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Analyzing Microbial Disease at High Resolution: Following the Fate of the Bacterium During Infection

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Abstract

The study of bacterial pathogens has historically been viewed with a wide lens, providing a picture of how bacterial populations act as groups, but with insufficient resolution to see how microorganisms act as individuals. For most bacterial pathogens, we do not know the minimal number of microbes that initiate infection in a particular organ site, the number that spread outside the site of initial colonization, and how many persist over time. Recent studies have begun to shed light on these points, and the development of new techniques has dramatically increased the ability of researchers to interrogate these problems. With new approaches, the field of bacterial pathogenesis is on the verge of understanding the role and fate of individual bacteria during infection.

Introduction

The most common way to assess the ability of a bacterial pathogen to infect, grow, and persist in a host is to measure the number of bacteria within different host organs over time. This extremely productive method has been used countless times to investigate host/pathogen interaction in animal infection models. For example, quantification of bacterial populations has been used extensively to assess bacterial and/or host mutations that affect the overall microbial burden in different organs. This method has been an important strategy for investigating innate and adaptive immune responses, as well as the function of bacterial virulence factors. Although critical for the progress of infectious diseases research, this strategy is severely limited. For example, if there is a 10-fold increase in the bacterial population over a specific time period, it is unclear whether 99% of the clones that initiate infection are killed, with 1000-fold growth of 1% of the population, or if every bacterium multiplies itself by 10. These are radically different scenarios that would greatly alter our view of a particular bacterial disease. This extremely basic question is still largely unanswered for most bacterial diseases.

Given our lack of understanding of this fundamental issue, it should not be surprising that many other questions about the dynamics of the disease process remain almost completely untouched. If we see an increase in bacterial number in an infected organ, is this due to bacterial growth in that organ, or large scale seeding from another infected organ? If we see

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a decrease in bacterial number, is this because the host is finally able to wipe out the invading microbes, or are the bacteria simply migrating to another organ? If we see a stable bacterial population in an infected organ, does this represent a quiescent, metabolically inactive bacterial population content to establish a quiet, persistent infection? On the other hand, does a stable bacterial population represent a furious battle between the bacteria and their host, and the “stable” population is the tally of bacterial replication combined with bacterial destruction by host immune cells? By measuring the total bacterial population we are measuring a combination of growth, death, persistence, influx, and egress of bacteria, without distinguishing between these events. In this review, we summarize the different experimental approaches that resolve the fate of individual bacteria, and discuss some preliminary findings derived from these methods.

Methods used to study the fate of individual clones during bacterial infections

1) Plasmid or Fluorescence Dilution—Relative amounts of bacterial growth and death within a host can be measured by using a bacterial strain harboring a conditional nonreplicating plasmid. Animals infected with bacteria containing a plasmid that is temperature-sensitive for replication and encodes antibiotic resistance allows quantitative information on the amount of replication that takes place in the bacterial population shortly after entering into an organ site [1]. Loss of the antibiotic resistance within a site in the animal is due to bacterial division, resulting in dilution of the plasmid because it is incapable of replication at physiological temperature. Conversely, if no bacterial division takes place, the bacteria remain resistant to the antibiotic, as there is no dilution of the plasmid. A recent, conceptually similar approach is to measure the dilution of fluorescent protein after shutting down expression of the protein (Figure 1). If the entire population of bacteria in an organ site is competent to replicate, then there will be uniform dilution of fluorescence in the absence of continued expression of the protein (Fig. 1, panel A). Helaine and colleagues measured the decrease in emission of a fluorescent protein after inoculation of *Salmonella enterica* (serovar Typhimurium) into mice, providing a quantitative picture of the amount of bacterial replication and death during infection [2]. This approach has the remarkable potential for quantifying bacterial growth dynamics during infection (compare various fates of bacteria, Fig. 1, panels A–C). The limitations of these methods are that neither plasmid nor fluorescent dilution can precisely determine how many bacteria initiate disease, how many of the original clones survive over time, or what percentage of the bacteria in an infected organ spread to other sites in the host.

