when inoculation density exceeds 10^7cfu) is followed closely by bacteremia and meningitis; mimicking the invasive disease process seen in children.

While there are a multitude of animal infection models for *S. aureus*, *S. pneumoniae* and *H. influenzae*bacteria (including mice[34] and cotton rats[36]), the neonatal rat model of colonization is unique in that it allows for all three of the species to reliably colonize their natural habitat, the nasopharynx, with low inoculums. One of the most important considerations (especially to the relevance) of a particular animal model of disease is whether the immune responses will be similar to those that occur in the pathogen's natural host. In the case of neonatal rats their immune development may be considered to follow that of a young infants (less than 6 months) [46, 55, 25, 54, 18], Figure 1.5. The infant rat model has also been employed to investigate *N. meningitidis* and *Streptococcus agalactiae* [72, 74].

Figure 1.5: Comparison of Neonatal Rat and Human Immune Development.



In this dissertation I have extend the model to be used for both *S. aureus* and *S. pneumoniae* nasal colonization.

1.4 Outline of the Thesis

The main aim of the research described in this dissertation is to obtain a better understanding of the bacteria- and host-mediated factors determining nasal colonization and invasive disease due to normally commensal bacteria, such as *S. aureus*, *S. pneumoniae* and *H. influenzae*.

Part I: Ecology of Nasal Colonization

While colonization is the necessary first step to both invasive disease and transmission to another host, little is known about the factors that determine the density and structure of commensal bacterial communities, in short, the ecology of colonization. In **Chapter 2**, I evaluate the role of competition from the same species and different species in determining colonization of *S. aureus*, *S. pneumoniae* and *H. influenzae*in the nasal passages of neonatal rats. Understanding the forces that maintain and disturb the density and structure of colonizing populations can inform efforts to develop probiotics and understanding why co-infection with viruses, such as influenza, promotes bacterial invasive disease. More importantly by investigating the ecology of colonization of these species, predictions can be made about the potential ecological sequelae of the wide-scale use of vaccines directed at specific strains or species, like their replacement by other potentially invasive strains and species.

In this vein, it has been proposed that the reduction of *S. pneumoniae* colonization due to the pneumococcal-conjugate vaccine has lead to a loss of H_2O_2 -mediated interference competition and in turn an increase in staphylococcal invasive infections. In **Chapter 3**, I directly test whether hydrogen peroxide production by *S. pneumoniae* affects the nasal colonization of *S. aureus* .

Part II: Evolution of Virulence

One of the most important questions in infectious disease is why do microbes cause damage to their hosts. While there has been extensive theoretical and some

experimental work on the evolution of virulence, the majority has ignored the role of the immune response. In **Chapter 4**, the different hypotheses for the evolution of virulence are summarized and the role of the host's immune system over-responding is considered. The technical aspects of a mouse wound abscess model is described in **Chapter 5** as I had intended to use this model to test whether abscesses can be considered immune over-responses.

Why do commensal bacteria cause invasive disease in sites from which they can not be transmitted and why do they do so in only a small minority of colonized hosts? While differences in host factors (e.g. age, genetic background, etc.) undoubtedly contributes to some hosts being more susceptible to invasive disease by commensal bacteria, variation (whether genetic or phenotypic) in the colonizing population of bacteria may contribute. One explanation is that the colonizing population of bacteria includes members that are selected because they have or acquire heritable modifications that enable them to invade new sites. In **Chapter 6**, I tested this within–host evolution hypothesis by evaluating whether *H. influenzae* isolated from the blood is more likely than nasal isolates to establish in the blood.

In addition to increasing a pathogen's virulence, within-host evolution can allow for the emergence of antibiotic resistant mutants during antibiotic treatment. Theoretical models of antibiotic treatment have provided hope that treatments can be designed rationally to not only treat infections but also to prevent the emergence of antibiotic resistance. However, most antibiotic treatments are still empirically designed. One limitation of theoretical models of antibiotic resistance is they have either not included the host's immune response or assumed that its only role is to reduce the bacteria's growth rate. In **Chapter 7**, models that explicitly consider how different immune responses can effect the within-host selection of antibiotic resistance during antibiotic treatment are presented.

Ecology of Nasal Colonization and Competition

Elisa Margolis, Andrew Yates and Bruce Levin
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Abstract

Background: Although nasal colonization is the necessary first step in many invasive bacterial infections, little is known about the colonization process. Here, we evaluate the role of competition from the same and different species in the colonization of *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Haemophilus influenzae* in the nasal passages of neonatal rats.

Methods/Results: When neonatal rats are colonized by one of any of these species the density of bacteria in the nasal passage rapidly reaches a constant steady–state density. To investigate intra– and inter–species competition, forty-eight hours after colonizing neonatal rats with one species we invaded with a second inoculum of a marked strain of the same or different species. During intra–species competition, both the established and invading populations co-existed for *H. influenzae* and *S. pneumoniae*. However established populations of *S. aureus* inhibit invasion of new *S. aureus* populations. In inter–species competition, both in neonatal rats and *in vitro* we found that *H. influenzae* reached higher densities when *S. aureus* or *S. pneumoniae* were resident.

Conclusions: The inter–species and intra–species competition results suggest that resource limitation may influence both *S. aureus* and *H. influenzae* colonization.

2.1 Introduction

The first step in a bacterial disease is the successful establishment of a bacterial population in a host, colonization. Although a great deal is known about the course of bacterial diseases and factors contributing to their virulence, relatively less is known about the necessary conditions for and dynamics of this essential first step. Under what conditions can a bacterial population colonize a particular site in a host? How are those conditions affected by prior colonization by bacteria of the same or different species? What determines the number and location of bacteria in the colonizing population and thereby the likelihood of invasive disease, transmission to other hosts and the presence of mutants resistant to antibiotics?

The answers to these questions are of more than just academic interest. They are needed to evaluate the potential ecological and evolutionary sequelae of the wide-scale use of vaccines directed at specific strains or species, resulting in their replacement by other potentially invasive strains and species [16]. In addition, the answers to these questions are critical to understanding the mechanisms of action and towards evaluating the consequences of probiotics (like the use of *Corneybacterium* species to eradicate *Staphylococcus aureus* [39]) as well as potential negative consequences of antibiotic prophylaxis and treatment. Finally, these answers are central to understanding the pathogenesis of bacteria like *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Haemophilus influenzae* that colonize and persist in a large numbers of people without causing disease[32, 1].

In accordance with ecological theory, whether bacteria can colonize or not is determined by the availability of resources (i.e. nutrients, space, attachment space), host immune responses and the presence of toxins or harmful substances.

As the presence of bacteria can influence all of these factors, co-inhabitants often determine whether colonization can occur. Classical ecological theory has suggested that colonization (i.e. stable coexistence) of ecologically similar strains or species is unlikely as the one that grew faster on the available resources should exclude the others [11]. However numerous theoretical and experimental studies suggest that coexistence may occur in many scenarios, for example where there is differential utilization of multiple resources [25, 37, 15, 12, 38, 3]or spatial or temporal variability in a habitat [6]. In addition to resource competition, colonization is influenced by whether the resident bacteria population produces harmful substances (like bacterocins [5, 31]) or induces a host immune response to which the invading population is more sensitive than the resident [10, 26]. Therefore colonization investigations must consider the co-inhabitants' influences on the habitat, namely resource availability and predation by the host's immune response, toxins or other harmful substances.

The nasal mucosa harbors a diverse prokaryotic microflora, including both endogenous harmless and potentially pathogenic bacteria. For bacteria in this latter category (including *S. aureus*, *S. pneumoniae* and *H. influenzae*) successful colonization of this often already occupied habitat is the first step for further transmission, invasive disease (i.e. meningitis, bacteremia or endocarditis) and infection of adjacent tissues (i.e. otitis media or pneumonia) [42, 9, 2]. Here we investigate the role of inter– and intra–species competition in the nasal colonization of neonatal rats by *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*. First we consider the population dynamics of nasal colonization for each strain separately and demonstrate that by 48 hours a steady-state density has been reached that is independent of inoculum density. To characterize the nature of intra–specific competition, 48 hours after colonizing neonatal rats with one species we pulsed with a second inoculum of a marked strain of the same species. These pulse experiments suggest that resident *S. aureus* prevents co–colonization of the same strain; while for both *H. influenzae* and *S. pneumoniae* the steady-state density is increased to allow for the co-existence of pulsed and established popu-

lations. We also found inter–species interactions between these three potentially pathogenic species which in one case increased and in another decreased the density of the colonizing species.

2.2 Methods and Materials

2.2.1 Bacterial strains, media and inoculum preparation

S. pneumoniae TIGR4 [36] and Poland(6b)–20[24] were provided by Lesley McGee. Tr7 was selected as a spontaneous rifampicin resistant mutant of TIGR4. *S. aureus* PS80 (serotype 8 ATTC 27700) was obtained from American Type Culture and Pr1 was selected as a spontaneous mutant of PS80 exhibiting resistance to rifampin. *H. influenzae* Eagan and its streptomycin resistant mutant Rm154 were provided by Richard Moxon. Em4 was selected as a spontaneous mutant of Eagan exhibiting resistance to nalidixic acid.

S. pneumoniae strains were grown in Todd-Hewitt broth supplemented with 0.5% w/v of yeast extract (THY) and plates were supplemented with 4% v/v of sheep blood (BBL). Broth cultures and agar plates of *S. pneumoniae* were incubated at $37^{\circ}\mathrm{C}$ with 5% CO_2. *H. influenzae* strains were grown in brain heart infusion broth supplemented with 10μg of hemin and 2 μg of βNAD per ml (sBHI). *S. aureus* strains were cultivated in Luria-Bertani (LB) broth cultures.

Equal fitness of antibiotic marked strains was confirmed by mixing equal densities of cultures in exponential phase and sampling the initial densities and the densities 6 hours later in broth or 48 hours later in nasal passages of neonatal rats. For all combinations (i.e. TIGR4/Tr7, PS80/Pr1, Rm154/Em4), there was no significant fitness difference *in vitro* or *in vivo* (data not shown).

Inoculum for all the infant rat experiments were prepared by initially growing strains to late logarithmic phase (OD_{620}:0.35-0.8). These were stored at $-80^{\circ}\mathrm{C}$ and then thawed before suspending in 2 ml of either LB, THY or sBHI. Mid-exponential phase cultures were centrifuged (5,000 g x 3 min) and resuspended in

phosphate-buffered saline with 0.1% gelatin (PBSG). Note the addition of gelatin did not lead to an increase in the inoculation density for any of these bacteria. Bacterial densities were estimated by plating dilutions of *S. aureus* on LB Agar plates or LB plates supplemented with rifampicin (40 mg/L); *S. pneumoniae* on THY blood plates supplemented with either streptomycin (40 mg/L) or rifampicin (50 mg/L) or *H. influenzae* on sBHI plates supplemented with bacitracin (0.3 g/L) and either streptomycin (4 mg/L) or nalidixic acid (5 mg/L).

2.2.2 Infant Rat Model

All *in vivo* experiments were performed under the guidelines approved by the Emory Institutional Animal Care and Use Committee. Three-day-old pups, born of timed-pregnant Sprague–Dawley rats (Charles River Laboratories), were randomly reassigned to dams. At 3 or 5 days of age, rats were intranasally inoculated by touching a drop of 10^2 – 10^8 bacteria of either *S. aureus* , *S. pneumoniae* or *H. influenzae* (that had been spun down and re-suspended in 5 μl PBS supplemented with 0.1% gelatin (PBS-G)) to the right and then another 5 μl to the left external nares[20]. The nasal flora of un-inoculated neonatal rats, determined by colony morphology on blood plates, appeared to consist primarily of non-hemolytic streptococci and coagulase-negative staphylococci. No *S. aureus*, *S. pneumoniae* and *H. influenzae*colonies were isolated from un-inoculated neonatal rats.

Two days after the innoculation, nasal wash was collected from 200μl of PBS-G instilled into a 5 cm intramedic polyetylene tubing (PE50, intramedic, Clay Adams) placed into the trachea, and nasal epithelium was scraped from the nasal passages after a second wash of 200μl of PBSG and removal of the frontal bones. Up to 3 sequential nasal washes contained no significant decrease in the bacteria density compared to the first wash. The nasal epithelium was homogenized in 1 ml of PBS-G.

In all experiments, 100μl of the nasal wash and nasal epithelium samples were plated directly and serially diluted onto selective plates. The limit for detection

was 10 cfu/ml. Nasal wash densities were converted to cfu in rat by multiplying cfu/ml by 5 (200uL total vol.) and nasal epithelium by multiplying by 1 (1ml total vol.). With the exception of the *H. influenzae –S. pneumoniae* interaction, data from the nasal wash and nasal epithelium data are in agreement and only the nasal epithelium data are presented; as nasal epithelium likely represents the persistent colonizing population [4].

2.2.3 Experimental Design

For the population dynamics of nasal colonization, groups of 4–16 5-day-old rats were intranasally inoculated with either 10^4 or 10^7 cfu bacteria of *S. aureus*, *S. pneumoniae* or *H. influenzae*and sampled 12–144 hours after inoculation. Inoculum independence was confirmed by inoculating groups of 7–16 5-day-old rats with 10^2– 10^8 cfu bacteria of *S. aureus*, *S. pneumoniae* or *H. influenzae*and sampling at 48 hours.

For intra–species invasion, one marked variant of a particular strain was intranasally inoculated into two groups of 24–36 3-day-old rats. Fourty-eight hours later one group was intranasally inoculated with the same species with the alternative antibiotic marker while the other inoculated with PBS. At 0, 24, 48 and 96 hours after pulsing with the same species 6–8 rats were sacrificed and sampled. For each pairing between antibiotic marked strains of the same species (i.e. TIGR4/Tr7, PS80/Pr1, Rm154/Em4), this experiment was repeated with the reverse strain being established and pulsed.

For the inter–species invasion, experiments testing, groups of 8–12 3-day-old rats were inoculated in both nostrils with either one species (*S. aureus*, *S. pneumoniae* or *H. influenzae*) or with PBS. All of these rats were then inoculated 48 hours later with 10^6– 10^7 of another species (*S. aureus*, *S. pneumoniae* or *H. influenzae*).

2.2.4 Immune Depletion

For systemic complement depletion, cobra venom factor (CVF; Advanced Research Technologies, San Diego, CA) was administered to 4-day-old neonatal rat by intraperitoneal injection of 500 μg/ kg of weight (dissolved in 0.1 M PBS)[7]. Systemic complement depletion was confirmed by the EZ Complement CH50 Test kit (Diamedix, Miami, FL)[14].

For systemic neutrophil depletion, anti-neutrophil serum (ANS, absorbed rabbit anti-rat PMN; Accurate Chemical, Westbury, NY) was administered to 4-day-old neonatal rat by subcutaneous injection of 6 μL/g of weight (diluted 1:1 in PBS)[13]. Systemic neutrophil depletion was confirmed by FACS analysis of blood and local depletion confirmed in the nasal passages using a myeloperoxidase (MPO) assay of nasal epithelium[44].

2.2.5 Statistical Analysis

The bacterial densities (and the log_{10} transformed densities) during colonization were not normally distributed. To determine whether inoculum size altered the median bacterial density or whether the density varied from 48 to 96 hours post-inoculation, a Kruskal-Wallis rank sum test was used to compare the ranks for each inoculum size or time point. A Wilcoxon rank–sum test was used to evaluate the statistical significance in inter-species competitions or the myeloperoxidase results for different strains.

2.3 Results

2.3.1 Population Dynamics

All three species readily colonize the nasal passages of neonatal rats. Within 48 hours after one of the three species is inoculated, *H. influenzae*, *S. aureus* and *S. pneumoniae* reach and maintain for at least three days a population of 100-10,000

cfu in the nasal epithelium (Figure 5.1). This colonization occurs in spite of the fact that these neonatal rats' nasal passages already contain bacterial flora. As the population dynamics of nasal colonization did not differ in the nasal wash sample with the nasal epithelium, only the nasal epithelium data are shown.

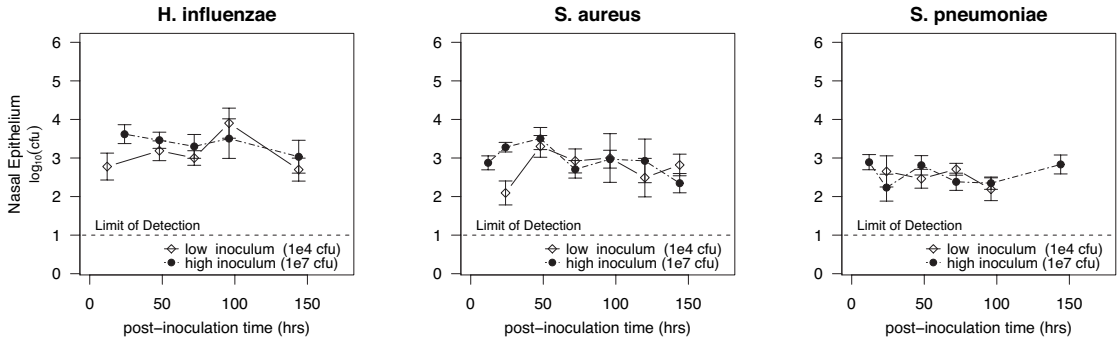

Figure 2.1: Population dynamics of nasal colonization. Five-day-old neonatal rats were inoculated with 10^7 (black circles) or 10^4 cfu (diamonds) of either *S. pneumoniae*, *H. influenzae* or *S. aureus*. The median bacteria density in the nasal epithelium of 4–16 rats at each time-point is plotted. Error bars represent SE.

The bacterial load for each of the species was not significantly different from 48 to 96 hours (p-values for each species determined by Kruskal-Wallis rank sum were <0.05). While the dynamics for both a low and high inoculum density appear to be similar, we ascertained whether bacterial load is inoculum-independent at 48 hours after inoculation. For all three species the bacterial load is invariant over a wide range of inocula (10^2-10^8 cfu) (Figure 2.2), suggesting that nasal colonization rapidly reaches a steady-state.

2.3.2 Invasion of Same Species in a Colonized Host

To test whether nasal colonization can occur in the presence of the same species, new populations of bacteria were pulsed (10^4 cfu inoculated) into rats that were

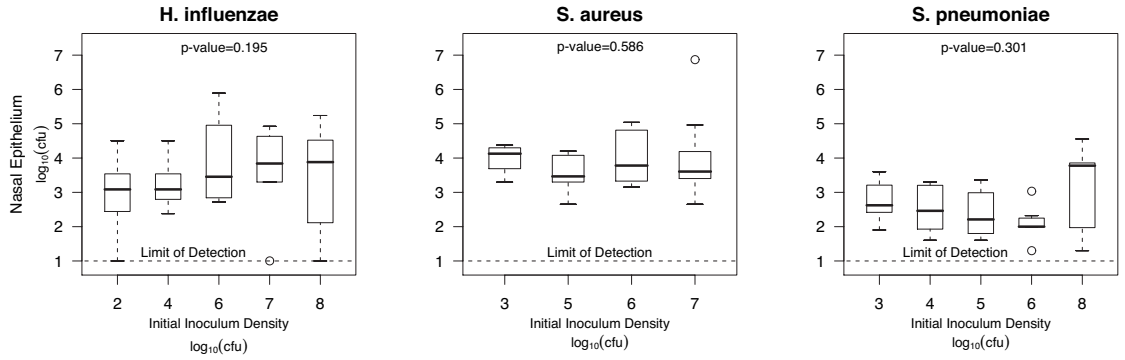

Figure 2.2: Bacterial load at 48 hours post-inoculation is independent of Inoculum Density. Groups of 7–16 five–day–old neonatal rats were inoculated with 10^2-10^8 cfu of either *S. pneumoniae*, *H. influenzae* or *S. aureus*. The 25th to 75th percentiles of nasal wash and epithelium samples taken 48 hours after bacterial challenge are represented by the box plots, with the bold horizontal bar indicating the median value, circles outlying values and dotted error bars SE. P values were determined by Kruskal-Wallis rank sum which tested the null hypothesis that the bacterial load are distributed the same in all of the inoculum groups.

already colonized by bacteria of that species. Antibiotic markers that conferred no *in vitro* or *in vivo* fitness costs were used to distinguish the resident and pulsed populations and each experiment was repeated reversing the strains as pulsed or resident to control for any fitness differences. As the population dynamics suggest that the bacterial load for each of these species is tightly controlled, we expected that the total density (resident+pulsed) would return to the bacterial load observed in rats without pulses. Because resident and pulsed strains of the same species utilize the same resource (and attract the same immune responses), co-existence of both strains is expected unless the limiting factor is available only on a first come first serve basis.

In the case of *S. aureus*, regardless of whether the marked strain is resident or pulsed, we find that the pulsed strain declines in density (faster relative to the established) over the course of 96 hours. As the pulsed strain declines (decrease in percent shown in dotted line) the total bacterial load of *S. aureus* in the rats with

the pulsed and established strain (+ pulse) doesn't differ from the total density of *S. aureus* in the rats with only the established strain (- pulse). For *S. aureus* the bacterial density does not exceed that observed in rats without a pulse and priority is given to the resident strain.

For both *S. pneumoniae* and *H. influenzae* there is an increase in the total density in the rats with the pulse (+ pulse) compared to rats with only the established strain (- pulse). As the population dynamics had suggested that bacterial load is under tight control, we ensured that the result was not a fluke by repeating the pulse experiments four times each for *S. pneumoniae* and *H. influenzae*. We saw the bacterial load increase to varying degrees (more so for *H. influenzae* and less for *S. pneumoniae*) in every replicate (data available upon request). In both of these species, we observe that the pulsed and resident strains co-exist with the pulse strain becoming 25-75% of the population.

For all the species, the pulse results obtain in reciprocal experiments (switching pulse and resident strains) confirming that the results are not due to fitness differences in the antibiotic marked strains.

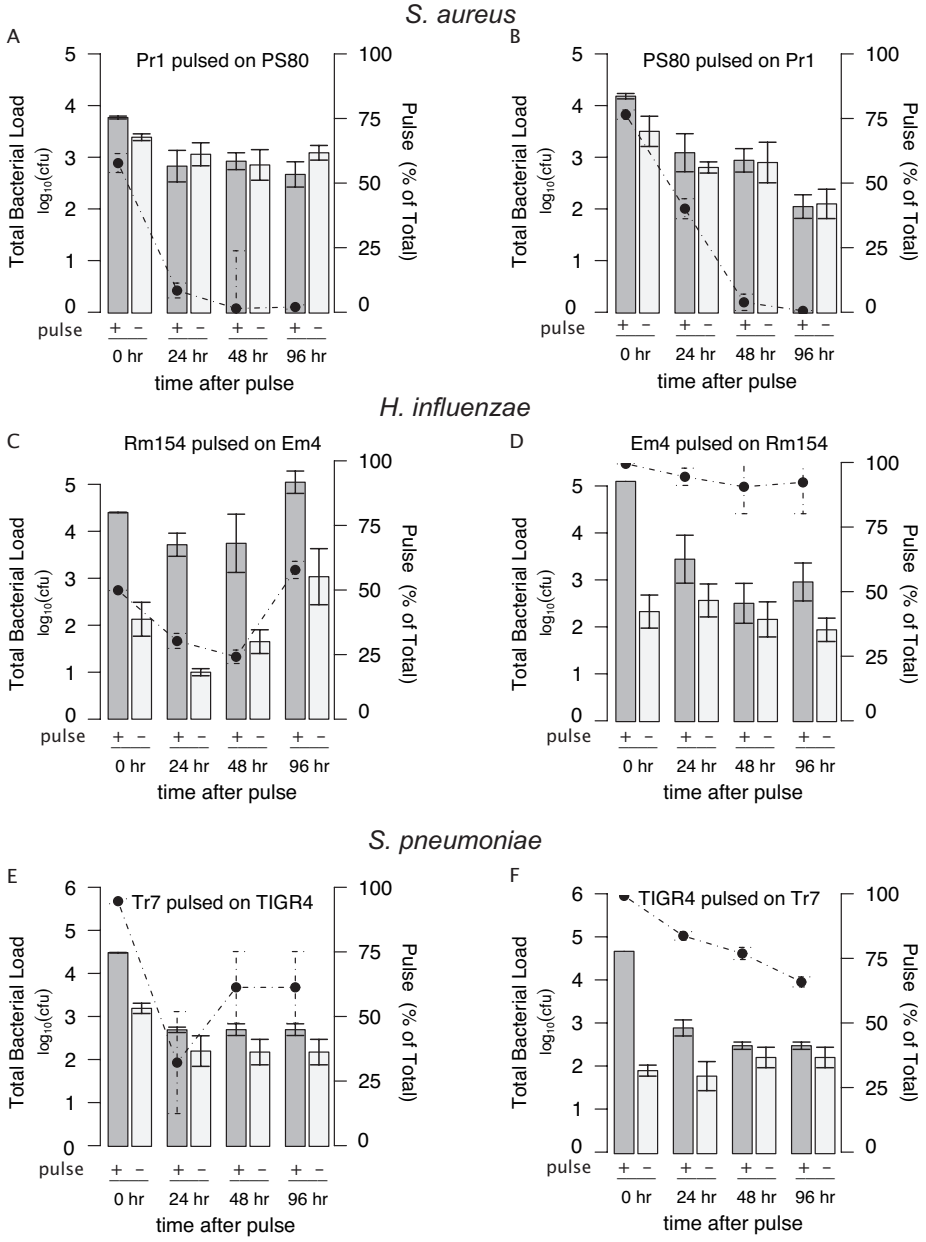

Figure 2.3: Pulse on established populations of same species. Established populations were inoculated into 3-day-old neonatal rats 48 hours prior to pulsing 10^4 cfu of a marked strain of the same species or PBS. The total bacterial density in nasal epithelium of 6–8 rats with the established and pulsed population (dark grey) and just the established population (light grey) were tracked over 96 hours after the pulse and expressed as the median with error bars indicating SE. In addition, the percent of the bacterial density that is pulsed is marked with points with dotted error bars indicating SE. Antibiotic marked strains were switched to be either pulsed or established for *H. influenzae* (in A and B), *S. aureus* (in C and D) and *S. pneumoniae* (in E and F).

