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GUEST COMMENTARY

Living in Stools Is Not as Dumb as You Think

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It has been difficult to know what might provide the best commentary for this series of articles commemorating the 100-year anniversary of the American Society for Microbiology (ASM). I have been an active medical bacteriologist for the past 46 years (or at least an ASM member for that time). Not unexpectedly, I think the most extraordinary advances in the last 100 years have taken place in understanding how bacteria cause infection and disease. I have worked on understanding *Salmonella* for a good part of the time since 1952, which was the first time in my life that I actually grew this organism from a clinical sample taken from the stools of an infected infant. Thus, I will use *Salmonella* as the touchstone for the discussion that follows.

In 1952, *Salmonella* was viewed as a bewildering group of microbes that possessed a common set of biochemical characteristics and unique cellular and flagellar antigens. The relationship of other members of the family *Enterobacteriaceae* to *Salmonella* was deduced from the presence of common *Salmonella* antigens, certain biochemical traits, like the failure to ferment lactose, and the incidence with which similar bacterial isolates were isolated from cases of diarrheal disease (5, 21). In those early days, we spent time trying to understand the distinction between the classic *Salmonella* species and a multitude of isolates with intermediate biochemical and antigenic properties. These strains were called the Paracolon bacteria, which included subgroups of microbes thought to be particularly *Salmonella*-like, including the Bethesda-Ballerup and Arizona groups. These groups of bacteria looked suspiciously biochemically and antigenically like *Salmonella* but had not yet been demonstrated unequivocally to be causative agents of human infection and disease. Also in those early days, a good deal of the time was spent in comparing collections of clinical isolates with one another in an attempt to distinguish between the virulent and avirulent isolates of the same species and to find out what might be the underlying basis for this difference in pathogenicity.

There was little thought about bacterial genetics until the end of the 1950s when bacterial conjugation and phage transduction provided the first means to look at gene exchange in pathogens. Work from Salvador Luria's lab showed that bacterial genes could be readily exchanged between members of the *Shigella* group and *Escherichia coli* K-12 (24). Transduction of genes between these two species was also effected by the bacteriophage P1 (23). In the 1960s, it was instructive to examine the loss of pathogenicity of mutant *Shigella* spp., which were created by transferring *E. coli* K-12 genes into the *Shigella* chromosome, thus sometimes disrupting the *Shigella* homolog (9). The assumption was that virulence loss in a guinea pig infection would accompany this substitution of K-12 genes for their allelic counterparts in *Shigella*. We did not have the slightest clue that *Shigella* possessed a plasmid essential for pathogenicity. Indeed, the word episome was not coined until 1959. The *Shigella* plasmid remained a mystery for another 20 years until Philippe Sansonetti, Dennis Kopecko, and Sam Formal showed that many virulence genes were on this plasmid (28, 29). Similarly, we were unaware that many so-called genetic recombinants were really merodiploids, and we made a number of other incorrect assumptions. Yet, the regions of the *Shigella* chromosome reported to be associated with virulence utilizing this technique were remarkably accurate. Like all new fields of inquiry, we were as often right in our ideas as we were wrong, but we did make progress.

A strategy similar to that employed with *Shigella* was used to identify virulence genes of *Salmonella* (8). However, *Salmonella* was less homologous to *E. coli* K-12 at the DNA level than it was to *Shigella* (1), and the apparent inheritance of large blocks of *E. coli* genes in *Salmonella* usually turned out to be unstable merodiploids. In short, we learned relatively little about the virulence traits of *Salmonella*. Moreover, our attempts to obtain *Salmonella* donor bacteria were thwarted in several ways. First, while *Salmonella typhi* Hfr donors could be isolated at that time, there was not a valid animal model in which to test potential *Salmonella* mutants for virulence. Second, the transfer of *S. typhi* genes to other *Salmonella* was of little use because of a major difference in restriction and modification of the DNA in the two mating partners. Although Helen Mäkelä and Ken Sanderson isolated both donor and recipient strains of *Salmonella abony*, these were not useful to dissect the genetics of virulence (25, 27). After several years of frustration, particularly in the 1965-to-1970 time frame, many of us gave up chasing the genetic basis of *Salmonella* virulence and focused on the technically more attractive features of plasmid-mediated drug resistance and, thanks to the pioneering efforts of H. William (Willie) Smith, plasmid-mediated virulence factors of *E. coli* (31, 32). The genetic tools available to us then were simply not refined enough to be applied to a problem as broad as bacterial pathogenicity.