2) Independently tagged strains (barcoding)—In order to learn the fate of individual bacterial clones, it is necessary to generate markers that distinguish between clones in a mixed population during an extended infection (Figure 2). Following pools of individually mutated bacteria during the course of infection, a strategy originally intended to identify pathogen genes required for disease in animal models, has largely solved this problem. The original method, signature tagged mutagenesis, was dedicated to following the behavior of pools of mutated bacteria. This involves preparing a bank of individual mutants with transposon insertions, each of which contains a unique oligonucleotide barcode, allowing the fate of individual mutants to be followed [3–7]. This method has been used extensively, with numerous bacterial pathogens, to identify virulence factors essential for different stages of infection. In addition, it can allow investigators to follow the fate of individual bacteria during infection (Figure 2). To ensure that individual insertion mutations do not result in bacterial strains having different phenotypes, wild-type isogenic tagged strains (WITS) with unique DNA tags in the same noncoding sequence or plasmids with unique oligonucleotide tags have been used [8–10].

A later method, transposon site hybridization (TraSH), improved on STM by increasing the number of mutants that could be easily screened [11]. With TraSH, probes made from sequences contiguous to transposon insertions are hybridized to microarrays. With this method, theoretically 10^5 or more mutants can be screened. Unfortunately, this method does not allow for detailed information on the fate of individual clones, as several different clones with a transposon insertion in a single gene will be indistinguishable. However, with this limitation in mind, there is still significant information on clonality that can be gleaned from TraSH data. At the very least, TraSH data can provide a minimum number of clones that are likely present at a given time.

A recently developed method, TnSeq [12,13], combines the strengths of STM and TraSH, as it is able to distinguish between individual transposon insertions in pools of 10^4 – 10^5 individual insertion mutants. With Tn-Seq, the sites of all transposon insertions, as well as the relative abundance of each clone in the population, are determined using deep sequencing techniques. This extremely powerful method allows tens of thousands of individual transposon insertion clones to be analyzed from the same animal.

A final method for independently tagging strains is to use strains expressing different protein markers. For example, Oellerich and colleagues tagged *Yersinia enterocolitica* clones expressing either GFP or RFP, and looked at the invasion and subsequent growth and spread within mice of different clones by histology [14]. Using clones that express different antibiotic resistance markers has been similarly used, most recently to analyze the trafficking of *Listeria monocytogenes* [15]. The weakness of these strategies is that the number of available fluorescent proteins or antibiotic resistance markers limits the number of clones that can be analyzed.

Comparison of Methods

Each of the above methods has strengths and weaknesses, and a full understanding of a bacterial infection will almost certainly require a combination of strategies. STM and Tn-Seq represent the best methods for looking at large numbers of clones, but carry the caveat that each clone is also a uniquely altered strain that may be defective in the animal model being analyzed. Solutions to this problem include the development of wild type isogenic tagged strains [8,9], or passaging an STM or Tn-Seq library of mutants through model hosts to remove those clones that are defective for growth. However, even without this caveat, STM and Tn-Seq do not provide definitive information on bacterial replication. STM and Tn-Seq can provide relative abundance of the different clones, but not the quantitative amount of replication and death that can be provided using plasmid or fluorescence dilution. In addition, WITS, STM and Tn-Seq do not provide any spatial information about where replication is occurring, nor how different clones interact with one another during infection.

Discussion of fate of clones during bacterial infection

Using the methods discussed above, several groups have uncovered unexpected findings regarding the fate of individual bacteria during infection. We will focus on recent advances in understanding clonal fate during infection with *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, and *Salmonella enterica* serovar Typhimurium, three diverse enteric pathogens.

One of two enteropathogenic *Yersinia* species, *Y. pseudotuberculosis* immediately invades the Peyer's patches lining the intestine after oral inoculation, temporally followed by colonization of the mesenteric lymph nodes, before detection of bacteria in the liver and spleen. This led to the logical hypothesis that the Peyer's patch serves as a beachhead for bacterial entry into deeper tissue sites, and that the subsequent colonization of other internal

organs depends upon entry into this organ. Using mixed infections with individually tagged strains after oral infection of mice, it was shown that this was not the case, with colonization being more complex [9]. Bacteria colonized the liver and spleen completely independently of prior colonization of the Peyer's patch, despite the early and robust bacterial growth in the Peyer's patch. *Y. pseudotuberculosis* managed to colonize internal organs (e.g. spleen) extremely rapidly following infection, but this infection was quickly cleared. Instead, bacteria that productively established replication in the liver and spleen were descendents of bacteria that had previously replicated in the lumen of the intestine. The intestine appeared to provide conditions that allowed effective colonization of these deep tissue sites [9]. The mechanism of how *Y. pseudotuberculosis* traffics to internal organs directly from the intestine, or what transformation the bacteria undergo in the intestine to allow them to survive in internal organs after their second wave of infection, is unknown.