2.3.3 Invasion of Different Species in a Colonized Host

Competition among the same species and particularly in the case of the same strain (as in the above pulse experiment) is usually mediated through a limiting resource. Competition between species, in addition to partitioning of a shared resource, can be mediated through inhibitory agents/toxins (allelopathy) or predators (the immune system). Previous studies suggest that *S. pneumoniae* production of hydrogen peroxide may affect the densities of other species[27, 30] and that immune-mediated competition reduces *S. pneumoniae* density in the presence of *H. influenzae* [17]. To evaluate the contributions of these different competitive mechanisms we performed invasion experiments (with one strain of each species: Eagan, TIGR4 and PS80), in which one species was resident and a second was introduced (an invader).

Table 2.1: Density ($\log_{10}$ cfu) in nasal epithelium when invading a host colonized with another species

	Resident[a]						
	Uninfected	*S. pneumoniae*		*H. influenzae*		*S. aureus*	
Invader	Density[b]	Density[b]	p [c]	Density[b]	p [c]	Density[b]	p [c]
S. pneumoniae	3.30±0.20			3.30±0.32	0.49	3.45± 0.22	0.51
H. influenzae	3.08±0.28	5.17±0.36	**0.01**			4.26± 0.18	**0.03**
S. aureus	3.53±0.18	3.31±0.18	0.20	3.21± 0.30	0.42		

[a] Resident bacterial density was not significantly different from un-invaded rats in any combination of species.

[b] Median (± SE) bacterial density of invader in 8–16 rats in units of $\log_{10}$ cfu

[c] P value from Mann Whitney U test comparing the bacterial density of previously uninfected rats and in those with another species already resident. Values considered significant (< 0.05) are in bold.

We found that *H. influenzae* reaches a higher density when invading resident populations of either *S. aureus* or *S. pneumoniae* (Table 1). A similar increase in the bacterial density of *H. influenzae* was observed *in vitro* ; when mixtures of these strains were grown in broth for 6 hours *H. influenzae* bacterial density was

20%(±14) greater with *S. pneumoniae* and 19%(±3) greater with *S. aureus* present than when grown alone.

Immune–mediated Competition

We had expected to detect immune–mediated competition between *H. influenzae* and *S. pneumoniae*, as had been observed in a mice model of colonization by Lysenko and colleagues [17], however we saw no evidence with TIGR4 and Eagan (Table 1). To follow up on why we detected no interaction between *H. influenzae* and *S. pneumoniae* in neonatal rats, we tested an additional *S. pneumoniae* strain (Poland(6b)–20). We found that this particular strain of *S. pneumoniae* had a reduced density in the nasal wash (but not the nasal epithelium) when invading in a neonatal rat with an established *H. influenzae* population (Figure 2.4). This reduction in Poland–20's population did not occur in neonatal rats which had been depleted of complement or neutrophils.

To explain why we could only observe this in one of the two strains tested and only then in the nasal wash, we hypothesized that either induction of or sensitivity to the immune system must differ in these strains and locations. We quantified the neutrophil infiltration in the nasal epithelium by measuring the Myeloperoxidase (MPO) activity at 48 hours after inoculation with each strain/species alone or when Poland(6b)–20 was inoculated on an established *H. influenzae* population (Figure 2.7 A).

No difference in neutrophil infiltration is observed between rats colonized by the two different *S. pneumoniae* strains (TIGR4 and Poland(6b)–20). The neutrophil infiltration observed 48 hours after Poland(6b)–20 invaded on an established *H. influenzae* population (when immune mediated competition was observed in the nasal wash) is significantly higher than rats with just Poland(6b)–20 colonizing alone, however it was not significantly higher than in rats with only *H. influenzae*. While these results suggest that *H. influenzae* is primarily responsible for the neutrophil infiltration that reduces the nasal lumen populations of some strains of *S. pneumoniae*, *S. pneumoniae* may still have a role in eliciting the immune response.

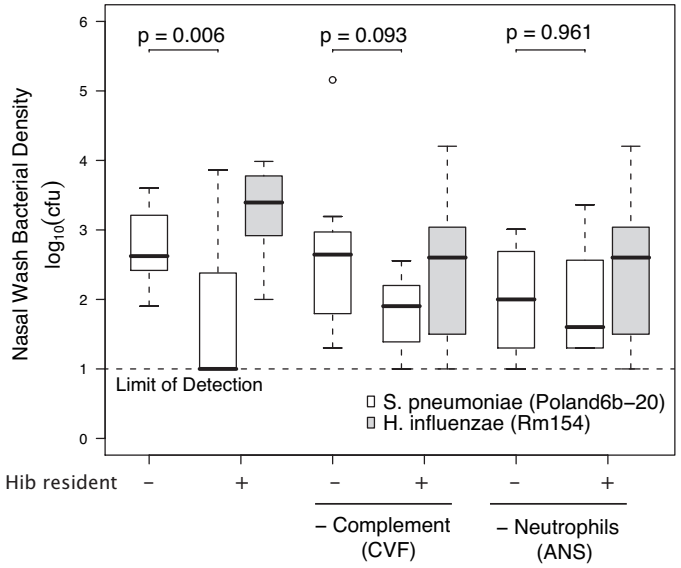

Figure 2.4: Immune Mediated Competition. Three-day-old neonatal rats were treated with either anti-neutrophil serum (–neutrophil) or cobra venom factor (–complement) or PBS and inoculated with either 10^6cfu of *H. influenzae* or PBS (alone). Forty-eight hours later, 10^4 cfu of Poland(6b)–20 *S. pneumoniae* was inoculated. The 25th to 75th percentiles of nasal wash samples taken 48 hours after *S. pneumoniae* inoculation are represented by the box plots, with the horizontal bar indicating the median value and circles outlying values. Error bars indicate the standard error. *P*-value from Mann Whitney U test comparing the bacterial density of previously uninfected rats and those with established populations of *H. influenzae*.

We observed that the neutrophil infiltration in response to *S. pneumoniae* colonizing alone increases from 48–96 hours after inoculation, compared to the constant neutrophil presence with *H. influenzae* (Figure 2.7 B).

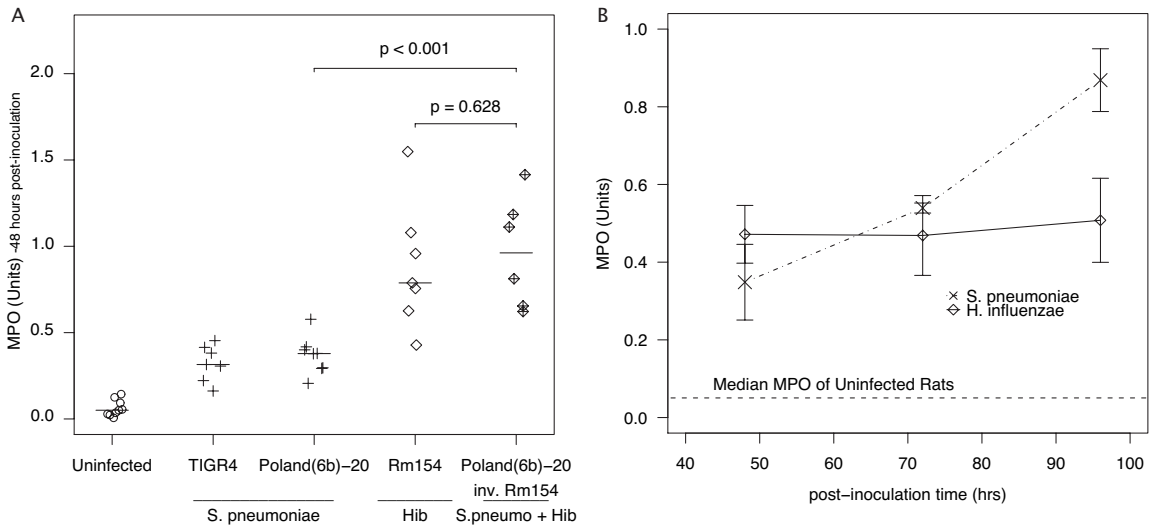

Figure 2.5: Neutrophil infiltration: comparison of strains and species at 48 hours and dynamics over 96 hours. A) Neutrophils in the nasal epithelium from rats inoculated 48 hours earlier with 10^4 cfu of bacteria from a single species (Rm154, TIGR4 and Poland(6b)–20) or from rats inoculated 96 hours earlier with 10^6 cfu of *H. influenzae* and 48 hours earlier with 10^4 cfu of Poland(6b)–20 were quantified using the MPO assay. Lines indicate median MPO values. P-value is calculated by the Wilcoxon rank sum test. B) Dynamics of neutrophil infiltration in response to nasal colonization by *S. pneumoniae* (TIGR4) or *H. influenzae*. Following inoculation groups of 5–8 rats were sacrificed and neutrophil infiltration was measured by MPO assay. Median MPO Units are plotted. Error bars represent SE.

2.4 Discussion

2.4.1 Population Dynamics

Our experiments indicate that all three species we studied (*S. aureus* , *S. pneumoniae* and *H. influenzae*) can colonize the nasal passages of neonatal rats and each reaches a bacterial load that is independent of the initial inoculum size – whether inoculated at densities above or below their respective levels, the population reaches and maintains at this density. The wide-spread variation in bacterial load and the relatively low total densities observed mirrors what has been seen in

human hosts [34]. That the bacterial load is invariant to changes in the initial inoculum size indicates that the bacteria population is tightly controlled– perhaps by a limiting resource or a host's immune response.

2.4.2 Invasion of Same Species in a Colonized Host

As the nasal passages are almost always coated with endogenous flora, newly inoculated bacteria likely face stiff competition. To investigate competition within and between each species, we performed a series of pulse experiments with genetically marked colonizing (pulsed) and resident (established) bacteria. We expected that the bacterial density in the rats with the pulse would return to the steady-state density with either the pulsed population being competitively excluded or co-existing with the established population.

During intra–species competition, for both *H. influenzae* and *S. pneumoniae* , the resident and the pulsed strains coexist. Presumably this means there is sufficient access to the limiting resource for the pulsed strain to colonize or that the limiting host's immune response doesn't distinguish between resident and pulsed bacteria. Surprisingly following the pulse the bacterial load of *S. pneumoniae* and *H. influenzae* increased and this result occurred in every replicate of this experiment. While clearly baffling in light of the steady state densities we observed in following the population dynamics of single inoculations of these strains, these results might be attributable to an expansion in the colonization area, increased immune suppression or the release of new resources– perhaps associated with an inflammatory response. While the innate immune response can reduce the amount of iron available for bacteria [22] we are unaware of investigations into whether bacteria can take advantage of resources (i.e. dead cells, released metabolites) created by an inflammatory response.

More straight forward, our results clearly indicates that invading strains of *S. aureus* are at a disadvantage relative to the resident; in intra–species competition the pulsed strain is lost while the resident strain is maintained. It should be noted that in these experiments we controlled for fitness differences by reversing

the markers of the pulsed and resident strain. Moreover, as expected following the loss of the pulsed *S. aureus* strain, the total density returns to the set point rather than increase. These experiments suggest that *S. aureus* is limited by a localized resource available on a 'first-come, first-serve' basis – perhaps attachment sites [41, 33]. This result may explain why competing strains of *S. aureus* were excluded from burn wounds[43] and from nasal colonization in persistent human carriers[23].

2.4.3 Invasion of Different Species in a Colonized Host

We expected that inter–species competition between these three strains would reduce the bacterial density of these species due to overlapping resource requirements, allelopathy by harmful substances (i.e. bacterocins and hydrogen peroxide) and immune–mediated competition. However our results are consistent with the proposition that any pairwise combination of the three species can co-exist; as the presence of one species does not reduce the nasal epithelium colonization density of another. In fact in the only significant interaction observed *H. influenzae* reaches a higher density when invading resident populations of either *S. aureus* or *S. pneumoniae* than it achieves in rats not colonized by these bacteria. Since this result is also observed *in vitro* it seems likely due to some host-independent mechanism like *S. aureus* and *S. pneumoniae* providing nutrients that would otherwise limit *H. influenzae* . Indeed, in the past *H. influenzae* has been identified and cultured due to the fact that it grew as satellites off of *S. aureus* colonies [8]. To our knowledge this is the first evidence for *S. aureus* and *S. pneumoniae* providing nutrients to *H. influenzae* during nasal colonization. That we were unable to detect other previously reported/speculated multi-species interactions in the nasal epithelium of neonatal rats, provides some clues as to the conditions in which these interactions are likely to occur.

It has been proposed that the production of hydrogen peroxide by *S. pneumoniae* may affect the densities of *S. aureus* and *H. influenzae* as both are susceptible to hydrogen peroxide killing [27, 30, 35]. However in this and previous work [19] we

found no evidence that hydrogen peroxide produced by *S. pneumoniae* limits the colonizing populations of either of the two species. This may be because the density of *S. pneumoniae* is too low for sufficient hydrogen peroxide production or the nasal epithelium inactivates the hydrogen peroxide produced. That we found no ecological interaction between *S. aureus* and *S. pneumoniae* colonization suggests that the epidemiological observation that *S. aureus–S. pneumoniae* co-colonization is rarer than expected [1, 29, 21, 40, 18] may be due to the bacteria preferring different hosts.

2.4.4 Immune–mediated Competition

Previous experiments by Lysenko and colleagues in a mouse model have shown that when *H. influenzae* and *S. pneumoniae* co-colonize, *S. pneumoniae's* density in the nasal wash is lower than when inoculated alone due to immune-mediated competition[17]. While the results of our rat model experiments with *H. influenzae* and *S. pneumoniae* are consistent with their work [17], they also suggest that this immune-mediated competitive interaction may only affect the colonizing *S. pneumoniae* population in the nasal wash (not the population adhering to nasal epithelium) and be strain-specific. Having observed this phenomenon with the clinical strain of *S. pneumoniae* Poland(6b)–20 but not with TIGR4, we hypothesize that these strains must vary either in their ability to elicit or in their suscebtibility to the immune response that occurs in the presence of *H. influenzae* . We found that both strains elicited similar neutrophil infiltration during nasal colonization. Moreover, while we can't exclude the possibility that *H. influenzae* and *S. pneumoniae* may elicit a more intense innate immune response together than when alone [28], our results provide no evidence for this synergy. There is no difference in the neutrophil infiltration 48 hours after inoculation in rats co-colonized with *H. influenzae* and *S. pneumoniae* than with *H. influenzae* alone. However, these results may be due to differences in the dynamics of the immune response to *H. influenzae* and *S. pneumoniae* colonization, as *S. pneumoniae* doesn't elicit much neutrophil infiltration until 72-96 hours after inoculation. Together our results suggest that the

immune response primarily elicited by *H. influenzae* is responsible for reducing the density of *S. pneumoniae* in the nasal wash and that *S. pneumoniae* strains may vary in their susceptibility to this innate immune response. While we found limited evidence for immune mediated competition, that the nasal epithelium population is un–affected suggests that this competition may not effect the long–term carriage of *S. pneumoniae* in the nasal passage.

2.4.5 Limitations and Significance

The most significant limitation and caveat associated with this study, is that the neonatal rat immune system is rapidly changing during the course of these experiments, thereby, limiting our ability to draw inferences about the persistence of colonizing bacteria. While arguably a decent model for young infants, the neonatal rats are unlikely to be an accurate model of the nasal passages of older children or adults. The results obtained may be strain specific and only one or two strains for each species was tested. Furthermore, our ability to discern interactions was limited by the large amount of variation in densities observed in individual rats.

Caveats and limitations aside, we believe that the application of an ecological framework to the colonization of neonatal rat model with *S. aureus*, *S. pneumoniae* and *H. influenzae*is useful to our understanding the epidemiology of colonization, disease processes and the impact of vaccination on these bacteria species. These results are only beginning to address the mechanisms responsible for the dynamic process of nasal colonization with turnover and replacement of species, serotypes and strains in the complex community. For example the pulse experiments results suggest that for *S. pneumoniae* and *H. influenzae* the presence (and turnover) of multiple strains and serotypes would be expected in carriers. Or that *H. influenzae* colonization is likely to be more successful (and possibly more likely to cause disease if disease is density dependent) when preceded by either *S. aureus* or *S. pneumoniae*. Ultimately the ecology of nasal colonization will be able to inform whether vaccination (or antibiotic treatment) directed at one particular species will lead to the unintended consequences of increased colonization by competing

(and possibly more pathogenic) species, serotypes or strains.

2.5 Appendix

The following appendix contains experiments investigating the immune system's role in the population dynamics of each species when colonizing alone and as it is very preliminary will not be included in the final published version of this research.

To ascertain if nasal colonization is immune limited we tested whether impairment of specific components of the innate immune response, specifically neutrophils and complement, leads to an increase in colonization density. We found that the set points for *S. aureus* and *H. influenzae* in rats depleted of neutrophils or complement did not differ significantly from the corresponding set points in control animals (Figure 2.6, panel B). In contrast, the set point of *S. pneumoniae* in rats depleted of neutrophils or complement was significantly greater than in control rats when inoculated at densities in excess of 10^4 cfu (Figure 2.6, panel A). An effect of immunity on the *S. pneumoniae* set point was not apparent at lower inocula densities, however.

Why is *S. pneumoniae* limited by immunity only at high inoculum densities? One explanation is that there is a threshold inoculum size below which elements of the immune response are not induced. To test this hypothesis, the degrees to which *S. pneumoniae* (and the other 2 species) elicited neutrophil infiltration were measured using the myeloperoxidase (MPO) assay. In support of this threshold hypothesis, we found that low inoculum densities of *S. pneumoniae* elicited a significantly lower neutrophil infiltration than high inoculum densities. In addition, *S. aureus*-colonized neonatal rats had no greater density of neutrophils in the nasal epithelium than the uninfected controls. Colonization with *H. influenzae* elicited the strongest neutrophil infiltration (Figure 2.7).

These experiments suggest that initial inoculum levels of *S. pneumoniae* may determine immune response -possibly due to antigen delivery and that neither

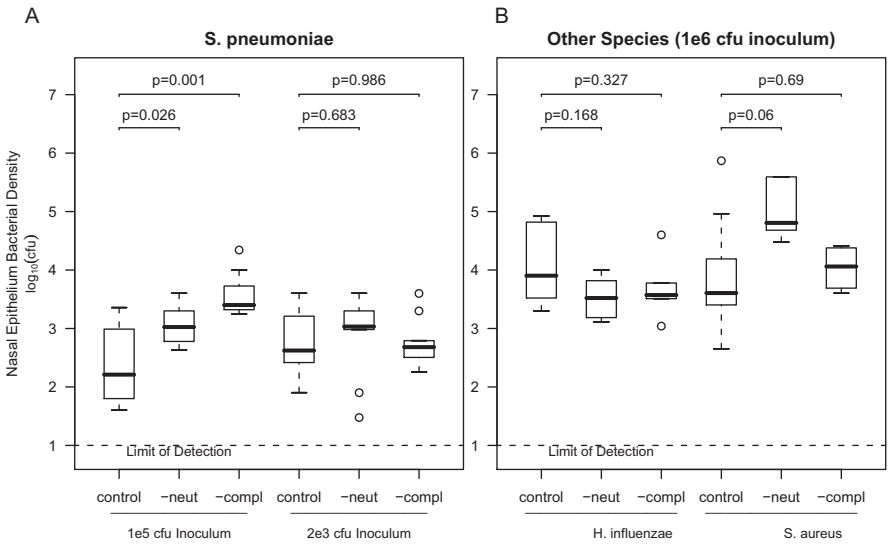

Figure 2.6: Nasal colonization in neutrophil or complement depleted neonatal rats. Twenty-four hours after treatment with either anti-neutrophil serum (neutrophil-depleted) or cobra venom factor (complement-depleted) or PBS (control) 10^6 or 10^3 cfu of either *S. pneumoniae*, *H. influenzae* or *S. aureus* was inoculated. The 25th to 75th percentiles of nasal epithelium samples taken 48 hours after bacterial challenge are represented by the box plots, with the horizontal bar indicating the median value. Error bars represent standard error.

H. influenzae nor *S. aureus* densities in the nasal passages are affected by neutrophils and complement. Further experiments looking into the inoculum density dependence- such as looking into the neutrophil infiltration elicited by heat killed *S. pneumoniae* would be interesting.

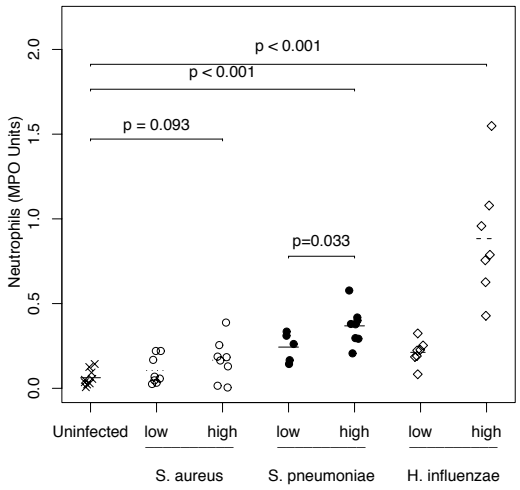

Figure 2.7: Neutrophil infiltration in response to nasal colonization of *S. pneumoniae*, *H. influenzae* and *S. aureus*. Neutrophils in the nasal epithelium from rats inoculated 48 hours earlier with either 10^3 (low) or 10^6 (high) cfu of one of the 3 bacteria species or from uninfected controls were quantified using the MPO assay. The p value displayed was determined by Wilcoxon Rank Sum test.

Hydrogen peroxide mediated interference competition by *Streptococcus pneumoniae* has no significant effect on *Staphylococcus aureus* nasal colonization of neonatal rats

Elisa Margolis

Journal of Bacteriology (*in press*)

Abstract

It has been proposed that the relative scarcity of *Staphylococcus aureus* and *Streptococcus pneumoniae* co-colonization in the nasopharynx of humans can be attributed to hydrogen peroxide-mediated interference competition. Previously it has been shown *in vitro* that H_2O_2 produced by *S. pneumoniae* is bactericidal to *S. aureus*. To ascertain whether H_2O_2 has this inhibitory effect in the nasal passages of neonatal rats, colonization experiments were performed with *S. aureus* and *S. pneumoniae*. The results of these experiments in neonatal rats are inconsistent with the hy-

pothesis that hydrogen peroxide - mediated killing of *S. aureus* by *S. pneumoniae* is responsible for relative scarcity of co-colonization by these bacteria. In mixed inocula colonization experiments and experiments where *S. aureus* invades the nasopharynx of rats with established *S. pneumoniae* populations, the density of *S. aureus* did not differ whether the *S. pneumoniae* strain was H_2O_2 secreting or non-H_2O_2 secreting strain (SpxB). Moreover, the advantage of catalase production by *S. aureus* in competition with a non-catalase producing strain (KatA) during nasal colonization is no greater in the presence of H_2O_2 producing *S. pneumoniae* than non-H_2O_2 producing *S. pneumoniae*.