A recent study, examining placental colonization during oral infection with two independently tagged *Listeria monocytogenes* strains, highlighted several fascinating features of *L. monocytogenes* infection [15]. First, it appeared that infection of the placenta generally arises from a single clone (Figure 2C), a remarkably narrow bottleneck. In addition, there was extensive transport of bacteria between placental tissues and maternal organs, as evidenced by a skewing of the bacterial population in maternal organisms toward the single clone present in the placenta, and the mixing of the clonal population in the placenta with the population from the maternal organs. The number of bacteria in the bloodstream was unaffected by extracellular antibiotic treatment, indicating that these bacteria are being transported inside host cells. In order to achieve this dramatic alteration of the bacterial population in maternal organs, there must be hundreds of thousands of bacteria transiting, inside of host cells, from one organ to another throughout infection.

One of the original studies focusing on individual clones during infection was performed on *Salmonella enterica*, using plasmid dilution to measure growth vs. killing in vivo [1]. A modern improvement on this method is fluorescence dilution, which was recently used to more thoroughly quantify *Salmonella* replication in vivo [2]. Using this method, it was determined that a large percentage of the *Salmonella enterica* entered a "dormant-like" state during infection, characterized by survival without replication and resistance to β -lactam antibiotics (Figure 1C). It is unclear why so many clones survive but do not replicate *in vivo*. These dormant clones could play a role in the long-term persistence of *Salmonella* in mammalian organs, in addition to allowing increased antibiotic resistance.

Future Directions

Efforts to understand the fate of individual bacteria during infection have begun to offer fascinating glimpses of mammalian and bacterial biology, raising numerous unanswered questions. Why is it that the first wave of intestinal *Yersinia pseudotuberculosis* clones that make it to the liver do not survive, and that replication in the intestine is required for successful liver colonization? How does *Listeria monocytogenes* spread from the liver to the placenta of infected animals, and how does it return to the liver after growing exponentially in the placenta? Are the *Salmonella enterica* clones that do not replicate during the acute infection the same clones that go on to establish chronic infection? Increased study of the fate of individual bacteria during infection will greatly enhance our understanding of both the host and the pathogen, and lead us to other questions we would not have known to ask.

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Highlights

- Counting bacterial number during infection provides poor resolution of bacterial fate.
- New methods, fluorescence dilution and barcoding, allow study of individual bacteria.
- Recent advances in understanding bacterial infection using these methods.

Figure 1. Fluorescence Dilution

Bacteria are charged with fluorescent protein during growth *in vitro* (dark green rods), but upon infection the protein is no longer made. This allows dilution of the stable fluorescent protein upon each successive bacterial division (successively lighter shades of green). Shown are three possible scenarios. (A) A single clone, and its daughters, may replicate at a constant rate, resulting in a uniform population of bacteria showing low levels of fluorescence. (B) Alternatively, a subpopulation of daughters of an original clone establish a successful niche, resulting in low fluorescence in the replicating population (pale green), and high fluorescence in the slowly replicating population (intermediate level of green). (C) Finally, some clones from the original infection might not replicate but remain viable, maintaining high fluorescence over extended periods of time (dark green rods).

Figure 2. Barcoding

Multiple clones are tagged with unique barcodes that can be used to track the fate of individual clones within a mixed population. Each clone may survive and replicate identically (A), indicating a wide bottleneck of infection and equal selection on each clone. Alternatively, each clone may survive, but the infection could be dominated by one of two clones (B), indicating that the bacterial counts in a particular organ are due primarily to one or two clones that have been extremely successful. Finally, the infection could have an extremely narrow bottleneck, characterized by loss of all but one clone, followed by its rapid replication (C).