3.1 Introduction

Recent epidemiological investigations of the carriage of *S. aureus* and *S. pneumoniae* suggest that co-colonization by these two commensal (and occasionally invasive) bacteria is negatively correlated [1, 16, 11, 25, 7]. One corollary of this observation is that a reduction in the frequency of colonization in one of these species would lead to a corresponding increase in the frequency of the other. In fact it has been proposed that the reduction of *S. pneumoniae* colonization due to the pneumococcal-conjugate vaccine has played a role in the increase in *S. aureus* acute otitis media and bacteraemia [24, 5]. The mechanism proposed to account for this co-colonization pattern is interference competition, or allelopathy, mediated by the killing of *S. aureus* by *S. pneumoniae* produced hydrogen peroxide (H_2O_2)[19]. In broth, high densities of *S. pneumoniae* mixed with *S. aureus* results in *S. aureus'* demise, and killing does not occur if the pneumococci genetically lack pyruvate oxidase (SpxB) or catalase is present to neutralize the H_2O_2 [19]. While much attention has been given to the appealing hypothesis that loss of H_2O_2 - mediated interference competition by *S. pneumoniae* is responsible for the increase in invasive *S. aureus* infections, there has been only indirect confirmation [14] that this allelopathic mechanism operates during nasal colonization.

In this report, I test the effect of H_2O_2 production by *S. pneumoniae* on nasal col-

onization of *S. aureus* in two scenarios where interspecies interference may occur in a neonatal rat model: the invasion of an established population of *S. pneumoniae* by *S. aureus* and mixed inocula of both species. By using isogenic strains of *S. pneumoniae* that either produce or do not produce H_2O_2 (SpxB) I demonstrate that H_2O_2 production by *S. pneumoniae* has no effect on *S. aureus* colonization. Furthermore, the advantage of catalase producing *S. aureus* in the presence of *S. pneumoniae* is manifest whether *S. pneumoniae* produce or do not produce H_2O_2. I discuss some potential reasons why *S. pneumoniae* - mediated H_2O_2 allelopathy is effective against *S. aureus in vitro* but not *in vivo*.

3.2 Methods and Materials

3.2.1 Bacterial strains, media and inoculum preparation

S. pneumoniae TIGR4 [22] and an SpxB-negative variant of TIGR4 (designated SxpB) [19] were provided by Marc Lipsitch. *S. aureus* PS80 (serotype 8 ATTC 27700) and Newman (NCTC 8178) were obtained from the American Type Culture collection. A catalase-deficient KatA-negative variant of Newman (designated KatA) [14] was provided by George Liu. *S. aureus* strains were cultivated in Luria-Bertani (LB) broth cultures and agar plates incubated at $37°C$. *S. pneumoniae* strains were grown in Todd-Hewitt broth supplemented with 5 g of yeast extract (THY) and plates were supplemented with 40ml of sheep blood (BBL). Broth cultures and agar plates of *S. pneumoniae* were incubated at $37°C$ with 5% CO_2.

Inoculum for all the infant rat experiments were prepared by initially growing strains to late logarithmic phase (OD:0.35-0.8). These were stored at $-80°C$ and then thawed on the experiment day before suspending in 2 ml of either LB or THY. Cultures that reached mid-exponential phase were centrifuged (5,000 g x 3 min) and resuspended in phosphate-buffered saline with 0.1% gelatin (PBSG). Inocula and animal specimen densities were estimated by plating dilutions on LB Agar plates for Newman or PS80, LB plates supplemented with spectinomycin (100mg/L) for KatA or *S. pneumoniae* on THY blood plates supplemented with

either streptomycin (40mg/L) for TIGR4 or kanamycin (75mg/L) for SpxB.

3.2.2 Infant Rat Model

All *in vivo* experiments were preformed under the guidelines approved by the Emory University Institutional Animal Care and Use Committee. Three-day-old pups, born of timed-pregnant Sprague–Dawley rats (Charles River Laboratories), were pooled, randomly reassigned to dams and maintained in microisolator cages in a biocontainment facility. At 3 or 5 days of age, rats were intranasally inoculated by touching a drop of 10^6 – 10^7 bacteria of either a mixture of *S. aureus* and *S. pneumoniae* strains or one strain alone suspended in 5 μl PBS supplemented with 0.1% gelatin (PBS-G) to the right and then another 5 μl to the left external nares[12, 13, 9].

Two days after the innoculation, nasal wash was collected from 200μl of PBS-G instilled into a 5 cm intramedic polyetylene tubing (PE50, intramedic, Clay Adams) placed into the trachea, and nasal epithelium was scraped from the nasal passages after a second wash of 200μl of PBSG and removal of the frontal bones. The nasal epithelium, which has been suggested to represent a distinct population [2], was homogenized in 1 ml of PBS-G.

In all experiments, 100μl of the nasal wash and nasal epithelium samples were plated directly and serially diluted onto selective plates. Plates were incubated overnight at $37^{\circ}\mathrm{C}$ and all colonies were counted. The limit for detection at any site was 20 cfu/ml.

3.2.3 Experimental Design

In the mixed inoculum experiments , 5-day-old rats were intranasally inoculated with 10^6– 10^7 bacteria of a mixture of a *S. aureus* strain (Newman, PS80, KatA or mixture of Newman and KatA) and a *S. pneumoniae* strain (TIGR4 or SpxB) at a ratio of 1:5 (*S. aureus* : *S. pneumoniae*). For each pairing between a *S. aureus* and *S. pneumoniae* strain, this experiment was replicated in three different groups of 6–12

rats; a single replicate is shown (all others are available on www.eclf.net).

For the experiments testing whether *S. aureus* can invade when *S. pneumoniae* is established, groups of 6 3-day-old rats were inoculated in both nostrils with 10^6 *S. pneumoniae* (TIGR4 or SpxB) or with PBS. All of these rats were then inoculated 48 hours later with 10^6– 10^7 *S. aureus* (Newman, PS80, KatA or mixture of Newman and KatA). For each pairing between a *S. aureus* and *S. pneumoniae* strain, this experiment was replicated in two different groups of 6–12 rats; a single replicate is shown (all others are available on www.eclf.net).

3.2.4 Statistical Analysis

A Welch's *t* test was used to evaluate the statistical significance of H_2O_2 production by *S. pneumoniae* on the bacterial density of *S. aureus* following growth on agar surfaces or during nasal colonization. To ascertain if catalase producing *S. aureus* was being selected during nasal colonization the selection rate constant (r_{nk}) was calculated. The selection rate is a measure of the relative recovery of the catalase producing *S. aureus* (Newman) and the catalase deficient *S. aureus* (KatA) inoculated into the nasal passages over the 48 hours and is given by

$$r_{nk} = ln\frac{N_n(48)}{N_n(0)} - ln\frac{N_k(48)}{N_k(0)} \tag{3.1}$$

where N_n (0) and N_k (0) are the initial densities in the inoculum of Newman and KatA, respectively and N_n (48) and N_k (48) are the densities detected at the specific site after 48 hours in the rat [23]. A selection rate of 0 indicates that there is no selection for catalase production and a positive rate indicates an advantage for the catalase producing strain. The P values given for the selection rates were determined using the two-tailed probability (from the t-distribution with n-1 degrees of freedom) of rejecting by chance the null hypothesis that the selection rate constant equals zero, indicating equal fitness for the catalase producing (Newman) and catalase deficient (KatA) strains.

3.3 Results

Previously it has been shown that *in vitro* H_2O_2 production by *S. pneumoniae* limits the growth of *S. aureus* in liquid culture [19]. To determine whether this occurs *in vivo*, neonatal rats were used because both *S. aureus* and *S. pneumoniae* readily colonize the nasal passages of neonatal rats in single clone and mixed culture (with low inoculum densities). Within 48 hours after a single species is inoculated alone, both *S. aureus* and *S. pneumoniae* reach and maintain for at least 5 days a population of 10^3 cfu/ml in the nasal wash and epithelium (data not shown). Despite large individual variation in bacterial densities with either species, the average bacterial density in neonatal rats are similar to the bacterial loads reported for humans [21]. To determine if H_2O_2 plays a role in competition during colonization when both *S. pneumoniae* and *S. aureus* are introduced in a mixed innoculum, 5-day-old neonatal rats were challenged with a mixture of *S. pneumoniae* and *S. aureus*. The three strains of *S. aureus* were separately mixed with either a H_2O_2 producing *S. pneumoniae* strain (TIGR4) or a non-H_2O_2 producing *S. pneumoniae* strain (SpxB). If H_2O_2 produced by *S. pneumoniae* killed the *S. aureus* one would expect that *S. aureus* would reach a statistically significant lower density in the presence of the H_2O_2 producing strain (TIGR4) compared to when inoculated with the SpxB. This is not observed, Figure3.1.

Since the amount of H_2O_2 produced is proportional to the number of bacteria, the maximum amount of H_2O_2 would be present when the population of producing strain is at a high density. Consequently one would expect that H_2O_2 production would be most effective in preventing invasion in habitats that are already colonized by the H_2O_2-producing population. To ascertain whether this is the case for *S. pneumoniae* H_2O_2 production, *S. aureus* was inoculated intranasally into neonatal rats with established populations of *S. pneumoniae* (inoculated 48hrs earlier) of either H_2O_2-producing (TIGR4) or non-H_2O_2 -producing (SpxB). At 48 hours, the nasal wash and epithelium were sampled and the density of both *S. pneumoniae* and *S. aureus* estimated. The results of this experiment suggest that *S. aureus* is equally able to invade populations of *S. pneumoniae* in the nasal passages

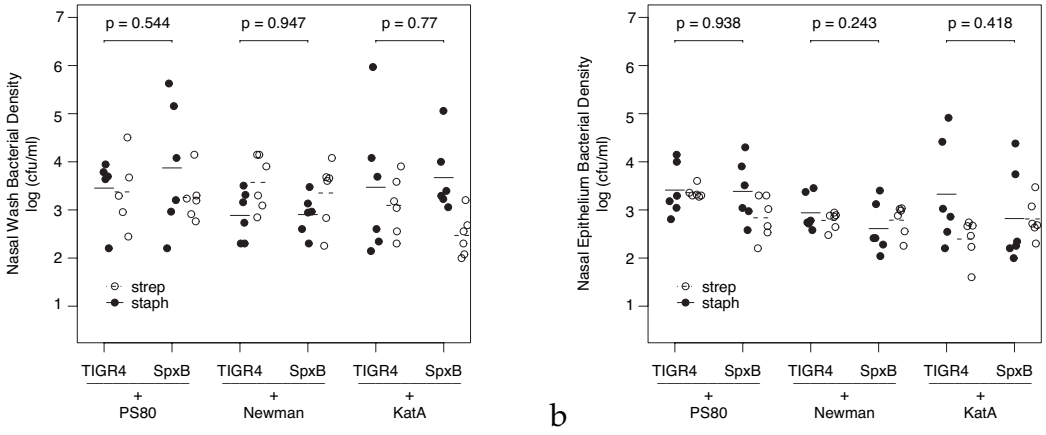

Figure 3.1: Forty-eight hour densities of *S. aureus* and *S. pneumoniae* from nasal wash and epithelium of colonized rats. Five -day-old neonatal rats were colonized with 5 x 10^6 cfu of a *S. pneumoniae* strain which produces H_2O_2 (TIGR4) or one that does not (SpxB) in the left nostril and 1 x 10^6 cfu of *S. aureus* either the PS80, Newman or catalase deficient Newman strain (KatA).

of rats whether the *S. pneumoniae* strain was capable of producing H_2O_2 or not and whether the *S. aureus* produced catalase or not, see Figure**??**.

One explanation for why *S. aureus* is uninfected by *S. pneumoniae* produced H_2O_2 during co-colonization and is able to invade established populations of H_2O_2 producing *S. pneumoniae* is that wild-type *S. aureus* produces a sufficient amount of catalase to neutralize the H_2O_2. Recent results by Park and colleagues [14] suggest that this may be the case. In their experiments with a mouse co-colonization model *S. pneumoniae* producing H_2O_2 selects for catalase - producing *S. aureus* in mixtures with otherwise isogenic KatA mutants that do not produce catalase.

To discern whether H_2O_2 production by *S. pneumoniae* selects for catalase producing *S. aureus* in the neonatal rat model, mixtures of catalase producing (Newman) and non-producing (KatA) *S. aureus* were inoculated either in co-inoculation with or invading on an established *S. pneumoniae* population of either H_2O_2 producing (TIGR4), a non-producing (SpxB) or a PBS buffer control. The selection

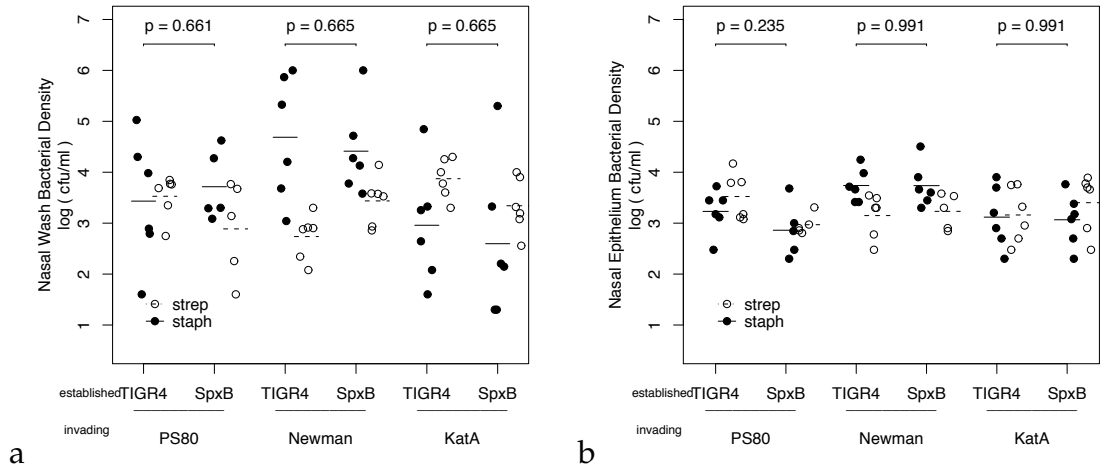

Figure 3.2: Forty-eight hour densities of *S. aureus* and *S. pneumoniae* from nasal wash and epithelium of colonized rats. Three-day-old neonatal rats were colonized with 10^7 cfu of a *S. pneumoniae* strain which produces H_2O_2 (TIGR4) or one that does not (SpxB). Five-day-old neonatal rats were colonized with 10^6 cfu of *S. aureus* either the PS80, Newman or catalase-deficient Newman strain (KatA).

rate, which compares the relative recovery of the two strains in the nasal epithelium, measured the competitive performance of the catalase producing *S. aureus* and the non-catalase producing *S. aureus* . If the selection rate constant is zero there is no advantage for the catalase producer, while a value in excess of 0 would mean catalase production is favored. The nasal epithelium results of this experiment are presented in Table 3.3.

Table 3.2: Selection for catalase production in *S. aureus* in the Nasal Epithelium

	mixed inoculum		*S. pneumoniae* **established**	
	Selection Rate [a] (KatA+ relative to KatA-)	P value[b]	Selection Rate [a] (KatA+ relative to KatA-)	P value[b]
PBS	0.48± 0.83	0.583		
TIGR4	0.48 ± 0.42	0.296	5.52 ± 0.59	0.006
SpxB	1.63 ± 0.39	0.006	3.40 ± 0.64	0.002

[a] Mean (± sem of the mean)

[b] Two-tailed probability from the *t*–distribution of rejecting by chance the null hypothesis that the selection rate constant equals zero, indicating equal fitness for catalase producing (new) and catalase deficient (KatA).

The catalase producing strain has a marked advantage over the non-producer when co-inoculated with a non-H_2O_2 -producing strain (SpxB), however there was no detectable selection for catalase production without *S. pneumoniae* present (PBS control) or when co-inoculated with the H_2O_2 producing strain (TIGR4) 2.3.3. The selective advantage for catalase production is even more pronounced when the mixture of *S. aureus* strains is invading neonatal rats with established populations of *S. pneumoniae*. In this case the fitness advantage of the catalase production is signigicant regardless of whether the established *S. pneumoniae* strain produced H_2O_2. Similar results obtained when fitness was estimated from densities determined from the nasal wash (results not shown).

3.3.1 Discussion

The hypothesis that hydrogen peroxide - mediated killing of *S. aureus* by *S. pneumoniae* is responsible for the relative scarcity of co-colonization of these two species of bacteria is appealing. It provides an explanation for why the increase in invasive *S. aureus* infections could be due to the vaccine associated decrease in pneu-

monoccal colonization. This hypothesis is supported by not only epidemiological studies[1, 16, 11, 25, 7] but also by *in vitro* experiments[19] that show H_2O_2 produced by *S. pneumoniae* is bactericidal to *S. aureus* and limits the density which *S. aureus* reaches in liquid culture. The neonatal rat nasal colonization results of this study are inconsistent with this hypothesis. Hydrogen peroxide production provided no advantage to *S. pneumoniae* in competing with *S. aureus* either in colonizing the nasal mucosa of these rats or preventing established populations of *S. pneumoniae* from being colonized by *S. aureus*. Although the results of our experiments that the production of catalase (which neutralizes H_2O_2) provides a competitive advantage to *S. aureus* in the presence of *S. pneumoniae* - that advantage occurred whether *S. pneumoniae* was H_2O_2 producing or not.

I propose three classes of explanations for why *S. pneumoniae* - mediated H_2O_2 killing of *S. aureus* is effective *in vitro* but fails to prevent colonization of *S. aureus* in the nasal passages of neonatal rats: 1) *S. pneumoniae* does not produce H_2O_2 in sufficient quantities *in vivo* ; 2) H_2O_2 produced in the nasal passages is inactivated either by the host or other members of the nasal flora or 3) the rate of replication of *S. aureus* more than makes up for killing by *S. pneumoniae* produced H_2O_2 . In support of the first explanation is the observation that in broth when *S. pneumoniae* is at low densities relative to *S. aureus* there are insufficient amounts of H_2O_2 to inhibit the growth of *S. aureus*. [16] The same would be expected on surfaces (plates or nasal epithelium) if the *S. aureus* and *S. pneumoniae* colonies are too far apart for *S. aureus* to come into contact with the zone of inhibition formed by *S. pneumoniae*. [3] This may have been the case in the nasal passages of neonatal rats if *S. aureus* and *S. pneumoniae* are not co-localized in the nasal passages or if their densities were too low. It should be noted that in the neonatal rat at least the recovered density of *S. aureus* or *S. pneumoniae* was never greater than 10^5 cfu/nose. It would be of interest to ascertain whether the densities of *S. pneumoniae* in the nasal passage of humans is greater than that observed in these rats. In support of the second explanation is the observation that the nasal epithelium produces both catalase and glutathione peroxidase (a scavenger of H_2O_2) [4]. As for the third explanation, I am unaware of estimates of the exponential growth rates of *S. aureus*

in the nasal passage much less the extent to which that growth rate is reduced by H_2O_2 killing.

Although the results of these experiments are consistent with earlier observations that catalase production provides a fitness advantage to *S. aureus* when co-inoculated with *S. pneumoniae* in a mouse model [14], they suggest that this advantage is not due to the production of H_2O_2 by *S. pneumoniae* . In my experiments, this advantage of catalase producing *S. aureus* over otherwise isogenic strains that did not produce this enzyme occurred whether the *S. pneumoniae* in the nasal epithelium of the rats can produce H_2O_2 or not. Why then would catalase production provide a fitness advantage to *S. aureus* only when *S. pneumoniae* is present? Perhaps in this habitat catalase production by *S. aureus* increases the survival rate in neutrophils by reducing oxidative stress [8, 6]. Or *S. pneumoniae* may indirectly select for catalase production in *S. aureus*; as the presence of both species could synergistically elicit a stronger innate immune response (particularly neutrophil infiltration) as has recently been observed for *H. influenzae* and *S. pneumoniae* [15, 10].

The results of the study can be seen as a cautionary tale; the moral being that what occurs in a flask may not predict what occurs in a bacteria's natural habitat. They also support the recent epidemiological evidence by Regev-Yochay and colleagues [17] that H_2O_2 is not the major determinant to explain the pattern of co-colonization. Why then is *S. pneumoniae* - *S. aureus* co-colonization rarer than expected? One can speculate that the scarcity of co-colonization may be due to either different host preferences by these bacteria species or to competitive interactions other than H_2O_2 allelopathy - perhaps resource or immune-mediated competition[18]. In support of the possibility of immune-mediated competition, there was a negative association between *S. pneumoniae* and *S. aureus* colonization only in HIV- children and not in HIV+ carriers.[11] Distinguishing between bacterial interactions and host preferences is especially important to vaccination efforts; reducing the incidence of one species with a vaccine may have the undesired consequences of increasing the incidence of a competing commensal or pathogenic species.

3.4 Appendix

The following appendix contains a demonstration of the *in vitro* effects of H_2O_2 produced by *S. pneumoniae* on *S. aureus* and the sensitivity of the various strains used to H_2O_2 and will not be included in the final published version of this research.

Two lines of evidence support previous results[19] that *in vitro* H_2O_2 production provides an advantage to *S. pneumoniae* in competition with *S. aureus* . First, when paper disks containing 10^8 cfu of the TIGR4 *S. pneumoniae* are placed on lawns of *S. aureus* of each of the three strains, there are clear zones of inhibition. And as demonstrated by Park and colleagues [14] the zone of inhibition is greater for the *S. aureus* strain that is deficient in the production of catalase (KatA) than for the isogenic strain Newman or another catalase producing *S. aureus* strain PS80. These zones of inhibition are not observed when the *S. pneumoniae* strain is defective for the production of H_2O_2 (SpxB) or when 1,000 units per ml of catalase is added to the lawn. The results of these experiments also suggest that at low densities the amount of H_2O_2 produced by *S. pneumoniae* may not be sufficient to engender much killing of *S. aureus* see fig 3.3(a). No zones of inhibition are observed when the density of TIGR4 on the disk was 10^6 cfu.

Earlier results by Regev-Yochay and colleagues [16] indicated that H_2O_2 production by *S. pneumoniae* in liquid culture at high densities was able to limit the growth of *S. aureus* . To ascertain whether this competitive inhibition also occurs when *S. aureus* and *S. pneumoniae* are competing as colonies on surfaces (where diffusing toxin can only act locally) rather than planktonic bacteria (where toxin and cells are well mixed) I performed competition experiments using the SSS protocol [20]. On agar surfaces, hydrogen peroxide - mediated killing by *S. pneumoniae* also reduces the density of *S. aureus* see fig 3.3(b). This limitation of the density of *S. aureus* on agar surfaces by TIGR4 does not occur when catalase (1,000 U/ml) or 4% sheep red blood cells are added to the agar or when the plates are incubated in anaerobic conditions (data not shown).

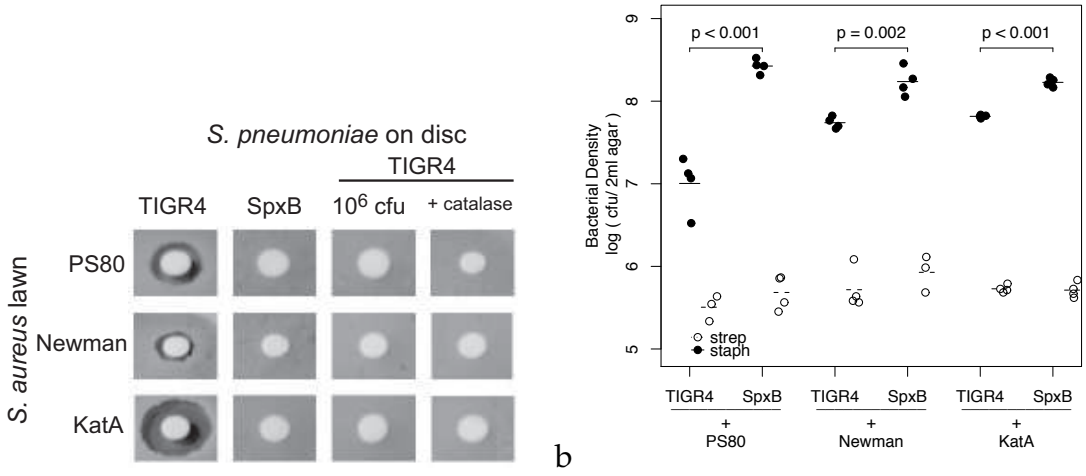

Figure 3.3: a) Zones of inhibition due to H_2O_2 produced by *S. pneumoniae* . Paper disks containing 10^8 CFU TIGR4, 10^8 CFU SpxB or 10^6 cfu TGR4 were placed on lawns of either PS80, Newman or KatA *S. aureus* on THY plates with or without catalase (1,000 U/ml). b) Bacterial density of *S. aureus* growing on agar surfaces limited by H_2O_2 produced by *S. pneumoniae* . A mixture of 10^4 cfu *S. aureus* and $5X10^4$ cfu of either *S. pneumoniae* TIGR4 or SpxB was spread on 2ml THY agar slides and allowed to grow for 12 hours before being sampled.

Evolutionary Biology of Bacterial and Fungal Pathogens
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Chapter 1

Evolution of Bacterial-Host Interactions: Virulence and the Immune Overresponse

ELISA MARGOLIS AND BRUCE R. LEVIN

While many people may not believe in evolution, for those of us with the great taste and good fortune to work with bacteria, viruses, and single cell fungi, evolution is not a matter of belief, and much less one of faith. Evolution is something we constantly see whether we want to or not. For those who are evolutionary biologists by training, inclination, or aspiration there is an obligation to be more than just witnesses and historians of evolution. We have to provide explanations for the origin and maintenance of all biological phenomena. There can be no exceptions.

Coming up with these explanations and better yet with testable evolutionary hypotheses is not hard for characters that provide obvious fitness advantages to the organisms that express them. The ascent of resistance following the introduction of antibiotics came as no surprise to evolutionary biologists. In the presence of antibiotics, bacteria that are resistant to their action have an obvious selective advantage relative to their susceptible ancestors. More challenging to account for are situations where it is not clear how the character in question could have evolved by natural selection favoring the individual organisms. While the interactions between parasitic bacteria and their mammalian hosts include many characters that can be explained by natural selection operating at the level of individual bacteria or individual hosts (Burnet and White, 1972), there are many that cannot. Virulence is one of these traits that is hard to account for by simple evolutionary models; why would bacteria harm the hosts they need for their survival?

In this chapter (speculative rant, if you prefer) we focus primarily on aspects of the evolution of the bacterium-host (mostly human) interactions that cannot be readily accounted for by simple, advantage-to-the-individual evolutionary scenarios. We postulate and provide evidence that much of the virulence of bacterial infections can be blamed on the seemingly misguided overresponse of the immune defenses, what is sometimes referred to as "friendly fire" (Levin and Anita, 2001; Whitnack, 1993) or immunopathology (Graham et al., 2005). We consider how this perversity of the immune system fits with current hypotheses for the evolution of virulence, the evolution of the so-called virulence factors, and speculate on the reasons natural selection has failed to or is unable to blunt the immune overresponse to bacterial infections. We conclude with a brief discussion of the implications of this perspective on virulence for the treatment of bacterial infections.

BACTERIAL VIRULENCE AS AN IMMUNE OVERRESPONSE

We define virulence as the magnitude of the morbidity and the increase in the likelihood of mortality resulting from the colonization and proliferation of bacteria in or on a host. To facilitate our consideration of this virulence and its evolution we use the gross simplification, a cartoon, of the bacterium-host interaction presented in Color Plate 1. Bacteria enter a site, the blue box, where they replicate and establish a population and colonize the host, but in which they do not generate perceptible symptoms. Virulence requires their passage into a second site, the red box, where the presence of bacteria (or their products) can, but need not, cause symptoms, e.g., for a *Streptococcus pneumoniae* bacteremia the blue site is the nasopharynx and the red is the bloodstream. In this model the red site needn't be a different physical location. It could be a different state of the bacteria in the site of their colonization, e.g., for a *Staphylococcus aureus* skin infection, the blue site would be the skin and the red a boil.

Elisa Margolis and Bruce R. Levin • Department of Biology, Emory University, Atlanta, GA 30322.

In Color Plate 1 as well as in mammals, virulence occurs in two ways, both of which require the bacteria to enter the red, potentially symptomatic site or state: (i) direct damage to the host tissue is caused by the replication of the bacteria and/or the production of specific products (toxins), or (ii) indirect damage to the host occurs through an inappropriate or overresponse of the immune system. Both types of damage are represented by the "!" within a triangle. In this scheme the immune defenses can prevent virulence in one or more of seven related ways:

1. Limiting the entry of bacteria into the asymptomatic site
2. Limiting the proliferation of the bacteria in the asymptomatic site
3. Increasing the rate of clearance of bacteria and their products from the asymptomatic site
4. Preventing entry of bacteria or their products into the potentially symptomatic site or state
5. Reducing the rate of proliferation of the bacteria within the potentially symptomatic site or state
6. Increasing the rate of clearance of bacteria and their products from the potentially symptomatic site or state
7. Preventing an immune overresponse to the bacteria or their products in the potentially symptomatic site or state.

The first three of these immune responses maintain the density of bacteria and concentrations of their products in the asymptomatic site at levels where they are unlikely to spill over or otherwise enter the site or state where they can generate symptoms. Whether they do generate symptoms and the magnitude of those symptoms given passage into the red site or state also depends on how well the immune system limits their densities and the concentrations of their products. In Color Plate 1, the number 8 is the infectious transmission of bacteria promoted by the generation of symptoms, and the number 9 is the transmission of bacteria from the asymptomatic site. All of these enumerated steps in which the immune system limits the virulence of the bacteria can be classified as appropriate responses. However, inappropriate responses, for which we use the term *overresponse* when they lead to host damage, may occur due to defects in one of these steps or as a secondary consequence of mounting an immune response.

In the following we focus primarily on the virulence resulting from the overresponse of the immune system. There are, however, examples of virulence that can be attributed to the direct damage of host tissue by the replication of bacteria or the secretion of their products. Included among these are (i) dental caries, resulting from the acid produced by metabolizing *Lactobacillius acidophilus or Streptococcus mutans* (Gibbons, 1964), (ii) paralysis due to the neurotoxins secreted by *Clostridium botulinum* or *Clostridium tetani* acting on the nerve and motor endplates (Schiavo et al., 1992), and (iii) diarrhea resulting from enterotoxins that inhibit resabsorption of sodium chloride or promote its secretion. Examples of virulence being a direct product of the interaction between bacteria and host cells appear to be rare relative to those in which the morbidity and mortality can be attributed to the indirect damage due to an immune overresponse.

As illustrated in Table 1, the morbidity and mortality of bacterial infections can be attributed to the host's immune system operating in one of three inappropriate ways: (i) being more vigorous than needed, (ii) being incorrect for that pathogen, or (iii) responding to the wrong signals. The best-investigated example of the immune system responding too vigorously is bacterial sepsis, where the entry of cytokines and bacteria into the bloodstream brings about widespread blood vessel injury and multiple organ failure (impaired pulmonary, hepatic, or renal function). Here the response to the bacteria is at one level appropriate, as the cytokines released play an important part in attracting neutrophils (immune cells that phagocytose bacteria) to the local infection site, but is also excessive (Kurahashi et al., 1999). The distinction between an inappropriate and appropriate immune response can be seen in the spectrum of illness associated with *Mycobacterium leprae* (Modlin, 2002; Sieling et al., 1999). Hosts that respond to infection predominantly with antibodies and very few CD4 T cells have infectious sites with large macrophages that contain numerous mycobacteria. These macrophages are responsible for the multiple skin lesions and nodules seen in lepromatous leprosy, while a host with T helper 1–type response (high interferon-γ production and low interleukin-4 [IL-4]) has numerous well-formed granulomas with very few mycobacteria that form minor skin lesions. Superantigens provide an example of the immune system responding to an incorrect signal. Superantigens are bacterial products that stimulate a large number of T cells(1–40% of T cells will react) by binding to major histocompatibility complex class II molecules and T cell receptors (beta chain) independently of their specificity for antigens (Rott and Fleischer, 1994). *S. aureus, Streptococcus pyogenes, Mycoplasma arthritidis*, and *Yersinia pseudotuberculosis* are among the bacteria that produce superantigens. In the case of toxic shock syndrome, the superantigens produced by *S. aureus* induce the indiscriminate and overwhelming activation of T cells leading to the production of cytokines that mediate shock and tissue injury. In all three of these cases the morbidity and mor-

Table 1. Some examples of virulence resulting from an immune overresponse

Disease	Bacteria	Red site[a]	Bacterium–host interaction[b]	Damage induced by immune response
Pneumonia	Multiple species, e.g., *S. pneumoniae*, *N. meningitidis*, *H. influenzae*	Lungs	Cell wall and other bacterial components → induce proinflammatory cytokines, edema, and fibrin deposition (Bergeron et al., 1998)	Recruitment of fluid and cells into the air spaces of the lungs
Toxic shock syndrome/scarlet fever	*Staphylococcus*, *Streptococcus*	Circulatory system, systemic	Superantigens → indiscriminate activation of T cells (McCormick et al., 2001)	Extreme inflammation leading to septic shock
Duodenal ulcers	*Helicobacter pylori*	Gastric and duodenal mucosa	Bacterial persistence → chronic inflammation (Czinn and Nedrud, 1997)	Mucosal atrophy
Septicemic plague	*Yersinia pestis*	Circulatory system, systemic	Endotoxin and/or unknown factors → systemic inflammatory response	Acute shock
Cutaneous anthrax	*Bacillus anthracis*	Skin	Anthrax lethal factor (toxin) → release of TNF-α and IL1β (Firoved et al., 2005)	Tissue necrosis
Petechiae, Waterhouse-Friderichsen syndrome	*Neisseria meningitidis*	Skin and adrenal glands	Endotoxin → release of TNF-α, IL-1, and IL-6 (Klein et al., 1996)	Blood vessel destruction
Rheumatic fever	*Streptococcus pyrogenes*	Heart, joints, skin and brain	M protein → activation of autoreactive (anticardiac myosin) antibodies (Cunningham, 2003)	Antibody-induced damage of heart muscle; immune complex deposited in large joints and skin
Reactive arthritis	*Chlamydia trachomatis*	Joints, eyes, urethra	Bacterial persistence → activation of autoreactive immune cells (Sieper, 2001)	Inflammation of large joints, eyes, and urethra
Tuberculosis	*Mycobacterium tuberculosis*	Lungs	Bacterial presence → release of high levels of TNF-α (Bekker et al., 2000; Kaushal et al., 2002)	Recruitment of fluid and cells into the air spaces of the lungs; necrosis
Meningitis	Multiple species, e.g., *S. pneumoniae*, *N. meningitidis*, *H. influenzae*	Meninges	Cell wall and other bacterial components → induce proinflammatory cytokines and chemokines (Braun et al., 1999)	Increased blood-brain barrier permeability; neutrophil recruitment; increased intracranial pressure; brain damage

[a]See Fig. 1 for an explanation of red site.
[b]TNF-α, tumor necrosis factor alpha.

tality of the host can be attributed to an apparently misguided response of the immune system, which we refer to as an overresponse.

THE EVOLUTION OF BACTERIAL VIRULENCE AS AN IMMUNE OVERRESPONSE

How does the observation that much of the morbidity and mortality can be attributed to a host overresponse to bacteria help in understanding the evolution of virulence? In a perspective written a decade ago, one of us (Levin, 1996) listed four hypotheses that account for the evolution of virulence: (i) the conventional wisdom, (ii) epidemiological selection, (iii) coincidental evolution, and (iv) short-sighted, within-host evolution. Since that time, although there have been a number of theoretical, experimental, and speculative articles on the evolution of virulence (for a small and admittedly biased sample see Andre and Godelle, 2006; Bonhoeffer and Nowak, 1994; Brown et al., 2006; Bull, 1994; Elbert and Bull, 2003; Ebert and Herre, 1996; Frank, 1996; Grech et al., 2006; Lipsitch et al., 1995, 1996; Lipsitch and Moxon, 1997; Mackinnon and Read, 2004; Regoes et al., 2000), we do not know of studies that have rejected any of these

hypotheses and only one adding what may be a new hypothesis: quasispecies evolution (Pfeiffer and Kirkegaard, 2005). This fifth hypothesis may only apply to viruses with high mutation rates and arguably could be subsumed under the broader rubric of within-host evolution. In this section we consider how the observation that morbidity and mortality of bacterial infections can be attributed to the hosts' immune overresponse fits each of these hypotheses for the evolution of the virulence of bacteria.

The Conventional Wisdom

This phrase, which the late John Kenneth Galbraith coined to describe ideas and explanations that are widely accepted as true by the public, was applied by Bob May and Roy Anderson (May and Anderson, 1983b) to describe the then-prevailing view of the evolution of the virulence. According to that wisdom, virulence is an artifact of the relative novelty of parasite's association with its host. As the relationship between the parasite and host matures, natural selection in either the parasite or host population or both will lead to the extinction of one or the other species or the evolution of symbiosis or mutualism.

While the original theory behind this hypothesis for the evolution of virulence of infections amounts to little more than the adage "don't bite the hand that feeds you," the evidence in support of it was and remains compelling. Many of the bacteria responsible for morbidity and mortality of humans were acquired from other species in the not-so-distant past (after the advent of agriculture), and some are continuously acquired in this way. Included among these zoonotic (and protozoonotic) infections are plague, tuberculosis, Legionnaires' disease, botulism, anthrax, brucellosis, tularemia, Rocky Mountain spotted fever, cholera, and other diarrheal diseases. The bacteria responsible for some of these infections, such as *Mycobacterium tuberculosis*, are transmitted between humans and can be maintained without the animal source. Others such as *Legionella pneumophila* are not. Also consistent with the conventional wisdom is the correlated observations that only a very small minority of the vast numbers of species of bacteria that colonize mammals cause disease.

It may seem that the proposition that the virulence of bacterial infections can be attributed to host immune overresponse fits quite well with this conventional wisdom. To wit, the immune system has not yet had the time to evolve to moderate the response to these novel bacteria and their products and/or these bacteria have not yet evolved into being nice. Eventually, or as it was once referred to, on "equilibrium day," (Levin et al., 2000), mutualism will prevail and the immune overresponse will be tempered.

Epidemiological Selection

The conventional wisdom is an observation rather than a mechanism, an observation that focuses on the interactions between bacteria and the individual hosts they colonize. To fully understand the evolution of commensal and pathogenic bacteria, however, it is necessary to consider their lifestyle outside the host and, in particular, their transmission between hosts. One approach to this more comprehensive picture of the evolution of parasitic microbes has been to draw inferences about the nature and direction of selection from epidemiological models (Levin et al., 1982; Levin and Pimentel, 1981; May and Anderson, 1983a). In accord with this perspective, the fitness of a particular strain of bacteria is given by its basic reproductive number, R_0, the number of secondary infections caused by a single infected individual in a wholly susceptible population of hosts; the higher the value of R_0, the greater the fitness of the bacteria. In these traditional epidemiological models, virulence is only expressed as mortality. Morbidity and other more subtle effects of infections are not directly considered in these epidemiological models.

As long as the transfer to new hosts requires viable hosts, selection will favor bacteria that are not only infectiously transmitted at ever-higher rates but also persist longer in colonized hosts (i.e., are less likely to kill the host). In other words, selection will favor ever-more-benign, symbiotic, or better yet, mutualistic bacteria. Evolution in the host population will also be for reduced virulence; hosts that are less subject to infection-associated morbidity and mortality will be favored. As long as transmission occurs from the blue asyptomatic site (8 in Color Plate 1) rather than the red site (9 in Color Plate 1), these epidemiological models can be seen as the theoretical basis of the conventional wisdom (also see Lenski and May, 1994). If, however, transmission and the morbidity and mortality of the host are coupled so the more virulent bacteria are transmitted at higher rates than the more benign, there is a trade-off between the loss of the host and gain to the bacteria; virulence would be favored in the bacterial population (Ebert and Bull, 2003).

On first consideration it may seem that this transmission and virulence trade-off is inconsistent with the proposition that the morbidity and mortality of the infection is a product of the host's immune overresponse. We suggest this is not necessarily the case. The host overresponse could be a by-product of selection operating on individual bacteria to promote their transmission. While we don't know of overwhelming, quantitative, empirical evidence of this being the case for any pathogenic bacteria (viruses are

another matter; see Fenner and Ratcliffe, 1965), this interpretation is supported by reasonable plausibility arguments. Here we consider two of the more compelling of these examples of pathogenic bacteria of humans.

The first is the diarrheal diseases in which humans play a significant role in the transmission process. Because of the massive output of bacteria, diarrhea is likely to increase the density of bacteria in water and food products and thereby the transmission rate of these bacteria. Thus, as long as transmission is promoted by diarrhea, selection in the bacterial population will favor mechanisms that cause diarrhea. In some cases the induction of diarrhea is attributed to what can be seen as immune overresponse. The dysentery bacteria *Shigella flexneri* induces the release of the cytokine interleukin 1 (IL-1) in infected macrophages, which leads to extensive injury of the colon mucosa, which in turn results in fluid and protein loss into the intestinal lumen and the ensuing diarrhea (Hilbi et al., 1997). This hypothesis for the evolution of diarrhea to increase transmission requires that the transmission advantage more than makes up for the loss in transmission due to host mortality. To our knowledge, there are no quantitative empirical studies demonstrating that this trade-off obtains for any diarrheal disease.

The second example is plague. Albeit not yet as well documented as the oft-told mother of all trade-off stories, myxoma and the Australian rabbits (Fenner, 1965), the emerging tale of the evolution of the virulence of the plague bacillus, *Yersinia pestis*, has parallels to that story. There is compelling evidence that this flea-transmitted pathogen evolved from a not very virulent enteric, oral-fecal transmitted *Yersinia* relatively recently by the acquisition of a couple of plasmids and a few chromosomal genes (Achtman et al., 1999; Carniel, 2003). Since fleas acquire these bacteria from the blood of rodents, the density of bacteria in circulating blood would be directly associated with the likelihood of their transmission to other rodents (or humans). Also directly associated with this density of bacteria in the blood is sepsis, the virulent manifestation of *Y. pestis* infections. Elisabeth Carniel (personal communication) has suggested that the capacity to generate lethal sepsis is not just a by-product of the proliferation of bacteria in the blood, but may be selected for in the bacterial population. Although the cost-benefit calculation has not been made, it may be that the rate of transmission of the bacteria is augmented by their killing infected rodents, as fleas move to new hosts when their original host dies. For both diarrheal diseases and plague, the virulence resulting from the host overresponse is associated with transmission. Clearly more empirical work would be necessary to confirm the existence of a trade-off between bacterial transmission and an immune overresponse and the postulated exploitation of this overresponse for the epidemiological advantage of the parasite.

Coincidental Evolution

In accord with this hypothesis there is no advantage to the bacteria to make the host sick and certainly no advantage for the host to be ill; virulence is a consequence of the bacteria being in the wrong host or in a wrong site in the right host (Levin and Svanborg Eden, 1990) (the arrow above 7 in Color Plate 1). The bacterial products responsible for the morbidity and/or mortality of the host, virulence determinants, evolved in response to selection for some function other than virulence.

Reasonable candidates for coincidental virulence due to an immune overresponse are diseases associated with *Helicobacter pylori*. These bacteria colonize and maintain populations in the stomachs of the majority of humans for most of their lives without generating symptoms and appear to have done so since prehistoric times (Falush et al., 2003). However, it wasn't until Marshall and Warren (1984) presented evidence that a curved bacteria we now know as *H. pylori* was an etiologic agent for gastric and peptic ulcers that this seemingly commensal bacteria was elevated to the status of pathogen. This distinction was further enhanced by evidence that *H. pylori* was also associated with gastric cancers (Moss and Blaser, 2005; Tatematsu et al., 2005). *H. pylori* colonization can result in a chronic inflammatory state that is generated when the host responses (such as the release of IL-8 and other chemokines, the attraction of neutrophils, macrophages, and the local stimulation of T cells) fails to clear the bacteria and lymphoid aggregates form in the lamina propria of the stomach and duodenum. This continued stimulation of the immune and inflammatory cells (termed chronic atrophic gastritis) results in the destruction of the gastric epithelium, formation of peptic ulcers, and increased risk for gastric cancers. Presumably, but not yet formally demonstrated, the induction of the inflammatory response and the subsequent diseases provides no advantage to *H. pylori* in a colonized host or its transmission to new hosts. In this sense, the virulence of *H. pylori* in colonized humans is coincidental.

While they are commonly described as pathogens, especially in grant proposals and by people suffering from the symptoms they can generate, a number of bacteria responsible for morbidity and mortality in humans also have good credentials as commensals.

Like *H. pylori* they are carried asymptomatically by many and cause disease in few. Included among the more prominent of these commensal pathogens for humans are *S. aureus*, *Haemophilus influenzae*, *S. pneumoniae*, and *Neisseria meningitidis*. From an evolutionary perspective, invasive disease seems to be the wrong thing for these bacteria to do—dead ends. The sites of their virulence, blood and meninges, are certainly not good for their transmission to new hosts by their normal route, through respiratory droplets. The rare virulence of these commensal bacteria can be accounted for by an immune overresponse in these sites (Bergeron et al., 1998; Braun et al., 1999). The occasional movement of bacteria into a site where they can cause disease (the red in Color Plate 1) may be due to chance or coincidental evolution or as we argue below may be a consequence of within-host evolution of the bacterial population.

Within-Host Evolution

In accord with this hypothesis, the virulence of bacteria is the product of selection favoring more pathogenic members of a population colonizing an individual host (Levin and Bull, 1994). The advantage gained by the bacteria by generating symptoms in a colonized host is restricted to that host and may be to its disadvantage in its transmission to a new host; this evolution is short-sighted. A mutant commensal bacterium with the capacity to establish and maintain populations in normally sterile sites, cells, or tissues could be favored within a colonized host because in those sites there is less competition for nutrients and/or those mutant bacteria are somewhat protected from the host immune defenses.

Although we can make a good case and even cite evidence for the virulence of some viruses, such as poliovirus and Coxsackievirus, being the product of within-host evolution (Gay et al., 2006; Levin and Bull, 1994), for bacteria the best we can do at this stage is present arguments founded on plausibility and consistency with observations (see, for example, Meyers et al., 2003). Central to these arguments are the results of studies with mice and rats demonstrating that the bacteria responsible for invasiveness (blood infection) are commonly derived from one of very few cells (Meynell, 1957; Moxon and Murphy, 1978; Pluschke et al., 1983; Rubin, 1987). One possible explanation for these observations is that the bacteria responsible for the blood infections are the products of single, mutant cells with an enhanced capacity to invade and proliferate in blood.

While supporting the within-host evolution hypothesis for virulence, these observations are also consistent with the coincidental evolution hypothesis: that, by chance alone, only one or a few cells establish blood infections can be attributed to very small holes in the host's defenses through which only one or very few bacteria traverse the arrow above 7 in Color Plate 1. Although the coincidental and within-host hypotheses could be distinguished by demonstrating that the bacteria establishing a blood infection have an inherited propensity for the invasion of blood, to our knowledge there are no published studies that have done this test. However, whether the invasiveness of the blood or other normally sterile sites is coincidental or due to within-host evolution, the virulence of bacteria in these sites can be attributed to a host's immune overresponse.

The Evolution of Virulence Determinants

Not all bacteria or even all members of the same species of bacteria capable of colonizing mammals are responsible for disease. One explanation for why some bacteria cause disease and others do not is what have become known as virulence factors or virulence determinants, the expression of which are, by definition, essential for that bacteria to cause disease in (or on) colonized hosts (Finlay and Falkow, 1989). Included among these are characters that facilitate adhesion to host cells, evade the host constitutive and inducible immune defenses, and produce toxins. Appropriately, much of contemporary bacteriology is devoted to understanding the molecular biology, genetics, evolutionary origin, and mode of action of virulence determinants as a way to understand bacterial diseases and ideally prevent or treat them. While virulence determinants (factors) are almost certainly the products of adaptive evolution in bacterial populations, not so clear are the selection pressures responsible for their evolution and maintenance. Are they favored because of virulence, i.e., the morbidity and mortality of the host promotes the colonization, persistence, and infectious transmission of bacteria that express these determinants? Are virulence factors by-products of selection for other functions, e.g., their expression provides protection against grazing protozoa (Wildschutte et al., 2004) and/or facilitates competition with other microbes? Or is the virulence attributed to these factors an inadvertent by-product of their normal function in a host, a primitive character that will be lost on or before equilibrium day. While these hypotheses may be mutually exclusive for any specific bacterium-host and virulence factor, they are clearly not so collectively. Whether they evolve in response to selection for virulence or not, some of these virulence factors are responsible for triggering the immune overresponse.

Why Does the Immune System Overrespond?

In the preceding, we have portrayed the host immune system as misguided, overresponding in ways that cause rather than prevent the morbidity and mortality of a bacterial infection. From the perspective of evolutionary biology, however, "misguided" is hardly an explanation. Colonization by bacteria is not a rare event but rather something mammals confront all the time, and overresponding in a way that results in their morbidity and mortality would almost certainly be selected against. In their review of "immunopathogy," Graham and colleagues postulated a number of reasons for this transgression of the immune response (Graham et al., 2005). Here we offer our perspective on this issue.

As we see it, there are two general classes of explanations for the maintenance of an overresponse of the immune system. (i) While infectious disease may be a major source of morbidity and mortality (Haldane, 1949), disease-mediated selection can be relatively weak, and extensive amounts of time would be required to evolve mechanisms to modulate the immune response to specific bacterial infections. (ii) Functional constraints on the immune system limit the ability of natural selection to totally prevent and maybe even partially mitigate an immune overresponse to bacterial infections.

(a) Even if selection universally favors tempering the immune overresponse to infections, and the favored genotypes could be generated (which we question below [b]), the time required for temperance to evolve could be considerable, especially if the overresponse is specific for particular bacteria and/or their products. This is due to two factors. (a) At its maximum the intensity of selection for modulating the immune overresponse to an infection would equal the fraction of the population with that infection. It would be substantially lower if the symptoms of the infection were not expressed in all colonized hosts, were rarely lethal or sterilizing, or were primarily manifest after reproductive years or if the magnitude of the reduction of the overresponse of the favored genotype was less than absolute. For most of the diseases listed in Table 1 virulence is a rare occurrence in colonized hosts (less than 1%), and therefore the intensity of selection against an immune overresponse would be relatively weak. (b) It can take a considerable amount of time for a rare beneficial mutant to ascend to substantial frequencies. For example, if the selection for a reduced overresponse is operating on genotypes at a single locus (the best case), the initial frequency of a favored allele is 10^{-3}, the favored genotype has a 1% selective advantage, and there is no dominance, it would take 1,381 generations (more than 20,000 years for humans) for that gene to reach a gene frequency of 50%. If the favored genotype is recessive, the corresponding number of generations would be 100,491 (Crow and Kimura, 1971).

What about the role of the bacteria in the evolution of a more temperate immune system? As a consequence of their vastly shorter generation times, haploid genomes, and propensity to receive genes and pathogenicity islands by horizontal transfer, it seems reasonable to assume that bacteria would have an edge in an evolutionary arms race with their mammal hosts. We suggest, however, that this edge contributes little if anything to the slowing pace at which mammalian evolution could modulate the immune overresponse. Although there maybe situations where virulence is positively correlated with the infectious transmission of bacteria, in most of these cases the morbidity and mortality associated with their transmission is not to the bacteria's advantage and may be to their disadvantage. Even greater transmission of these bacteria would be possible if the hosts were not debilitated or killed as a result of diarrhea or if the bacteremias required for vector-borne transmission did not result in sepsis. In this interpretation evolution in the bacteria population would not oppose the evolution of a more temperate host immune system. Of all the examples considered in this chapter, the only one in which evolution in the bacterial population might favor an immune overresponse is Carniel's suggestion that by killing their host, *Y. pestis* acquires a transmission advantage.

(ii) While the above realities of the ecology and genetics of natural selection may be part of the answer to the question of why evolution has not eliminated the immune system's overresponse to bacterial (and other) infections, we suggest it is not the most important reason. We conjecture that the primary reason mammalian evolution has not tempered and perhaps cannot temper the immune overresponse to bacterial and other infections is functional constraints that limit the extent to which the immune system can be modified. The immune system has roles other than clearing bacterial infections. It has been postulated that these other roles dominated the evolution of the mammalian immune system (Burnet, 1970). These different roles as well as the extraordinary diversity of organisms colonizing mammals, bacteria, viruses, fungi, and worms of various ilks and the variety of sites of colonization impose different and potentially conflicting demands on the immune defenses, phenomena referred to as antagonistic pleiotropy. An appealing hypothesis for the immunopathogy known as allergies is an overresponse of those elements of the immune system that in less-pristine times would otherwise be occupied with the control of helminth infections (Wilson and Maizels, 2004).

There is a fine line between responding (1–6 in Color Plate 1) and overresponding (7 in that figure), which may be difficult for the systems regulating the immune response to perceive, much less avoid. As suggested by Frank (Andre et al., 2004), the intensity of an immune response may be determined by a trade-off between increasing the strength and rapidity of an immune defense and the virulence from an immune system overresponse.

Is there evidence in support of these two hypotheses for why evolution has not eliminated the virulence resulting from the immune overresponse? Not much—at least not yet. We suggest, however, that some of the considerable amount of inherited variability in the susceptibility to infectious disease in human populations (Bellamy et al., 2000; Bellamy and Hill, 1998; Segal and Hill, 2003; Sorensen et al., 1988) can be interpreted as support for these hypotheses. To be sure, there is good and even overwhelming evidence that some of this variation is maintained by disease-mediated balancing or frequency-dependent selection, but this is not the case for all or even the majority of it. We suggest that much of the standing genetic variation in disease susceptibility in human populations is a reflection of the myopia and limitations of natural selection: (i) the relative weakness of selection for modulating the immune overresponse and (ii) even more, the impotency of natural selection due to the constraints on the immune system—antagonistic pleiotropy. Genetic variation that is not or is poorly perceived by natural selection will build up and persist (Crow and Kimura, 1971).

Implications

While the morbidity and mortality of most bacterial infections can be attributed to an immune overresponse, virtually all of our efforts to treat these infections are directed at controlling the proliferation and clearing the bacteria, primarily with antibiotics. This approach has been and continues to be effective, but not completely so. Antibiotic treatment commonly fails, and patients die or remain ill for extended periods. Resistance of the pathogen to the antibiotics employed for treatment is only one of the reasons for this failure and for some infections is not the major one, at least not yet (Levin and Rozen, 2006; Yu et al., 2003).

The obvious alternative approach to treating infections is to reduce the morbidity and prevent the mortality by modulating the immune system's overresponse. There have been attempts to do just that for the treatment of bacteria-mediated sepsis. Clinical trials have evaluated the use of glucocorticoids(Bone et al., 1987), drugs designed to neutralize endotoxins (Ziegler et al., 1991), tumor necrosis factor α (Fisher et al., 1996), and IL-1β (Fisher et al., 1994), but none of these treatments was effective. The most successful trials in humans to date have been with a component of the natural anticoagulant system, activated protein C, which has substantial anti-inflammatory properties along with being a potent anticoagulant (reduces the formation of clots that are responsible for organ failure in late stages of sepsis) (Fourrier, 2004). In addition, new agents redirect the immune response and hold promise as effective future therapies for sepsis, such as IL-12 (O'Suilleabhain et al., 1996) and antibodies against complement (C5a) (Czermak et al., 1999). However, understanding the specifics of the immune overreaction and the intricacies of the feedback mechanisms that control an immune response is necessary for therapies to be directed at enhancing or inhibiting the patient's immune response.

At this time, taken at large, the success of these immune modulating methods in preventing the morbidity and mortality of bacterial infections can at the very best be described as modest. However, in maintaining the speculative nature of this rant, and desiring an optimistic conclusion, we suggest that as we learn more about the regulation of the immune response and develop procedures to monitor as well as administer regulatory immune molecules in real time, these methods will become increasingly effective for the treatment of bacterial infection.

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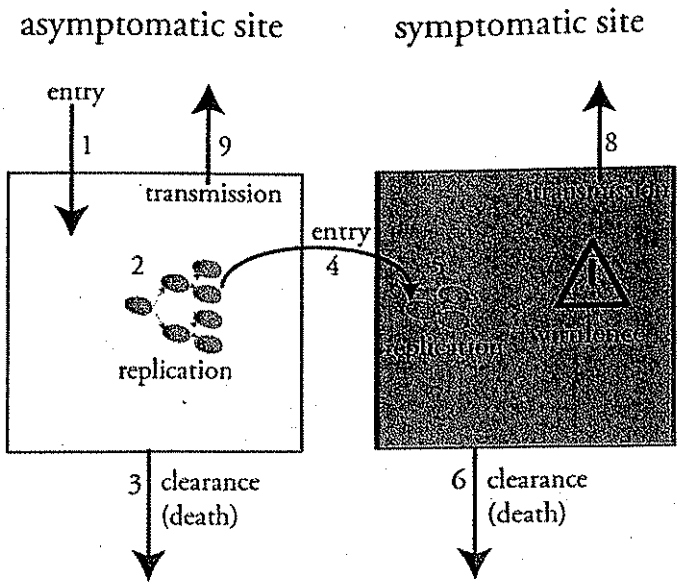

Color Plate 1. Artist's conception of the infection process and the host's immune response and overresponse: (Blue) site where the presence of bacteria does not result in symptoms–asymptomatic. (Red) site or state where the presence of bacteria can result in symptoms. See the text for more details.

Mouse Wound Model

5.1 Introduction

Although the immune system has received good press for protecting innocent people from the ravages of infectious diseases, immune 'over–responses' in fact are responsible for the majority of morbidity and mortality of most bacterial infections. It is therefore inappropriate to assume that all aspects of the immune responses are evolutionarily adaptive. In this chapter, I focus on the formation of abscesses in response to bacteria. On first consideration it would seem that abscesses would be evolutionarily adaptive because they limit bacterial dissemination. However there is evidence that abscess formation protects *S. aureus* and other pathogens from being rapidly cleared and allows the bacteria to persist at high densities for considerable periods of time.

The possibility that phagocytes, particularly neutrophils, and their role in forming abscesses could contribute to *S. aureus* pathogenesis, increase bacterial load and lengthen time necessary to clear infections has been hypothesized and investigated since the 1950s[8, 7, 4] More recently, evidence has been presented that implicated increased CXC chemokine production (primarily by T cells) followed by increased neutrophil transmigration in higher bacterial loads and increased morbidity. These order of events have been demonstrated in CD14 (-/-) knockout mice infected with *S. pneumoniae* [1] and in mice infected with *S. aureus* that had been administrated CXC chemokines systemically[3, 5] or with CXC chemokine receptors blocked [6].

I had planned to test two hypotheses on the evolutionary benefit of abscess formation; namely whether abscess formation (i) is essential to controlling high density inocula or mixed infections; or (ii) is necessary to prevent systemic or metastatic infections. First using a realistic (i.e. accurately mirrors clinical infection processes) animal model for *S. aureus* infections I intended to collect data on the dynamics of both the bacteria population and the immune cellular components. The dynamics of both the bacteria and the immune response would offer clues as to what prevents bacteria from being cleared in an abscess and what allows bacteria to be rapidly cleared when an abscess does not form. In addition, I had planned to manipulate the immune system to prevent abscess formation and see if mice deficient in immune components required for abscess formation had difficulty controlling high density inocula or mixed bacterial species infections or had a higher incidence of metastatic (infections at sites distant from primary infection site). I was unable to repeat the previously published results that suggested abscess formation could be blocked.

5.2 Material and Methods

5.2.1 Bacterial strains, media and inoculum preparation

S. aureus PS80 (serotype 8 ATTC 27700) was obtained from the American Type Culture collection and pr1 (Rif–R) was selected as a spontaneous mutant of PS80 exhibiting resistance to rifampin. *S. aureus* strains were cultivated in Luria-Bertani (LB) broth cultures and agar plates incubated at $37°\mathrm{C}$.
Inoculum where prepared either by the broth method or the plate metod. Aliquots of late logarithmic phase growth frozen at $-80°\mathrm{C}$ were thawed and either plated on LB (plate method) or suspended in 2 ml of LB. For the broth method, cultures that reached mid-exponential phase were centrifuged (5,000 g x 3 min). For the plate method, colonies from plates which had been incubated for 24 hours were scraped. In both cases the bacteria were resuspended in phosphate-buffered saline (PBS). Inocula and animal specimen densities were estimated by plating dilutions

of *S. aureus* on LB Agar plates or LB plates supplemented with rifampicin (40 mg/L).

5.2.2 Mouse Wound Model

All *in vivo* experiments were performed under the guidelines approved by the Emory University Institutional Animal Care and Use Committee. Mice were anesthesized with 0.11mg/ml solution of ketamine/xylazine IP. Hair was removed of rear leg with an electric razor. Mice were placed in sterile area exposing only leg which was disinfected with propidium iodine. Skin was cut with a scapel (perpendicular to leg muscle following from front knee to hip). A 1 cm long incision to the depth of the bone was made in the thigh muscle and a single 4-0 silk suture was placed. 5μl of staph was placed below suture with pipeteman. Skin was closed with 3–5 4-0 prolene continuous sutures. Mice were placed on heating pad and received 100μl of 0.05 mg/ml Meloxicam.

At given time points, tissues were sampled and weighed; blood drawn by cardiac puncture and the wound removed by cutting around muscle (.25 cm square margins around wound and to depth of bone). Tissue was either disassociated by homogenizing or by mincing with scissors, resuspended in 1ml PBS and drawn through a 45micron cell filter. No difference was noted in the bacterial load for the two tissue disassociation methods. Dilutions of tissue were either plated directly on LB agar, frozen for MPO assay or spun for FACS.

5.2.3 Attempts to Manipulate Abscess Formation

For systemic thymocyte depletion, anti-thymocyte serum (ATS; IgG fraction goat anti-mouse thymocyte; Inter-cell Technologies, FL) was administered to 25 g mice by intraperitoneal injection of 0.3 mg/ g of weight (dissolved in 0.1 M PBS). Thymocytes in blood collected from cardiac puncture 24 hours after treatment as quantified by FACS (%CD45+CD3+) were 13.46 % in ATS treated mice and 0.44 % in mice treated with IgG fraction control serum.

For systemic neutrophil depletion, anti-neutrophil serum (ANS, IgG fraction rabbit anti-rat PMN; Inter-cell Technologies, FL) was administered to 25 g mice by intraperitoneal injection of 150μL of 10.3 mg/ml serum. Granulocytes in blood collected from cardiac puncture 96 hours after treatment as quantified by FACS (%CD45+Gr1+) were 18.79$\pm$2.76 % in ANS treated mice and 43.63$\pm$0.11 % in mice treated with IgG fraction control serum.

T cell receptor-α knockout mice (designated TCRαKO) are mice breed from B6.12952Tcratm1mom/J from Jackson Laboratories and are on a C57BL6/J background with a deficiency in T cell receptor α chain. Thymocytes in blood collected by retro-orbital collection were quantified by FACS analysis and show that while not all thymocytes are deficient, there is a deficiency in T cells of the $\alpha\beta$ background (see **??**).

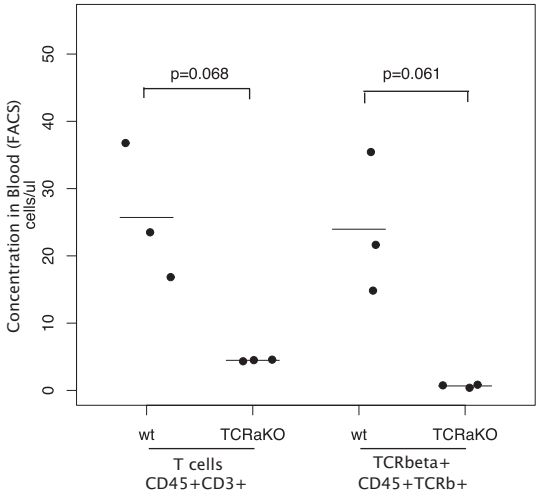

Figure 5.1: Thymocytes in TCRαKO and C57BL6/J control mice. The line represents the mean thymocyte density in blood (cells/μL) for CD45+CD3+ cells in the first two columns and CD45+TCRβ+ in the third and fourth columns. P-value is the two-tailed probability form the t-distribution of rejecting by chance the null hypothesis that the mean thymocyte densities are the same.

5.3 Results

5.3.1 Mouse Wound Model

Bacterial Population Dynamics: Abscess

S. aureus readily colonized the sutures left in the murine thigh muscle wounds. Within 3 days after *S. aureus* is inoculated, the bacterial density exceeded 10^4 cfu/g continuing to divide and reach the maximum bacterial load at 6 days (ranging from 10^5 to 10^8 (Figure 5.3). At 9 days the bacterial load had already decreased at least a hundred fold and by 14 days many of the mice had cleared the infection.

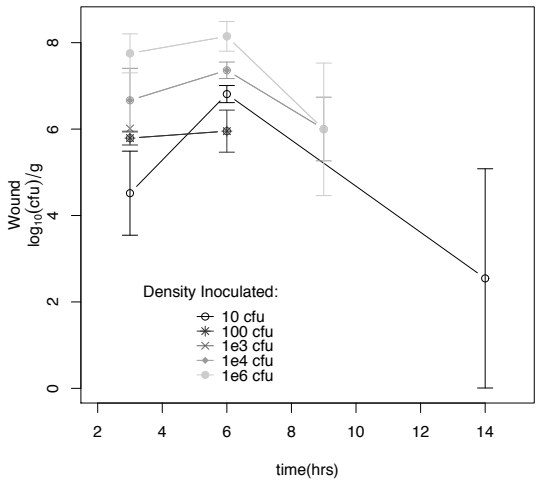

Figure 5.2: Population dynamics of muscle wound infection. CD-1 Mice were inoculated with 10 – 10^6 cfu of *S. aureus*. The mean bacteria density in the muscle wound of 3–11 mice at each time-point is plotted. Error bars represent standard error.

The number of *S. aureus* bacterium needed to cause infection is dramatically lower at the site of a suture compared with healthy skin.[2] Here I found that inoculums with as low as 10 cfu were sufficient to cause muscle wound infections.

While the dynamics do not appear to differ for a wide range of inoculums the peak bacterial load did increase with increasing inoculum.

Bacterial Population Dynamics: Metastatic Infections

To determine whether bacteria migrated from the primary site of infection (muscle wound) to other sites in the mice, the blood, spleen, kidney, liver were sampled and cultured for *S. aureus* bacteria. *S. aureus* was never detected in the blood-stream. However, within 3 days after infection of the muscle wound *S. aureus* could be found in the spleen, liver and more consistently the kidney (Figure **??**).

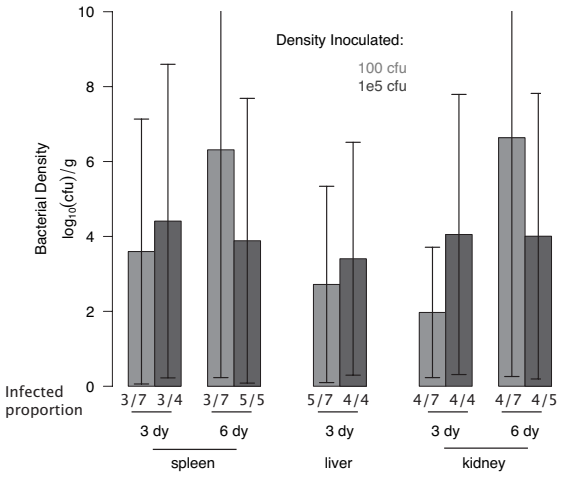

Figure 5.3: Metastatic *S. aureus* infection. CD-1 Mice were inoculated with either 10 or 10^5 cfu of *S. aureus*. The mean bacteria density in the muscle wound of 3–6 mice at each time-point is plotted. Error bars represent standard error.

5.3.2 Attempts to Manipulate Abscess Formation

The objective was to determine whether abscess formation prevents systemic disease (as commonly believed) or simply allows for bacteria to persist within the host (prevents clearance). The hypothesis that the immune response (specifically abscess formation) leads to bacterial persistence within the host was based on a

McLoughlin et al. PNAS 2006 paper in which α-TCR (T cell receptor) Knockout Mice did not form abscesses and rapidly cleared *S. aureus* wound infections. I tried to extend and understand these results by testing whether anti-thymocyte or anti-neutrophil serum would reduce abscess formation when *S. aureus* was inoculated into muscle wounds.

The first manipulation that was attempted was depletion of neutrophils using Anti-neutrophil serum (ANS). While neutrophils were decreased (data in materials and methods) in treated mice, there was no difference noted in their bacterial load at 3-9 days after inoculation (Table 5.3.2).

Table 5.3: Bacterial Load in ANS or Control Mice

Time Point:	**3 days**		**6 days**	
	Log_{10} (cfu/g)[a]	p [b]	Log_{10} (cfu/g)[a]	p [b]
ANS	6.53±0.45	0.62	6.38±0.29	0.54
Control	6.85±0.44		5.96±0.49	

[a] Mean (± standard error) bacterial density of 3–13 mice inoculated with 10^2 cfu in mouse wound.

[b] P value from t-test comparing the bacterial density of control mice and those treated with Anti-neutrophils Serum (ANS).

Next the immune system was modified to deplete thymocytes using Anti-thymocyte serum (ATS). While T cells were decreased (data in materials and methods) in treated mice, there was no significant difference noted in their bacterial load at 3-9 days after inoculation (Table 5.3.2).

Table 5.4: Bacterial Load in ATS or Control Mice

Time Point:	**3 days**		**6 days**		**9 days**	
	Log_{10} (cfu/g)[a]	p [b]	Log_{10} (cfu/g)[a]	p [b]	Log_{10} (cfu/g)[a]	p [b]
ATS	8.09±0.18	0.59	7.20±0.30	0.18	6.88±0.42	0.66
Control	7.75±0.45		8.14±0.34		5.99 ± 1.53	

[a] Mean (± standard error) bacterial density of 3 mice inoculated with 10^6 cfu in mouse wound.

[b] P value from t-test comparing the bacterial density of control mice and those treated with Anti-thymocyte Serum (ATS).

As neither anti-thymocyte serum or anti-neutrophil serum reduced the bacterial density in the mouse wound model, I had to go back to the original system in which abscess formation was shown to allow bacteria to persist within the host: TCRαKO mice [6]. Unfortunately, using the specific knockout mice as in the McLoughlin and et al. paper and repeating their experiments– same strain, same method of inoculation preparation (in table referred to as 'plates'), same method and same knockout mice– gave a very different outcome. Specifically I saw no reduction in the bacterial load in TCRαKO mice compared to control mice (C57BL/6J) (Table 5.3.2).

Table 5.5: Bacterial Load[a] in TCRαKO or Control Mice

	inoc. (cfu)	**method[b]**	**C57Bl/6J**	**α TCR (-/-)**
margolis	50	plates	3.63 ± 1.21 (n=3)	3.24 ± 0.94 (n=3)
margolis	65	broth	3.94 ± 0.79 (n=3)	4.44 ± 0.85 (n=3)
mcloughlin et al [c]	100	plates	6.81 ± 0.23 (n=7)	2.85 ± 0.95 (n=4)
margolis	4000	broth	6.01 ± 0.35 (n=8)	5.89 ± 0.49 (n=8)

[a] Mean ($\pm$ standard error) bacterial density of 3 mice inoculated with 10^6 cfu in mouse wound.

[b] Method of inoculum preparation bacteria were either resuspended colonies from plates or washed bacteria from broth.

[c] Data taken from [[6]]

In order to discern whether the discrepancy was due to our knockout mice not being deficient for α TCR+ T cells, I ran a FACS analysis of blood collected from these infected mice and determined the density of CD3+ T cells and α TCR cells. The knockout mice were indeed deficient in α TCR+ T cells (data presented in materials and methods). The McLoughlin et al 2006 paper mentions that abscess formation occurs in the knockout mice when the inocula is greater than 1000 cfu, however repeated experiments at lower density inoculum did not give the result of the paper. Through extensive email correspondence with Jean Lee (corresponding author of McLoughlin et al 2006) we could find no differences in our methodologies that could explain the differences observed.

5.4 Summary

The wound infection model is an accurate model of *S. aureus* infections which occur after surgical procedures. The bacterial dynamics suggest that bacteria in these infections rapidly divide and that after a certain lag-time the bacterial density is controlled. However there is an additional lag time before the bacteria are cleared and metastatic disease (especially in the kidneys) is common while blood cultures are uniformly negative.

Blocking abscess formation by using TCRαKO mice is either not a robust result or is not reproducible. Other methods used to manipulate the immune response failed to affect abscess formation and the bacterial load in muscle wounds.

MAJOR ARTICLE

Within-Host Evolution for the Invasiveness of Commensal Bacteria: an Experimental Study of Bacteremias Resulting from *Haemophilus influenzae* Nasal Carriage

Elisa Margolis and Bruce R. Levin

Department of Biology, Emory University, Atlanta, Georgia

Background. Many bacteria responsible for clinically relevant disease reside harmlessly in a large fraction of humans. Three explanations have been proposed to account for why these normally commensal bacteria occasionally cause invasive disease: host susceptibility, stochasticity in the host-bacteria interaction, and the evolution of invasive mutants in colonized hosts. Here we test the third of these hypotheses for the rare invasiveness of commensal bacteria: within-host evolution.

Methods and Results. Using neonatal rats intranasally colonized with pairs of marked *Haemophilus influenzae* type b strains, we demonstrate that the resulting bacteremias are derived from single organisms. To test the within-host evolution hypothesis we explored the relative ability of bacteria isolated from the blood and nasal passages of bacteremic rats to colonize the nasopharynx and invade the bloodstream.

Conclusions. Our results provide support for within-host evolution as one but not the sole explanation for the invasiveness of these bacteria. We discuss the implications of these results for both the rare invasiveness of commensal bacteria and the general observation that bacteria isolated from the sites of human invasive disease are almost invariably monoclonal.

A number of bacteria (e.g., *Staphylococcus aureus, Escherichia coli, Neisseria meningitidis, Streptococcus pneumoniae,* and *Haemophilus influenzae*) colonize and persist for extended periods of time in substantial fractions of the human population without causing symptomatic disease and thereby are considered commensals. However, these same species of bacteria are responsible for significant morbidity and mortality due to the occasional invasive infection [1–3]. Although there is genetic variability in these species and some variants are more likely to cause invasive disease than others, even for the most virulent strains colonization rarely results in disease [3–7]. How do these bacteria, which are maintained and transmitted from sites where they are harmless, pass through the host defenses and proliferate in sites where they cause symptoms but are unlikely to be transmitted? And why do they do so in only a small minority of colonized hosts?

There are 3 not-mutually-exclusive answers to these questions. First is host susceptibility; at any given time the immune defenses of a small fraction of hosts are not sufficient to prevent colonizing bacteria from invading. This variation in susceptibility can be physiological—due to age, concomitant infections (e.g., viral infections [8, 9]), or chronic conditions (e.g., diabetes mellitus [10])—or genetic (e.g., immune deficiencies [11–13]). The second explanation is stochasticity; as a consequence of chance alone, even in hosts with perfectly functional defenses there would be a low probability that bacteria enter and replicate in sites where they cause disease. The third explanation is within-host evolution [14, 15]; the colonizing population of bacteria may include members that are selected within colonized hosts because they have or acquire heritable modifi-

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Reprints or correspondence: Elisa Margolis, Dept. of Biology, Emory University, Atlanta, GA 30322 (emargol@emory.edu).

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cations that enable them to invade and replicate in new sites.

Although the first explanation is widely accepted [16], the latter 2 appear to have been given less consideration, despite evidence that can be interpreted in their support. Natural populations of the commensal bacteria responsible for rare invasive disease are genetically very diverse [17–20], and at any given time humans are commonly colonized with multiple types [21, 22]. Nevertheless, the bacteria isolated from sites of invasive disease in individual patients are almost invariably monoclonal, and these invasive clones differ among patients [23]. Furthermore, in experiments in which bacteremias occur in rodents that are colonized with pairs of marked strains of bacteria, only one or the other marked strain can be isolated from the blood [24–28]. These 2 observations suggest that the low frequency of invasive disease is due to rare events occurring in the bacterial population. Here, we ascertain whether these rare events are due to random chance encounters between the colonizing bacteria and holes in the host defenses or to the generation and ascent of invasive mutants in the colonizing population.

We used an *H. influenzae*–neonatal rat model similar to that developed by Richard Moxon and colleagues [26, 29] and demonstrate, through statistical analysis, that single organisms are responsible for founding the bacteremias that occur from nasal colonization. We test whether bacteria isolated from the blood of these bacteremic rats were more likely than those from nasal-wash isolates to establish populations in the blood of new rats. We interpret our results as evidence that within-host evolution is one but not the unique reason for the rare invasiveness of commensal bacteria and the monoclonality of invasive disease. We discuss the limitations of these finding and their implications for understanding the mechanisms responsible for invasive disease.

MATERIALS AND METHODS

Microorganism. *H. influenzae* Eagan strain and its spontaneous streptomycin-resistant mutant Rm154 (Str-R) were provided by R. Moxon (Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK). Em6 was selected as a spontaneous nalidixic acid–resistant mutant (Nal-R) of Eagan.

Inocula preparation and storage. Inocula for all of the infant rat experiments were prepared by resuspending a frozen aliquot of a midexponential-phase culture in 250-mL flasks that contained 50 mL of brain-heart infusion (BHI) broth supplemented with 10 μg of hemin and 2 μg of βNAD/mL. Shaking cultures that reached the midexponential phase were centrifuged (at 5000 *g* for 3 min) and resuspended in PBS with 0.1% gelatin (PBSG).

Bacteremia studies. All experiments were preformed under the guidelines of and approved by the Emory University Institutional Animal Care and Use Committee. Two-day-old pups, born to timed-pregnant Sprague-Dawley rats (Charles River Laboratories), were pooled and randomly reassigned to dams. Groups of 30 five-day-old rats were intranasally inoculated by touching a 10-μL drop of 10^7 or 10^8 bacteria of a mixture of equal densities of Str-r and Nal-r to the external nares [29, 30]. These doses were higher than that used in previous studies, because this hanging-drop method resulted in more initial loss of inoculum than did the scalp-vein needle method used by Moxon and Murphy [26]. Two days after the infection, 0.5 mL of blood was collected by cardiac puncture; nasal-wash samples were collected from 200 μL of PBSG instilled into a 5-cm Intramedic polyetylene tubing (PE50) placed into the thorax; and nasal epithelium was scraped from the nasal passages after a second wash with 200 μL of PBSG and removal of the frontal bones. The nasal epithelium, which has been suggested to contain a distinct, potentially invasive population [31], was homogenized in 1 mL of PBSG. Then, 100 μL of the blood, nasal-wash, and nasal epithelium samples were plated directly and serially diluted onto BHI agar plates with bacitracin (0.3 g/L) with either streptomycin (4 mg/L) or nalidixic acid (5 mg/L). Plates were incubated overnight at 37°C with 5% CO_2. The limit for detection at any site was 10 cfu/mL. The density of bacteria in bacteremic rats ranged from 500 to 4×10^5 cfu/mL. Bacteremias were considered to be of only one type if at least 50 colonies were observed on one of the antibiotic plates (Nal or Str) but no colonies on the other.

Rare invasion model. Details of the model and the statistical analysis can be found in the Appendix.

Invasiveness studies. For the experiments testing the within-host evolution hypothesis, single colonies were picked at random from antibiotic-containing plates of blood and nasal-wash samples from rats that were bacteremic 48 h after nasal inoculation. These colonies were grown to the midexponential phase in 2 mL of BHI, after which 200 μL of glycerol was added, and they were frozen at −80°C as 100-μL aliquots. In each of 3 independent replicates, 10–20 neonatal rats received intranasal inoculation (prepared as indicated above) of mixtures of blood and nasal isolates; invasiveness was determined from blood samples of these rats at 48 h.

The following statistical procedure was used to determine whether the blood isolates were significantly more invasive than the ancestral strains. The invasiveness of the ancestral strains (Em6 and Rm154) was determined from the relationship between the density of a mixed inoculum (10^5–10^9 cfu) and the frequency of bacteremias where [32]

$$\text{Fraction with bacteremia} = \frac{e^{-27.84 + 3.52\ [\log(\text{dose})]}}{1 + e^{-27.84 + 3.52\ [\log(\text{dose})]}}\ .$$

From this function we calculated the anticipated fraction of

invasions for the ancestral strain with the dose set for that used in each replicate, the null hypothesis. We then compared (using the χ^2 test) the observed number of bacteremias for each of the 6 blood isolates in each replicate to that anticipated from this null model. Each replicate of the same pair of blood and nasal isolates were analyzed separately because they represented different inoculum densities given their independent culturing from frozen aliquots. The frequency of bacteremias should be no different from that observed with the ancestral strains if the blood and nasal isolates were equally likely to invade. If the blood isolates were inherently more likely to invade, then the fraction of rats with bacteremias would differ from the null.

Relative fitness. Relative fitness of the ancestral strains, nasal isolates, and blood isolates was measured in 3 environments: in vitro, nasal passage, and bloodstream. In vitro fitness was determined by sampling initial and final densities after 8 h of growth from mixed (50:50) liquid cultures. Nasal passage fitness was determined by inoculating sets of 10–25 five-day-old rats with 50:50 inoculum; 48 h later we sampled nasal-wash and epithelium samples as described for bacteremia studies. Because there were no venous access points without surgical manipulation in rats of this age, intraperitoneal inoculation of ~100 bacteria suspended in 100 μL of PBSG into a set of 3 neonatal rats and subsequent 48-h blood cultures were used to determine the relative fitness in the blood. For relative fitness, we used the selection rate r_{sn}, which is a measure of the relative recovery of the Nal-r and Str-r bacteria inoculated into the nasal passages over the 48 h and is given by

$$r_{sn} = \ln\frac{N_s(48)}{N_s(0)} - \ln\frac{N_n(48)}{N_n(0)} ,$$

where $N_s(0)$ and $N_n(0)$ are the initial densities in the inoculum of Str-r and Nal-r, respectively, and $N_s(48)$ and $N_n(48)$ are the densities detected at the specific site after 48 h [33]. A selection rate of 0 indicates no fitness difference between the strains and a positive rate indicates an advantage for the Str-r strain.

RESULTS

Bacteremias derived from single organism. Rats were inoculated into the nasal passage with a mixture of 10^7 Nal-r and 10^7 Str-r *H. influenzae* type b Eagan strain. The design of this experiment is illustrated in figure 1*A*. Of 29 rats with mixed nasal colonizations, 5 were bacteremic at 48 h; of these, 3 yielded only Nal-r colonies and 2 yielded only Str-r colonies (figure 1*B*). With a 10-fold increase in the initial inoculum, 14 of 30 rats were bacteremic; of these, 4 bacteremias were due to purely Str-r organisms, 6 were due to purely Nal-r organisms, and 4 were due to mixed infections.

We compared these results with the predictions of a statistical model that assumes that bacteria enter and establish a population in the blood (invade) at random (table 1). In this model (detailed in the appendix), bacteremias with both Nal-r and Str-r bacteria are generated in 2 ways: by bacteria of both types establishing a population in the blood in a single invasion event or bacteria establishing populations separately in multiple invasion events. The expected number of independent invasions of the bloodstream in each rat for the 10^7 and 10^8 inoculation doses was calculated using Poisson distribution. The assumption in this model that both strains were equally likely to invade was confirmed, because both strains had similar invasion rates (data not shown), and we failed to detect differences in the in vivo fitness between the strains (table 2). By using binomial distribution, we determined for a certain number of bacteria invading in each invasion the expected number of blood infections caused by a single type of bacteria (Nal-r or Str-r) and the expected number caused by both types (Nal-r and Str-r). In table 1, we compared this expected number of rats with a

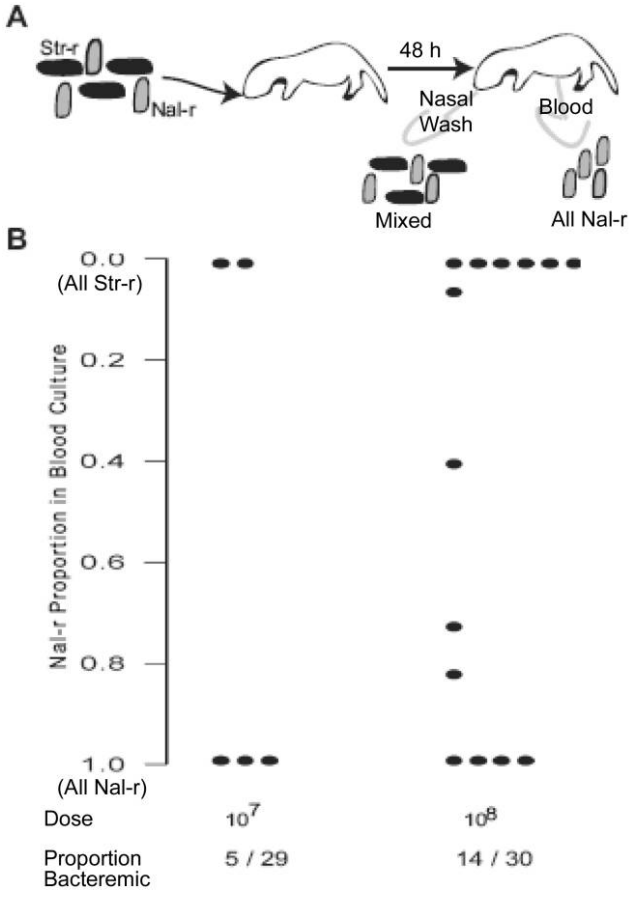

Figure 1. Mixed-culture colonization experiments. *A,* Experimental design. Equal densities of nalidixic acid–resistant mutant (Nal-r) and streptomycin-resistant mutant (Str-r) *Haemophilus influenza* bacteria were inoculated into nasal passages of 5-day-old rats. Then, 48 h later, nasal washes and blood were sampled. *B,* Results. The Nal-r bacteria fraction in positive blood cultures for 2 different inoculation densities (10^7 and 10^8) is shown.

Table 1. Comparison of the no. of blood cultures with 1 or 2 types of bacteria observed and expected from a statistical no.

	10^8 dose (14 bacteremic rats)			10^7 dose (5 bacteremic rats)		
Bacteria	Rats with 1 type	Rats with 2 types	*P*	Rats with 1 type	Rats with 2 types	*P*
Observed	10	4		5	0	
Expected from the statistical model if the no. of bacteria is						
1 bacterium	11.82	2.18	**.33**	4.76	0.24	**.52**
2 bacteria	5.45	8.55	.006	2.33	2.67	.014
3 bacteria	2.62	11.38	>.001	1.15	3.85	>.001
4 bacteria	1.28	12.72	>.001	0.57	4.43	>.001

NOTE. Rats with 1 type have either nalidixic acid–resistant mutant or streptomycin-resistant mutant bacteria in their blood cultures. *P* values where the model does not significantly differ from observes values are in bold type.

bacteremia of a single strain for different numbers of bacteria establishing blood populations to the observed numbers of bacteremias (with 10^7 and 10^8 inoculation doses). For both the 10^7 and 10^8 inoculation doses, the most probable number of bacteria establishing blood infections is 1.

Evidence for within-host evolution. To test whether the bacteremias were caused by a mutant in the bacterial population with a greater propensity to invade the blood, we isolated single colonies of bacteria (3 Nal-r and 3 Str-r) from the blood and nasal-wash samples of 6 separate bacteremic rats. These isolates were grown to the midexponential phase and stored in aliquots at −80°C. Each of the blood isolates was paired with nasal isolates with the alternative marker (from the same bacteremic rat) and introduced in equal frequency into the nasal passages of a new set of rats. The design of this experiment is illustrated in figure 2*A*.

Five of the 6 blood isolates provided no evidence for an increase or decrease in the likelihood of blood invasion relative to the nasal isolates (figure 2*C*). However, 1 blood isolate (Em091) showed significant increases in relative invasiveness in 3 independent trials ($P > .001$, $P > .031$, and $P > .004$, calculated from a comparison with the ancestor's invasiveness for that inoculation density) (figure 2*B*).

To determine whether the apparent increased invasiveness of Em091 was due to a competitive advantage in either the nasal passage or blood, we estimated its fitness relative to a nasal isolate in these sites. As can be seen in table 2, there was no evidence for an increase (or decrease) in Em091's fitness in the nasal wash or epithelium after nasal inoculation or in the blood after intraperitoneal inoculation. This was also the case for the 5 blood-isolated clones, for which we detected no enhanced ability to invade the blood (data not shown). We were unable to detect a trade-off between invasiveness and colonization in the strains tested.

DISCUSSION

We interpret the results of these experiments as evidence that the rare invasiveness of commensal bacteria and the monoclonality of the invasive populations can be attributed to rare events in the colonizing bacteria, either by the random success of one bacterium in establishing an invasive population or a random mutation in the colonizing population producing an invasive clone.

The results of our experiments are consistent with those of previous rodent model studies with *H. influenzae* as well as

Table 2. Selection rate of ancestral strains and the evolved blood isolate (Em091).

Strain (Str-r/Nal-r)	Nasal wash ($n = 25$)	Nasal epithelium ($n = 25$)	Blood ($n = 6$)	In vitro ($n = 6$)
Rm154/Em6 (ancestral)	−0.199 ± 0.159	−0.087 ± 0.205	0.070 ± 0.615	−0.151 ± 0.262
Em091/Em092 (blood/nasal)	0.129 ± 0.189	0.256 ± 0.190	0.069 ± 0.599	

NOTE. The selection rate is a measure of fitness calculated from

$$r_{sn} = \ln\frac{N_S(48)}{N_S 0} - \ln\frac{N_N(48)}{N_N(0)},$$

where $Ns(t)$ is the density of the streptomycin-resistant mutant (Str-r) strain at t h and $N_N(t)$ is the density of the nalidixic acid–resistant mutant (Nal-r) strain. A selection rate of 0 indicates no difference in the Str-r and Nal-r strain fitness. Data are mean ± SE. The blood isolate is in bold type.

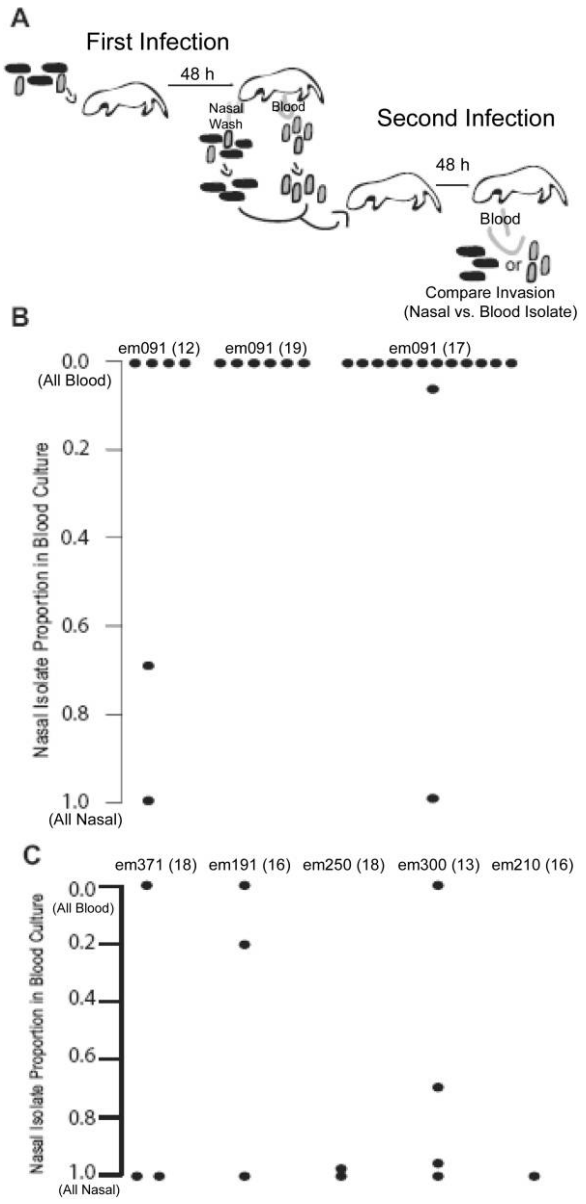

Figure 2. Test of within-host evolution. *A,* Experimental design. Blood and nasal isolates with alternative markers from a bacteremic rat were used to prepare a mixed inoculum for the second infection. At 48 h, the blood was sampled to determine the relative invasiveness of the blood and nasal isolates. *B,* Results. The nasal isolate fraction in positive blood cultures of rats for blood isolate Em091 (no. of rats colonized) from 3 separate sets of second infections with inoculation dose of 3.5×10^7 cfu is shown. *C,* Results. The nasal isolate fraction in positive blood cultures of rats for representative trial of the other 5 isolates (no. of rats colonized) is shown.

other bacteria (*Salmonella enterica, E. coli,* and *Streptococcus agalactiae*) [24–28]; bacteremias resulting from experimental colonizations with pairs of genetically marked strains can be attributed to one or the other strain but rarely to both. This has been interpreted as evidence that these bacteremias are the products of very few bacteria [26]. Our statistical analysis not only supports this interpretation but also suggests that the majority of the bacteremias are due to 1 rather than to a few bacteria. It should be noted that our results and analysis do not preclude the possibility that >1 bacterium enter the bloodstream but rather indicate that all of the bacteria recovered from the blood are derived from a single cell.

Our experiments also provide support for within-host evolution as one but not the unique explanation for why these bacteremias were founded by single bacterium. One of the 6 clones of bacteria that we isolated from the blood was more invasive than the corresponding nasal isolate. This genetic modification either occurred within a few generations after the bacteria were introduced into the nasopharynx or may have existed in the inoculum. Regardless of when these mutants arose, their ascent within the host was precipitous, and most likely a single genetic modification in the bacteria produced the invasive clone. This evolution is not the same as has been observed elsewhere [34, 35] in passage experiments in which selection in successive hosts favors bacteria that are ever more capable of replicating in the blood. Likewise, it is different from the evolution that takes place in chronic bacterial infections, such as those of *Pseudomonas aeruginosa* in patients with cystic fibrosis [36] or of *Helicobacter pylori* [37]. Although this phenomenon has been observed in viruses [38], to our knowledge, this is the first experimental evidence for precipitous virulence resulting from within-host evolution in bacteria. These results are consistent with observations that suggest that single point mutations can dramatically augment bacterial virulence [39]. For example, in *Streptococcus pyogenes*, mutations in the 2-component system, CsrR/CsrS, were found to evolve during experimental murine skin infections and lead to more necrotic lesions [40].

Our test of the within-host evolution hypothesis was stringent; the genetic modification had to be not only heritable but also stable. It was not lost during storage (at −80°C) or during the preparation of the inocula, which requires growth for >15 generations in liquid culture. This stability of the genetic modification makes a strong case for either a single point mutation, a transitory genetic mechanism (such as phase variation of contingency loci [41]) or a gene amplification [42] that was selected for during colonization. Interestingly, the increased likelihood of blood invasiveness that we observed could not be attributed to the evolved clone (Em091) having a superior fitness in the nose or in the blood. This suggests that invasion is not simply a byproduct of the bacteria's ability to maintain long-term carriage, as has been suggested for pneumococci [31].

Although supporting within-host evolution, the present results also indicate that it is not the sole reason for the rare virulence of commensal bacteria or the monoclonality of the invasive pop-

ulations. This would have been the case even if all rather than just 1 of the 6 blood isolates that we tested were inherently more invasive. That we were unable to detect heritable increases in invasiveness for 5 of the 6 clones points to the importance of stochastic processes other than mutation in the bacteria as responsible for the rare invasiveness of commensal bacteria and the monoclonality of the invasive populations.

We do not interpret the results of these experiments as evidence against the conventional wisdom that the rare invasiveness of commensal bacteria can be attributed to variation in host susceptibility [16]. There is good evidence for inherited variation in human susceptibility to infectious disease [43, 44] and a plethora of noninherited mechanisms, including age and underlying disease [45, 46], that could account for how colonizing populations of bacteria become invasive. Although host variation may play a prominent role in whether colonizing bacteria will or will not lead to invasive disease, in itself variation in host susceptibility cannot account for the monoclonality of invasive bacterial populations. It seems unlikely that, when confronted with millions of bacteria, only a single bacterium would pass through the hole(s) in the defenses of a host with physiologically or genetically compromised immune defenses. The invasion process is likely to involve a number of steps: the bacteria must adhere to and pass through the epithelium and replicate within the bloodstream, all the while avoiding opsonization and evading the host's other immune responses. It is easy to imagine why at each of these successive steps there are fewer and fewer bacteria; this may well explain why bacteria normally do not succeed in establishing populations in the blood. But if the invasion of the blood is due to an even transitory defect in one of these barriers, it is hard to explain why many bacteria would not exploit this defect.

That is not to say that the monoclonality of infections excludes host susceptibility from playing a prominent role in the rare invasiveness of commensal bacteria. The monoclonality in the invasive population of bacteria could be due to secondary processes, such as selective or random purging of the variation from the invading population. Another possibility for this secondary process is a host response to the invasion. For example, it has been proposed that inflammation induced during the invasion of one clone may preclude the invasion of others [47].

We set out to test the hypothesis that the rare virulence of commensal bacteria can be attributed to selection for mutants within the colonizing populations that are capable of invading sites where they cause disease: within-host evolution. Although the results of these experiments provide some evidence in support of this hypothesis, they also point to the importance of alternative mechanisms. Most important, the results of our experiments indicate that whatever mechanisms are responsible for commensal bacteria becoming invasive, they must account for the monoclonality of the invasive population.

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APPENDIX

STATISTICAL MODEL FOR NUMBER OF INVADING BACTERIA

To statistically evaluate the number of bacteria involved in establishing blood populations, we constructed a model that assumes that bacteria enter and establish a population in the blood—invade—at random. In the first part of our analysis, we calculated the expected number of independent invasions of the bloodstream in the same rat for the 10^7 and 10^8 inoculation doses. The number of independent invasion events, k, can be calculated from the observed fraction of infections that did not yield bacteremias and from that anticipated from a Poisson distribution. Let λ be the expected number of bacteremias in a group of rats inoculated with a given dose of bacteria placed in the nasal passages. In accord with Poisson distribution, the probability of not having bacteremia is $\lambda = -\ln[P(k = 0)]$ or $P(k = 0) = e^{-\lambda}$. With the 10^7 inoculum, our estimate is $P(k = 0) = \frac{24}{29}$ or $\lambda = -\ln(\frac{24}{29}) = 0.19$, and with the 10^8 inoculum, the estimate is $\lambda = 0.63$.

From these estimates of λ and the full Poisson distribution, we can calculate the distribution of rats with different numbers of invasion events ($k = 0, 1, 2$):

$$P(k;\lambda) = e^{-\lambda}\frac{\lambda^k}{k!} .$$

From a binomial distribution, we then calculate the expected number of blood infections caused by a single strain (Nal-r or Str-r) and the expected number caused by both strains (Nal-r and Str-r) for different numbers of bacteria invading in each event. As in the case of heads and tails in a coin toss, we assume that the Nal-r and Str-r strains are equally likely to invade. The parameter w is the number of bacteria that are responsible for establishing the blood population in each invasion event, equivalent to the number of tosses of the coin. From the binomial

distribution, the probability of either all Nal-r or all Str-r (all heads or all tails) in the blood is then

$$P(\text{all Nal-r or all Str-r; } k) = \frac{1}{2} + \frac{1}{2} wkwk \ .$$

We then can determine the expected number of rats with bacteremia caused by a single (Nal-r or Str-r) strain or both (Nal-r and Str-r) strains for different numbers of bacteria establishing blood populations by multiplying the probability of having a bacteremia due to k invasion events by the probability that k events are all of one strain.

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Exploring the role of the immune response in preventing antibiotic resistance

Immune Response Preventing Antibiotic Resistance Journal of Theoretical Biology (*in press*) **Andreas Handel, Elisa Margolis and Bruce R. Levin**

7.1 Abstract

For many bacterial infections, drug resistant mutants are likely present by the time antibiotic treatment starts. Nevertheless, such infections are often successfully cleared. It is commonly assumed that this is due to the combined action of drug and immune response, the latter facilitating clearance of the resistant population. However, most studies of drug resistance emergence during antibiotic treatment focus almost exclusively on the dynamics of bacteria and the drug and neglect the contribution of immune defenses. Here, we develop and analyze several mathematical models that explicitly include an immune response. We consider different types of immune responses and investigate how each impacts the emergence of resistance. We show that an immune response that retains its strength despite a strong drug-induced decline of bacteria numbers considerably reduces the emergence of resistance, narrows the mutant selection window, and mitigates the effects of non-adherence to treatment. Additionally, we show that compared to an

immune response that kills bacteria at a constant rate, one that trades reduced killing at high bacterial load for increased killing at low bacterial load is sometimes preferable. We discuss the predictions and hypotheses derived from this study and how they can be tested experimentally.

7.2 Introduction

By the time bacterial infections cause symptoms and thereby call for antibiotic treatment, the bacterial population is often so large that it likely includes mutants that are resistant to the treating antibiotic [21, 19]. One might therefore expect treatment with single antimicrobial agents to fail. One reason why this is frequently not the case is the host's immune defense, which contributes to bacteria clearance [51, 26]. Despite the general recognition of the important role of the host's defenses, most studies of the within-host dynamics of bacteria and antibiotics focus almost exclusively on the pharmacokinetics (PK) and the pharmacodynamics (PD) of the drug and bacteria, without explicitly considering the immune response [41, 48, 43, 21, 57, 16, 47, 10, 2, 1].

Such omission of the immune response also applies to models that are specifically intended to develop treatment protocols to prevent the ascent of resistant mutants, like those based on the Mutant Selection Window (MSW) theory [61, 30, 18, 62]. The MSW is defined as the range of drug concentrations for which the drug is strong enough to remove the sensitive population, but not strong enough to remove the (partially) resistant population. In the absence of an immune response, this is expected to lead to selection of the resistant mutant, which can ascend to high levels [49, 20]. As we show here, the presence of an immune response can alter the MSW. Several theoretical and experimental studies have addressed the role of the immune response during antimicrobial treatment, and the issue has also been studied in the context of viral infections [14, 34, 58, 12, 29, 4, 11]. But, to our knowledge, the interactions between antibiotics and the host's immune response as it affects the emergence of resistance during bacteria infections has not

been addressed.

While the lack of data precludes the development of detailed and quantitatively accurate models of the contribution of the immune response for the antibiotic treatment of specific bacterial infections, simple mathematical models can provide a way to generate hypotheses for the design and interpretation of future experiments. We develop such simple mathematical models and use them to analyze the effects of different types of immune responses on the ascent of resistance during antibiotic treatment. We show that an immune response that retains its strength despite a strong drug-induced decline of bacteria numbers considerably reduces the emergence of resistance, narrows the mutant selection window, and mitigates the effects of non-adherence to treatment. Additionally, we show that compared to an immune response that kills bacteria at a constant rate, one that trades reduced killing at high bacterial load for increased killing at low bacterial load is sometimes preferable. We discuss the implications of these theoretical results to antibiotic treatment and how the hypotheses generated from our analysis can be tested experimentally.

7.3 The Mathematical Models

7.3.1 Bacteria and drug dynamics

To describe bacterial growth, pharmacokinetics (PK) and pharmacodynamics (PD), we use a model that has previously been shown to successfully fit data from *in vitro* experiments on the emergence of fluoroquinolone (ciprofloxacin) resistance in *Staphylococcus aureus* [8, 9]. We consider two populations of bacteria, one susceptible to the antibiotic (B_s) and the other resistant (B_r). The susceptible and resistant bacteria grow at rates g_s and g_r. Growth slows down as the total number of bacteria approaches a maximum population size, N_0. During growth, susceptible bacteria generate resistant mutants at a rate μ; back mutations to the sensitive genotype are ignored. The antibiotic is administered at a dose C_0 every T hours. It decays according to a standard first-order pharmacokinetic function with an ex-

ponential decline in concentration at rate d. The antibiotic kills bacteria according to a hyperbolic (Monod like) E_{max} function with maximum kill rates k_s and k_r and half-maximum antibiotic concentrations C^s_{50} and C^r_{50}, for the sensitive and resistant bacteria respectively. (This corresponds to a Hill function with Hill-coefficient of 1 [55]). Note that the resistant bacteria are not fully resistant to the drug, instead they require higher drug concentration for clearance, which reflected in the values of the kill and half maximum kill concentrations, i.e. $k_r < k_s$ and $C^r_{50} > C^s_{50}$. This is the type of resistance usually found in experimental or clinical situations. The model is expressed by the set of coupled differential equations (a dot denotes differentiation as a function of time)

$$\dot{B}_s = (1-\mu)g_s B_s(1 - \frac{B_s + B_r}{N_0}) - \frac{k_s C}{C + C^s_{50}} B_s, \tag{7.2}$$

$$\dot{B}_r = (g_s \mu B_s + g_r B_r)(1 - \frac{B_s + B_r}{N_0}) - \frac{k_r C}{C + C^r_{50}} B_r, \tag{7.3}$$

$$\dot{C} = -dC, \qquad C(t) = C(t) + C_0 \text{ every T hours.} \tag{7.4}$$

While the model was shown to describe *in vitro* data, it is not clear how it applies to an *in vivo* situation. However, since not enough is known about the *in vivo* dynamics of bacteria and drugs during the process of resistance generation, we decided to choose the present model because of the availability of experimentally measured values for the model parameters (Table 7.6).

7.3.2 Immune Responses

The important and novel aspect of our study is that we explicitly model the dynamics of an immune response. The immune response is immensely complex; many interdependent players, such as different cell types and cytokines, participate at varying degrees. This complexity, combined with the experimental difficulty in accurately measuring all the different immune response components, leads to a lack of detailed, quantitative data. Because of this lack of data, we do not try to create a detailed model of the immune response for a specific infec-

symbol	meaning	values
$B_s(0)$	susceptible bacteria inoculum	10^3
$B_r(0)$	resistant bacteria inoculum	0
N	total bacteria	$B_s + B_r$
N_0	carrying capacity	10^9
g_s	maximum sensitive growth	1h^{-1}
g_r	maximum resistant growth	0.65h^{-1}
k_s	maximum kill rate of sensitives	1.5h^{-1}
k_r	maximum kill rate of resistant	1.1h^{-1}
C_{50}^s	half-maximum kill rate of sensitives	0.25 μg/ml
C_{50}^r	half-maximum kill rate of resistant	5 μg/ml
μ	mutation rate	10^{-8}
d	drug decay rate	$0.15h^{-1}$
T	times at which drug is administered	varied
C_0	drug dose administered at times T	varied

Table 7.6: Variables and parameters for the part of the model describing bacteria and drug dynamics. Parameter values are chosen in accordance with [8, 9].

tion, such as *S aureus*. Instead, we employ several simple, heuristic models that are meant to capture known aspects of immune response dynamics, while at the same time realizing that these are simplified caricatures and the obtained insights are therefore conceptual. We consider the following four models:

Immune Response Model 1. For the first model, we consider an immune response, I, that is triggered upon onset of infection and increases exponentially at rate g_i (clonal expansion [6, 3, 15]), independent of the bacteria. Pathogen independent aspects of the expansion dynamics have recently been found for CD4 and CD8 T-cells [31, 46, 59, 5, 35], which are known to play important roles against obligate or facultative intracellular bacteria, such as *Listeria monocytogenes* and *S. aureus* [50, 44]. The immune response saturates once it reaches some maximum strength. Such dynamics seems to hold for CD4 and CD8 T-cells in the lung after tuberculosis infection [33, 32]. Since I is given in arbitrary units, we choose the maximum strength to be $I_{max} = 1$. This leads to a term for the immune dynamics given by $\dot{I} = g_i I(1 - I)$. We set the immune response at the beginning of the in-

fection to $I_0 = 10^{-6}$ and a rate of expansion of $g_i = 1\text{h}^{-1}$. These values was chosen based on data from CD4 and CD8 T-cells [6]. However, the exact choices are not important, the results presented below also hold for different values. Killing of bacteria by the immune system is assumed to occur at a rate directly proportional to the strength of the immune response, with a killing rate constant b. In all our immune response models, killing of sensitive and resistant bacteria occurs in the same manner. This leads to the terms $-bIB_s$ and $-bIB_r$ added to the equations for sensitive and resistant bacteria, respectively. This mass-action type killing term is consistent with observations from CD8 T-cells and neutrophils [39, 54, 60].

Immune Response Model 2. For the second model, we consider the same dynamics of the immune response as in model 1, but now the rate at which bacteria are killed saturates at some maximum level as the bacterial load increases. We implement this by scaling the killing term with the total number of bacteria present, so that these terms become $-bIB_s/(N+s)$ and $-bIB_r/(N+s)$, where $N = B_s + B_r$ and s is a saturation constant for the killing rate. This captures the observation that immune cells need time to kill and under these conditions, the mass-action formulation of model 1 breaks down at high pathogen load [53, 45]. This has been observed experimentally for neutrophils and is also likely to apply to CD8 T-cell mediated killing at high infected cell densities [36, 40, 54, 60].

Immune Response Model 3. For the third model, we change the dynamics of the immune response. We assume that the immune response grows proportional to the bacterial load and decays at a fixed rate. This leads to the term $\dot{I} = g_i N - d_i I$. If the decay of the immune response is reasonably fast – and we will focus on that situation in the following – this model approximates an immune response that closely tracks the bacterial load, i.e. $I \approx \frac{g_i}{d_i} N$. Such dynamics might apply to cytokines or highly activated immune cells [22, 27, 25]. For the killing, we again assume a mass-action term, as described for model 1.

Immune Response Model 4. The fourth model uses the same assumption for the dynamics of the immune response as model 3, and combines it with the saturated killing term of model 2.

We want to again stress that these four models we use here are simple and

IR model	IR term	killing term
model 1	$\dot{I} = g_i I(1-I)$	$-bIB_n$
model 2	$\dot{I} = g_i I(1-I)$	$-\frac{bIB_n}{N+s}$
model 3	$\dot{I} = g_i N - d_i I$	$-bIB_n$
model 4	$\dot{I} = g_i N - d_i I$	$-\frac{bIB_n}{N+s}$

Table 7.7: Summary and parameters of the immune response (IR) models. $n = s, r$ representing the sensitive and resistant subpopulations. Values for the parameters are given in the results section.

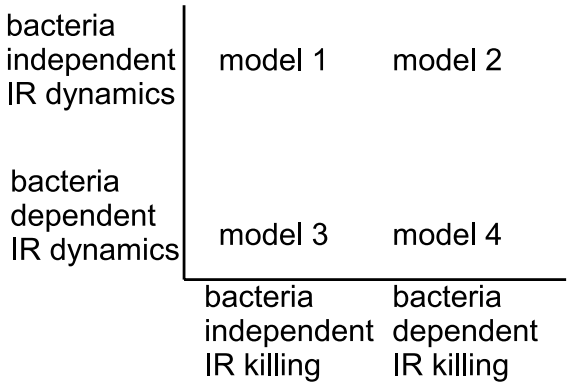

Figure 7.1: Graphical representation of the differences between the four models. IR = immune response.

heuristic, meant to capture aspects of the spectrum of possible immune response dynamics. Figure 7.1 shows graphically the different conditions for the models just described, Table 7.7 summarizes the model equations. All models were implemented in Matlab R2007a (The Mathworks), the code is available from the authors upon request.

7.4 Results

7.4.1 Resistance emergence in the absence of an immune response

We start by considering the dynamics of susceptible and resistant bacteria in a situation where there is no immune response. The infection starts with a few drug sensitive bacteria. As the bacteria grow, resistant mutants are generated and because they also replicate (at a lower rate compared to the drug sensitive population), they too increase. Both the resistant and susceptible populations level off when the total bacterial load reaches the saturation level. In the absence of drug treatment, the less-fit resistant population constitutes a small fraction of the total population. Once antimicrobial treatment is started, the sensitive population is cleared by the drug and the total bacterial load falls below the saturation level. This allows the resistant population to increase until its net growth rate equals the rate of killing by the antibiotic (Fig. 7.2). (Recall that resistance is not complete, the drug can kill the resistant population, albeit at a very low rate).

7.4.2 Resistance emergence in the presence of immune responses

In the worst case, even the combined effect of antimicrobial drug and immune response can not eradicate the susceptible population. While this will likely prevent the ascent of the resistant population, this scenario represents treatment failure not due to resistance but simply due to an ineffective drug. We do not consider this situation further. If the immune response alone is not strong enough to clear the susceptible population, but a combination of antimicrobial treatment and immune

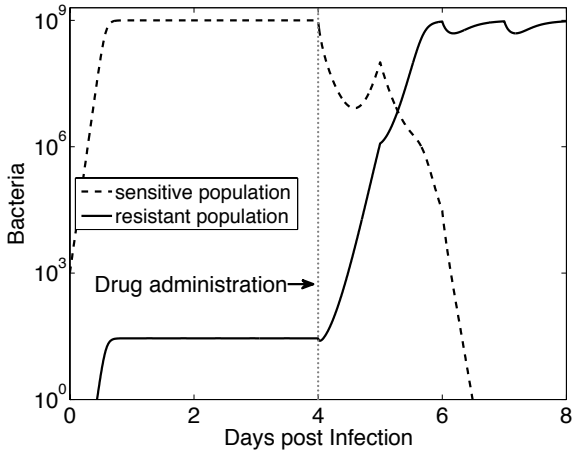

Figure 7.2: Dynamics of infection in the absence of an immune response. Antimicrobial treatment is started at day 4. Every T=24h, a dose of $C_0 = 4\mu$g/ml is administered. Parameters as given in Table 7.6.

response can do so, two possibilities exist for the resistant subpopulation: Either the immune response (together with the weak effect of the antibiotic on the resistant mutants) can prevent the emergence of the resistant population. This will likely occur if the cost of resistance is non-negligible (Fig. 7.3A, black lines). Or, alternatively, the immune response can not prevent the emergence of resistance. This might be the case if the fitness of the resistant strain is close to that of the sensitive strain (Fig. 7.3A, gray lines), or if the immune response is less potent (e.g. a value for b half that shown for the first scenario in Fig. 7.3A leads to resistance emergence. Graph not shown).

The type of killing can also impact the outcome. If the killing rate saturates for high bacterial load (model 2) and the rate is the same as for the non-saturating model at low bacterial load, the result is a weaker immune response and less good control of the resistant population (compare Figs. 7.3A and B, black lines). In contrast, if the killing at low bacterial load is more effective, it can lead to better clearance of the resistant subpopulation once the drug has reduced the sensitive subpopulation, even if the killing strength at high bacterial load is much weaker

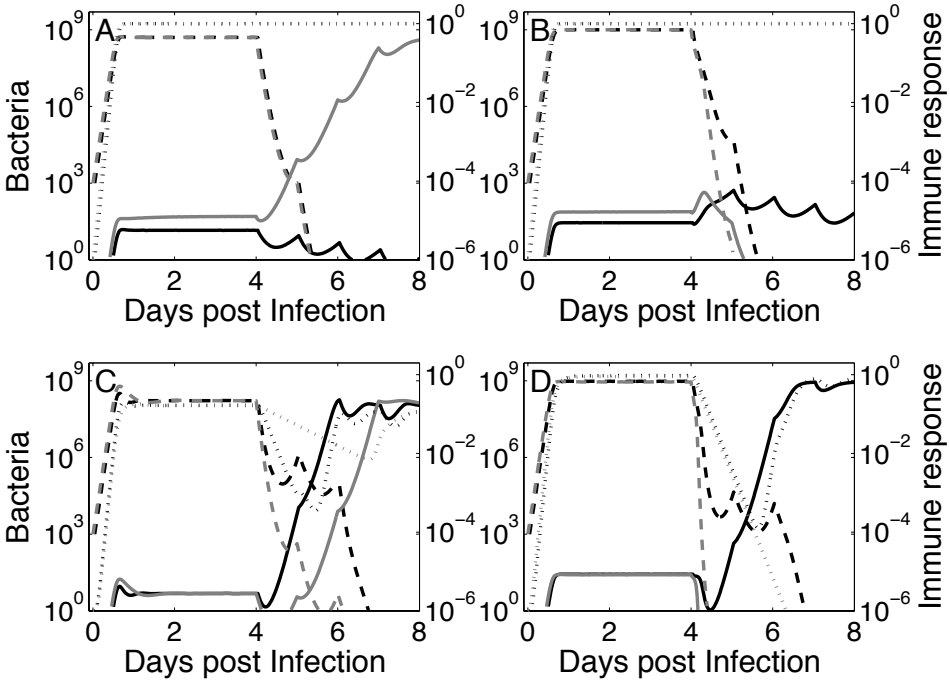

Figure 7.3: Dynamics of infection in the presence of immune responses. Dotted lines show immune response (with axis label on the right side), solid and dashed lines show the drug resistant and drug sensitive bacteria. Drug dosing as described in legend of Figure **??**. The parameters b, g_r, d_i, g_i are all given in units of 1/hour. All parameter values are as given in Table 7.6 unless otherwise stated. **A)** Model 1. Black: low-fitness (growth rate) resistant strain ($g_r = 0.65$), prevention of resistance. Gray: high-fitness resistant strain ($g_r = 0.9$), resistance emerges. (Killing rate $b = 0.5$). **B)** Model 2. Black: maximum killing at low bacterial load is the same as in model 1, killing rate declines once the bacteria increase beyond 1% of the carrying capacity and is about one-hundredth that of model 1 for $N \to N_0$ ($s = N_0/100$, $b = 0.5s$, $g_r = 0.65$). Gray: Maximum killing at low bacterial load is twice that of model 1 but still only one-fiftieth that of model 1 for $N \to N_0$ ($s = N_0/100$, $b = s$, $g_r = 0.9$). **C)** Model 3. Black: the immune response changes rapidly as bacterial load changes ($b = 5$, $d_i = 0.25$, $g_i = d_i/N_0$). Gray: the immune response changes less rapidly as bacterial load changes ($b = 5$, $d_i = 0.05$, $g_i = d_i/N_0$). **D)** Model 4. Black: maximum killing at low bacterial is the same as in model 3, killing rate declines once the bacteria increase beyond 1% of the maximum carrying capacity and is about one-hundredth that of model 3 for $N \to N_0$ ($s = N_0/100$, $b = 5s$, $d_i = 0.25$, $g_i = d_i/N_0$). Gray: Maximum killing at low bacterial load is twice that of model 1 but still only one-fiftieth that of model 1 for $N \to N_0$ ($s = N_0/100$, $b = 10s$, $d_i = 0.25$, $g_i = d_i/N_0$).

compared to the non-saturating, mass-action killing (compare Fig. 7.3A and B gray lines).

For the two models considered so far, the immune response quickly reaches a constant level and remains at this level, independent of the bacteria dynamics. We now turn to models 3 and 4 to investigate how an immune response that depends on bacteria numbers affects resistance emergence. If the immune response decays quickly (d_i large), a decline in bacteria due to drug treatment will result in a rapid decline of the immune response and subsequent emergence of resistance is likely, even if the immune response is very potent. The black lines in Figure 7.3C show this for a scenario where resistance emerges, even though the killing rate, b, is ten times larger compared to model 1. Note how closely the immune response (dotted line) tracks the bacterial load. If the decay of the immune response is slower, it will lead to less immediate tracking of the bacteria decline and improved ability to control the resistant population. the gray lines in Fig. 7.3C show a situation where resistance emergence is almost prevented. A further reduction in the decay rate leads to a situation that approaches the pathogen-independent immune response shown in panels A+B, where resistance emergence is prevented (not shown).

As seen for models 1 and 2, a saturating immune response that kills less efficiently at high bacterial load but more efficiently at low bacteria numbers can help to prevent resistance emergence. This is also true if the immune response declines almost immediately as the bacteria decline. The higher killing efficiency at low bacteria numbers can make the difference between resistance emergence or clearance (compare Fig. 7.3C and D, gray lines).

7.4.3 Immune responses can reduce the mutant selection window

So far, we have shown illustrative examples how different types of immune responses can affect the emergence of resistance. We now investigate the impact of the different immune responses in more detail. We begin by exploring how the immune response can change the size of the mutant selection window (MSW). The

MSW is defined as the range of drug concentrations for which the drug is strong enough to remove the sensitive population, but not strong enough to remove the resistant population. In the absence of an immune response, this is expected to lead to selection of the resistant mutant, which can ascend to high levels [49, 20]. Figure 7.4 illustrates the MSW idea.

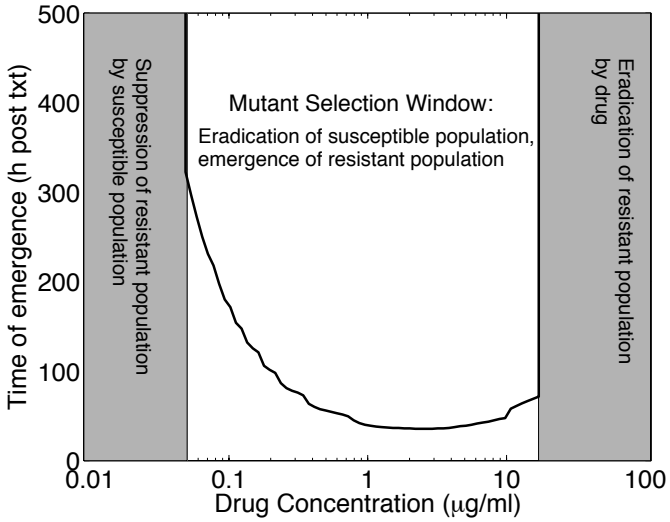

Figure 7.4: The mutant selection window (MSW) in the absence of an immune response. Shown is the time of resistance emergence following treatment (txt), as a function of drug concentration (C_0). Emergence is defined as the resistant population reaching 10% of the carrying capacity. The simulation is run until 14 days post treatment start. If the resistant population has not reached 10% by day 14, the time of emergence is set to infinity. At low drug concentrations, the drug sensitive population is not removed and the resistant population can not emerge. Very high drug doses kill both sensitive and resistant populations. Intermediate drug doses clear the sensitive population only, and thereby allow the resistant population to reach high levels.

The presence of an immune response is expected to change the MSW. One would expect to see the MSW shrink at the right side, for high drug concentrations, simply because in the presence of additional killing by an immune response, a lower drug dose is required to remove both the susceptible and the (partially)

resistant subpopulations. This is indeed what one finds (Fig. 7.5). Also, as expected, the immune responses that do not tightly follow the decline of bacteria (models 1 and 2) are able to shrink this part of the window by a larger amount, though model 4, which declines in strength as bacterial load declines, but at the same has an increasing killing rate, is able to perform almost as good as models 1 and 2.

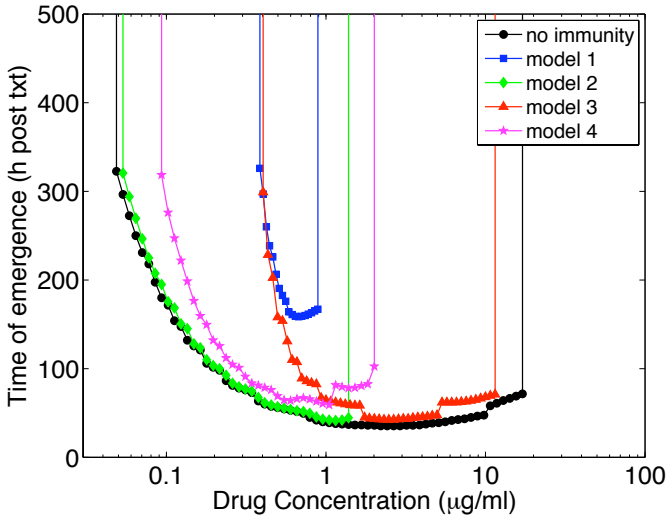

Figure 7.5: The MSW in the presence of the different immune responses. Time to emergence is defined as described in the caption for Figure 7.4. Immune responses are chosen as in Figure 7.2 with $g_r = 0.65$, $b = 0.5$ (model 1), $s = N_0/100$ and $b = s$ (model 2), $b = 5$, $d_i = 0.25$, $g_i = d_i/N_0$ (model 3), and $s = N_0/100$, $b = 10s$, $d_i = 0.25$, $g_i = d_i/N_0$ (model 4).

Less intuitively obvious is why the MSW shrinks at the left side, for low drug concentrations. Since the immune response acts together with the drug, the sensitive population is cleared at lower drug concentrations. One might therefore also expect resistance to emerge at lower, not higher, drug concentrations compared to the situation without an immune response. This would indeed be the case if the immune response were to act only on the sensitive population. However, it affects both populations equally. In the absence of an immune response, any

drug-induced decline of the sensitive population allows the resistant population to quickly increase in numbers. In contrast, if an immune response is present, it can prevent the resistant population from growing, even if some of the competitive pressure is removed by a reduction in the sensitive population. It therefore takes higher drug doses, corresponding to a stronger selective pressure in favor of the resistant population, before resistance can emerge. Since at high bacteria loads, the saturating immune responses (models 2 and 4) are rather weak, they are not very good in preventing resistance to emerge at low drug concentrations (Fig. 7.5, green diamonds and magenta stars). In contrast, the mass-action models retain a potent response at high bacteria numbers, therefore preventing the emergence of resistance (Fig. 7.5, blue squares and red triangles) and significantly shrinking the MSW at the left side.

7.4.4 Immune responses can dampen the effect of imperfect adherence to treatment

The emergence of resistance is often due to the failure of patients to follow a prescribed antibiotic treatment protocol. A prominent example is treatment failure of long-term infections like tuberculosis [42, 23, 24]. It is therefore of interest to understand how the immune response might modulate the effect of imperfect adherence to an antibiotic treatment regime. As our model for non-adherence we use a scheme similar to one of those considered previously [41]. In this scenario, patients are less likely to take the prescribed drug dose as symptoms reduce. We assume that symptoms are proportional to bacterial load. This is implemented by assuming that a dose is taken with probability $p = 0.25 + 0.75 \log_{10}(N)/\log_{10}(N_0))$. This means that for maximum bacterial burden ($N = N_0$), adherence is perfect. As bacteria load declines, so does the probability that the patient takes the drug. At very low bacteria load, the probability that the patient takes the antibiotic is reduced to ≈25%. We simulate 5000 infections using a Monte Carlo routine where at each scheduled dosing interval the drug will be taken with probability p. Figure 7.6 shows the MSW for perfect and imperfect adherence in the absence or presence

of immune responses. We plot the median value for the time to resistance emergence, defined as previously. As expected, in the absence of an immune response, non-adherence increases the MSW (Fig. 7.6, open and filled circles). The presence of an immune response dampens the impact of non-adherence. The immune response models with a bacteria load-dependent killing (models 2 and 4) seem to perform slightly better, compared with the bacteria load-independent killing models (models 1 and 3). This is presumably because killing in these models is improved at low bacteria numbers, which is exactly the regime where the probability of taking the prescribed drug dose is the lowest.

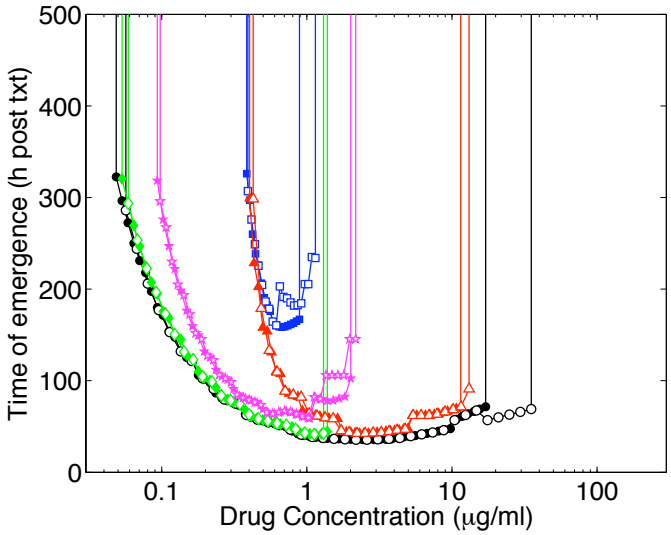

Figure 7.6: The mutant selection window for imperfect adherence (empty markers). For comparison, results for complete adherence are replotted from Figure 7.5 (solid markers). Everything else as described for Figure 7.5.

7.4.5 Immune responses can change optimal dosing strategies

The goal of an optimal treatment strategy is to achieve all specified goals (e.g. clearance, prevention of resistance emergence), while at the same time ensuring that the smallest possible amount of drug is used (to reduce toxicity and financial

costs). Additionally, one might want to reduce the frequency at which the drug is administered. If the killing action of the drug (the PD) is well described by an E_{max} model, such as the one we use here, overall killing of bacteria is larger if a given amount of drug is administered in frequent small doses instead of few large ones [41]. Because the E_{max} model applies to both the sensitive and resistant populations, we expect that in the absence of an immune response, more frequent drug doses are better at killing the resistant strain and clearing the infection. This is indeed the case (Fig. 7.7, black circles). We also find that this holds for the immune response models 1 and 3, which kill at a rate independent of bacterial load (blue squares and red triangles). Interestingly, the bacterial load-dependent killing models, model 2 and 4, behave differently. For model 2 (green diamonds), small frequent doses can prevent resistance emergence, but so can few large ones. The reason for this is that a high drug dose strongly reduces the sensitive population, which leads to improved per-capita killing by the immune response and subsequent clearance of the resistant population. For model 4, this latter mechanism works as well, while the improved overall killing by the drug due to the E_{max} PD has less impact.

Note that we chose the total drug dose administered over the 24 hour interval ($\hat{C}$) for the different models such that a change in dosing frequency lead to a switch between resistance emergence and resistance prevention. There are of course a wide range of values for the drug concentration that lead to less interesting results, namely resistance emergence or bacteria clearance, no matter how the dosing schedule over a 24 hour period is chosen. While it is not clear if for a realistic situation, any of the results shown in Fig 7.7 might occur, it is nevertheless important to illustrate what *could* happen, and how the complicated dynamical interactions between the antibiotic, bacteria and immune response can lead to unexpected outcomes, which depend on the details of the immune response.

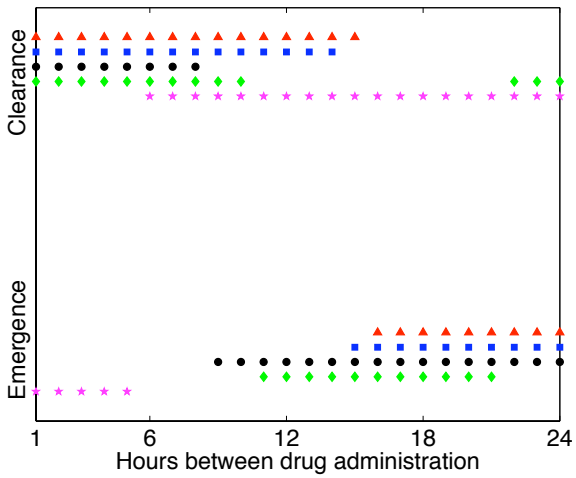

Figure 7.7: Bacteria clearance or resistance emergence as a function of dosing regime. Drug is administered at the indicated time intervals, in doses such that the total amount of drug administered over one day, $\hat{C}$, remains fixed. $\hat{C}$ for the situation without immunity and the four immune responses models are 10, 0.75, 1.5, 8 and 2.5 μg/ml (see text). Everything else as described for Figure 7.5.

7.5 Discussion

Help from the immune response is often necessary to clear bacterial infections, even in the presence of antibiotic treatment. An example is the success of drugs that – at least *in vitro* – are only bacteriostatic [52]. Here we developed mathematical models that combine the dynamics of bacteria and drugs with different models for the immune response. We used these models to analyze the emergence of resistance during the course of treatment.

7.5.1 Caveats and limitations

As with all mathematical (as well as verbal) models of biological systems, the models employed in this study represent strong simplifications of the complex interactions between bacteria, antibiotics and the host's immune response. Although the equations for bacteria and drug dynamics provided a reasonable fit to data for *S. aureus* and ciprofloxacin generated *in vitro* [8, 9], it is not clear how well such models apply *in vivo*. In fact, *S. aureus* readily evolves resistance to ciprofloxacin [13] which makes this fluoroquinolone a less than optimal drug for treating staphylococcal infections.

However, the focus of this study was not on direct clinical relevance but rather to generate a conceptual framework for addressing questions about the contribution of the immune response to preventing the evolution of antibiotic resistance during the course of therapy. With minor modifications, this same theory could apply to many different antibiotics and bacteria for which resistance can be generated by mutation. Of particular relevance in this regard is multi-drug therapy in situations where mutants resistant to single drugs are almost always present [42, 56]. Although our model of the immune response is a simplistic caricature of the plethora of host responses to a bacterial infection, it is based on biologically realistic assumptions and captures aspects of the anticipated dynamics of the host response. More specifically, we chose scenarios were the strength of the immune response is independent of or strongly dependent upon the bacterial load, and

where the rate of immune-mediated killing does and does not saturate at high bacterial load. We believe these models capture aspects of the dynamics of the induction, buildup and waning of the immune defenses and immune-mediated killing of bacteria.

Our models focused on resistance generated by genetic mutation through a one-step process. We did not formally consider non-inherited resistance due to physiological processes, biofilm formation or persistence, which can prolong therapy and promote the generation and ascent of inherited resistance [28, 24, 37, 17, 38]. We also assumed that a resistant population already existed prior to treatment start. As mentioned in the introduction, we chose this setup because it is likely to occur for many clinical infections, where the bacteria have reached such high numbers by the time symptoms occur and treatment starts that the existence of a resistant subpopulation is likely. For a situation where resistance had not yet emerged at the beginning of the treatment period, an immune response that helps to eradicate the sensitive population as quickly as possible – and thereby minimize the chance that a resistant mutant is created – is expected to perform best.

7.5.2 Predictions and hypotheses

Our results suggest that the presence of an immune response narrows the mutant selection window, helps to mitigate the negative effects of non-adherence, and influences the optimal dosing strategy. We find that if antibiotic drug concentrations can be maintained at relatively high levels (the right border of the MSW), the synergism between immune response and drug in reducing resistance emergence is best for immune response components that are largely independent of the dynamics of the pathogen (Fig. 7.5, models 1 and 2). Such dynamics applies probably most strongly to parts of the adaptive immune responses. For an immune response that is tightly linked to pathogen load (cytokines, parts of the innate immune response, highly activated CD8 T-cells), trading reduced killing at high bacterial load for increased killing at low bacterial load can be better at

preventing resistance emergence (Fig. 7.5, model 4). In contrast, if antibiotic drug concentrations cannot reach levels that are high enough to be above the MSW (for instance due to toxicity), immune responses that do not saturate in their strength of killing at high bacterial load will perform best (Fig 7.5, models 1 and 3). Saturated killing might be unavoidable due to biological constraints (e.g. time it takes to kill [53]), one can speculate that reduced killing at high pathogen load might be a "choice" made by the immune response in certain situations to prevent excessive immunopathology. It is worth pointing out that the type of killing function depends on the exact numbers of immune players and bacteria and the killing mechanism, and can switch from non-saturated to a saturated regime in some but not other situations. In general, a higher number of immune players (e.g. a certain type of immune cell) and a faster killing process will reduce the potential for killing saturation at high bacterial load.

The predictions from our models can be tested experimentally in laboratory animals. One can use a "resistance competition assay" [49, 7], whereby animals are inoculated with low numbers of bacteria resistant to the treating antibiotic and high numbers of the susceptible population, and the changes in frequency of resistance during the course of antibiotic treatment are followed. In addition to measuring the bacterial load and concentrations of antibiotics in these experiments, different components of the immune responses should also be quantified. Applying this protocol to laboratory animals with normal immune systems and animals with specific components of the immune system impaired can provide information into the role different components of the immune response play in affecting the mutant selection window, dosing strategies, etc. [19]. We would expect that animals with impaired immune response components which are largely independent of bacterial load, both in their dynamics and their killing behavior, will be most susceptible to resistance emergence, compared with healthy animals or animals that have bacteria load-dependent immune dynamics or killing. Of course, the main problem with such experimental tests (and the direct applicability of our results) is the fact that immune response components tightly interact, therefore it might be difficult to knock out certain components without affecting

the performance of others.

To summarize, we have shown that the host's immune defenses can play an important role in the emergence or prevention of drug resistance. This highlights the need for further studies that consider the joint impact of the immune response and antimicrobial drug treatment on the emergence of resistance. Eventually, such a combination of experimental and theoretical studies should allow us to design treatment protocols that prevent resistance emergence and lead to complete bacteria clearance, while also optimizing drug dose, robustness against non-adherence and treatment schedule.

Conclusions

The main aim of this research was to understand the bacteria- and host–mediated factors that determine nasal colonization and invasive disease due to normally commensal bacteria, specifically *S. aureus*, *S. pneumoniae* and *H. influenzae*. A few general conclusions emerge from this research such as the predominance of stochastic processes in colonization and invasion or the importance of host responses in both virulence and antibiotic emergence. But more often than not, this research has called attention to all the unknowns in our understanding of competition, invasive disease, antibiotic emergence and the specific role of the immune system in these bacterial processes. Here, I will attempt to highlight the numerous unanswered questions that may serve as future directions in this scientific endeavor.

8.1 Part I: Ecology of Nasal Colonization

Why is there large variation in the bacterial load of individual rats colonized with *S. aureus*, *S. pneumoniae* **and** *H. influenzae***?**
When inoculated alone the median bacterial load for each of these species appears to be maintained for at least 96 hours and invariant over a large range of inoculums (**Chapter 2**). However, there is a considerable amount of variability in individual rats. This variability among individual rats could easily be dismissed as resulting principally from heterogeneity in the susceptibility of the individual (out–bred) rats. However, it seems more likely that this variability arises

from (self-reproducing) bacteria acting independently (i.e. not synchronized or cooperating- which was confirmed in **Chapter 6**) as this has been demonstrated to have a much larger impact on bacterial infection experimental systems than differences in host susceptibility[4]. Further investigations into the relative role of host susceptibility and stochastic processes in determining the bacterial density of colonizing populations may be informative to disease prevention.

What is the mechanism for established *S. aureus* populations excluding subsequent *S. aureus* from colonizing?
During intra–species competition in the nasal passage, established populations of *S. aureus* are capable of excluding other *S. aureus* strains (**Chapter 2**). This confirms prior results that demonstrated that prior inoculated strains in burns excluded subsequent strains[9] and provides an explanation for why when human persistent carriers of *S. aureus* were incoulated with a mixture of strains, the strain they had originally out-competed the others[5]. Determining whether this exclusion is due to occupation of limiting resources like attachment sites or spatially deposited resources or other mechanisms may aid in designing means to limit *S. aureus* colonization.

Why does introducing a second population of *S. pneumoniae* or *H. influenzae* in rats with an established population increase the bacterial load?
Population dynamics, especially inoculum independence, had suggested that the bacterial density of the colonizing population for all three strains was in a steady-state where tight control (either by a limiting resource or an immune response) limited the colonizing population from exceeding this density. However, this apparent carrrying capacity increased after the introduction of a second *S. pneumoniae* or *H. influenzae* population (a pulse; **Chapter 2**). One explanation is that the apparent steady state observed in the population dynamics for these strains is the result of an extremely large bottleneck in the inoculating population, thus the colonizing population isn't being controlled as much as the size of the founding population is independent of the inoculum density. Alternatively the second

pulse could have modified the population size limiting factors- either freeing up a limiting resource or suppressing an immune response. These results were not straight-forward but are not likely to be artifacts as they occurred in multiple (at least 4) repeats.

In addition, these experiments could be repeated with different strains (preferably with known or no fitness differences) and serotypes to determine what is likely to occur during strain/serotype replacement.

Why and what is responsible for the relatively low density of bacteria in the nasal passages?

Clearly both host and bacterial factors influence the density achieved by colonizing populations. In **Chapter 2**, I investigated the role of already established bacterial populations but did not identify the mechanisms of resource or immune limitation in hosts without other species present. There are hints from the competition experiments: *S. pneumoniae's* may be limited by neutrophil and complement killing (from interaction with *H. influenzae*), *S. aureus* limited by a priority resource (pulse experiment) and *H. influenzae* limited by a nutrient (which can be increased by *S. pneumoniae* or *S. aureus* colonization). However more research is needed into the ecological conditions, including investigating the role of other immune components and identifying limiting resources that maintain and disturb the density and structure of colonizing populations.

How do *S. pneumoniae* strains differ in their susceptibility to *H. influenzae* induced immune responses?

Only one of the two *S. pneumoniae* strains tested (TIGR4 and Poland(6b)–20) was susceptible to neutrophil and complement–mediated reductions when invading the nasal passages with an established *H. influenzae* population, and this competition was only observed in the nasal wash (not the nasal epithelium sample; **Chapter 2**). The proposed mechanism for this immune–mediated competition is that the presence of *S. pneumoniae* and *H. influenzae* together elicits a synergistic immune response (neutrophils and complement) to which *S. pneumoniae* is sensitive

and this may occur through antigen (presumably of *H. influenzae* origin) delivery being increased in the presence of *S. pneumoniae's* pneumolysin. However this mechanism does not account for the differences in the two strains (both have the pneumolysin gene) or why the decrease in *S. pneumoniae* population was only in the nasal wash. That it could only be observed at one site and not in the nasal epithelium, presumably where the more tightly adhering bacteria are present, suggests that this competition may not have much effect on the persistence of *S. pneumoniae* in the nasal passages. In addition, the two strains did not differ in the neutrophil infiltration they elicited during nasal colonization (**Chapter 2**). This provides some support for the notion that *S. pneumoniae* serotypes/capsules (or at least 6b and 4) differ in their sensitivity to neutrophil/complement immune responses.

Why is *S. pneumoniae*–*S. aureus* co-colonization rarer than expected?
The results presented in **Chapter 3** are inconsistent with hydrogen peroxide production by *S. pneumoniae* reducing the nasal colonization of *S. aureus* . In addition to the relative scarcity of *S. aureus* and *S. pneumoniae* not being due to H_2O_2 –mediated interference competition, I could find no evidence for any other bacteria-bacteria interaction between *S. aureus* and *S. pneumoniae*(**Chapter 2**). An alternative explanation for the epidemiological observation is that *S. aureus* and *S. pneumoniae* have different host preferences and that the carriage population can be sub-divided into these host preferences. Distinguishing between bacterial interactions and host preferences is especially important to vaccination efforts, as bacterial interactions but not host preferences may lead to vaccination having the undesired consequence of increasing the incidence of a competing species.

Why does catalase production provide a fitness advantage to *S. aureus* only when *S. pneumoniae* is present?
I found that catalase production by *S. aureus* provides a fitness advantage during colonization of neonatal rat when co–inoculated with *S. pneumoniae* but that this is not due to the production of H_2O_2 by *S. pneumoniae* (**Chapter 3**). While catalase

protects against free radicals, why would it be selected for only when *S. pneumoniae* is present. Two explanations warrant more investigations: *S. pneumoniae* increases the free radical mileu indirectly through the immune response (principally neutrophils which use free radicals to kill bacteria) or directly through toxins.

If H_2O_2 does not provide a competitive advantage against *S. aureus* , why would *S. pneumoniae* produce it?
I found no evidence for hydrogen peroxide production by *S. pneumoniae* providing it with a competitive advantage against *S. aureus*. The gene coding for pyruvate oxidase, which produces H_2O_2, was identified in a screen directed at bacterial elements required to bind *S. pneumoniae* to epithelial cells of the nasopharynx and lung.[8] The mutant (Spxb) which does not produce H_2O_2 had a 70% reduction in binding to epithelial cells and specifically decreased adherence to glycogonjucates. Furthermore, pyruvate oxidase is implicated in protecting against oxidative stress[6] , competence[1] and spontaneous pneumococcal death during stationary phase[7]. There are multiple possible reasons for the higher fitness of H_2O_2 producing strains seen during colonization[7] and disease (pneumonia and sepsis)[8].

8.2 Part II: Evolution of Virulence

How much of virulence is due to microbial factors and how much is due to the host's immune response?
While **Chapter 4** offers multiple examples of where the host immune response causes damage during infection processes, it is not clear how wide–spread this is as a phenomenon. In addition to the three bacterial exceptions noted in **Chapter 4** (i.e. tooth caries, gastro-intestinal and neuronal bacterial toxins) most intracellular pathogens (whether bacteria, viral or parasites) have to burst out of the host cells as part of their life-cycle, which clearly causes virulence in the process. Whether most virulence is due to microparasite's life history traits or to the host's immune response (often an 'over–response') is still unresolved.

In the cases when bacterial virulence is due to a host's immune (over-)response, why hasn't selection on the host's immune system tempered the response?
I propose three hypotheses for why selection on the host has not tempered immune over-responses (**Chapter 4**). The first is that disease mediated selection can be relatively weak as not everyone exposed to a disease becomes infected (maximum intensity of selection is equal to the frequency of symptomatic infections) and even then it takes considerable time to evolve tempering mechanisms. Second hypothesis is that selection on the immune system may be constrained as it is primarily selecting for speed and breadth of immune defenses which may be in direct opposition to selection on the magnitude of reactions or tempering mechanisms. Lastly selection on the immune system may be constrained by the immune system's other roles— such as defending against cancer, maintaining blood-flow homeostasis (clotting) or its roles in pregnancy. In support of the first hypothesis, research into the TB-mediated selection on humans has found no evidence that selection by TB has been strong in the human population[3]. There is little evidence for either of the latter two hypotheses.

Is abscess formation an example of an immune over–response?
As I was unable to reproduce a blockade of abscess formation using a TCRαKO mouse model, the role of the immune response in abscess formation and prevention of bacterial clearance from wounds is still unsettled.

When within–host evolution for bacterial invasiveness does not occur, what determines whether invasive disease will occur?
In our test of within–host evolution for invasive *H. influenzae* infections (**Chapter 6**), I found that only 1 out of the 6 blood isolates I tested had acquired an increased propensity to cause bacteremia. What was responsible for the bacteremia in the other 5 isolates? Were these bacteremias linked to disruptions in nasal colonization ecology or (as discussed below) changes in densities of colonizing bacterial populations? What determines whether within–host evolution occurs? For example, during Coxsackie virus infections, within–host evolution occurred in elderly

mice but not in young mice [2]. The only factor I have elucidated is population size, as bacteremias only occurred with inoculum greater than 10^7 cfu but there likely are other factors.

What is the relationship between the density of colonizing bacteria and disease?
It seems quite logical that the more colonizing bacteria the likelihood of invasive disease or infections in adjacent sites would increase. In fact this is implied by the experiments in **Chapter 6** where the likelihood of bacteremia increases with the inoculation density. However the results of **Chapter 2** make the relationship more nebulous, as I demonstrated that the inoculation density is independent of the colonizing bacterial load. While an increased density of colonizing bacteria may increase disease likelihood, this hypothesis has to my knowledge not been conclusively demonstrated.

Does within–host evolution for invasive disease occur with other bacterial species?
I only tested the possibility of within–host evolution for *H. influenzae* and bacteremia, however there are many other pathogens where within–host evolution may play a role in disease pathogenesis. Given that *S. pneumoniae* readily colonize neonatal rat it would be a natural starting place to investigate the role of within–host evolution in pneumococcal bacteremia and pneumonia.

How do immune responses affect the likelihood of antibiotic resistance emergence?
The details of immune response dynamics and killing can have a strong effect on the ability of the immune response to prevent resistance emergence **Chapter 7**. Importantly for many bacterial infections the dynamics (expansion and contraction) and killing rate/mechanisms of specific immune compentents is not known. Therefore quantitative experimental studies which investigate both the immune response and bacteria dynamics are necessary.

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