Around 1980, we returned to examine the genetic and molecular bases of bacterial pathogenicity. Recombinant DNA methods and gene sequencing finally had provided the tools to focus on bacterial virulence. However, tackling facultative intracellular pathogens, like *Salmonella*, still seemed too complex to those of us in the field that was to become known as bacterial pathogenesis. (I personally dislike this term and much prefer to think that we study the biology of host-parasite relationships or simply the biology of bacterial pathogenicity.) For myself, the trepidation to take on *Salmonella* again was relieved by Ralph Isberg's successful cloning of the *Yersinia*

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pseudotuberculosis inv gene in 1985 (19) and his subsequent characterization of the invasin protein (20). It seemed that it would be possible to productively study facultative intracellular pathogens. We were not alone in this quest to be sure, but it was then confined to a relatively few laboratories around the world. In particular, several of us thought that the tools of the cell biologist would provide our best chance at understanding the factors involved in *Salmonella* virulence. Hence, cell culture models of infection were used for our genetic studies instead of depending on animal models of disease. In the 12 years that have passed, there has been a remarkable increase in the number of investigators focusing on *Salmonella* pathogenicity (4, 10–14). It is probably fair to say that in the past decade *Salmonella* has become the most studied enteric pathogen at the genetic and molecular levels. There has been an extraordinary explosion of information dealing with *Yersinia*, *Shigella*, enteropathogenic and uropathogenic *Escherichia*, *Listeria*, and mycobacterial pathogenesis as well (10, 11).

One of the most extraordinary findings has been just how often very different microorganisms use similar strategies to foil their host. Many of the enteric species possess large pathogenicity islands consisting of either a chromosome- or plasmid-mediated block of genes devoted to a type III secretion system designed to inject bacterial proteins into the host cell in response to a combination of environmental signals received from the host cell (15, 18, 22). The effector molecules injected into the host cell are remarkable in the sense that they are targeted to interfere with host cell signal transduction pathways. In many cases, the bacteria secrete pathogenicity island-encoded proteins into the host cell cytoplasm, which induce apoptosis (35), and almost universally they simultaneously subvert the host cell cytoskeletal apparatus (3) to permit the invading bacterium to subsequently multiply in the face of innate antibacterial defenses.

The bacterial pathogens *Salmonella*, *Yersinia*, and *Shigella* each cause distinguishable clinical syndromes or at least have a predilection for a particular host organ system. Enteric yersiniosis is characterized by mesenteric adenitis that resembles appendicitis. *Salmonella* elicits gastroenteritis in a wide variety of animals and birds. However, certain strains are adapted to specific mammalian species to cause a systemic infection beginning in the small bowel and eventually involving infection of the liver, spleen, and bone marrow, as well as establishing long-term intestinal shedding in many infected individuals. *Shigella* has a surprising number of pathogenicity genes (15, 18, 22) in common with the more ancient *Salmonella* and classically induces a distinct dysentery syndrome specifically in humans with inflammation of the large bowel characterized by acute diarrhea, with blood and mucus. *Shigella* also displays a higher level of transmissibility than seen in other pathogenic enteric species. Underlying these distinct clinical syndromes runs an eerie similarity in genes that teaches us that these bacteria have learned to take a similar arrangement of building blocks and fashion them by evolution to each do their thing in a different way (15, 18, 22). I think all of us in the field have been astounded by just how clever the bacteria have been to undermine the host cell signaling capabilities and the host cell cytoskeleton. I have suggested in the past that I thought this bacterial strategy is a reflection of the earliest interaction between bacteria and eukaryotic bacterial predators like amoeba and nematodes (7). Yet, it has always seemed to me that the final battle between the microbe and the host was most often not the result of a single virulence factor like a toxin but actually overlapping and redundant factors designed to overwhelm some facet of host defense. Similarly, it seemed to me that the mammalian immune system, particularly the innate immune system, possessed a wealth of different factors designed to thwart invading bacteria. Freedom from microbial infection must be a prime selective feature of the evolution of this branch of our immune system. Similarly, the evolution of bacterial virulence factors was no less driven by an increasing sophistication of host immunity.

Genetically altered transgenic and knockout mice have permitted the examination of particular host determinants in immunity to infection. Some of these findings have been surprising and almost counterintuitive from the way we have been taught to view antibacterial immunity. Hence, the recent discovery that caspase-1-deficient mice are resistant to *Salmonella* infection (17; D. Monack, D. Hersh, N. Ghori, A. Zychlinsky, and S. Falkow, unpublished data) helped us understand that bacterium-induced apoptosis and perhaps the induction of certain proinflammatory cytokines, IL-1β and IL-18, are absolutely essential for *Salmonella* to produce a successful infection after oral challenge; it is the key to getting through the initial mucosal defenses and apparently for spreading to adjacent tissue. One is perhaps not surprised to see that a bacterium mutant in inducing apoptosis is avirulent by oral challenge. Yet, such bacteria are still fully virulent if injected intraperitoneally in either a conventional or caspase-1 knockout mouse. Moreover, they enter the Peyer's patch of caspase-1-deficient mice in a way that is initially indistinguishable from that of wild-type bacteria. One might have thought then that once the bacteria breached the mucosal surface to enter the Peyer's patch, there were multiple facets of both host defense and bacterial virulence that might come into play. This is likely the case. However, we see that the absence of a single key host cell enzyme target of a single bacterial virulence gene is sufficient to thwart the microbe's advantage, suggesting that the interplay between the pathogen and host reflects a complex cascade of events, the order of which may be crucial. Clearly, caspase-1 exists in the inflammatory cascade because it plays a role in the inflammatory response. The fact that caspase-1 has not disappeared during evolution must mean that the selective advantage of being resistant to certain classes of bacterial infection must not be of sufficient selective advantage to mute its expression. The initial conclusion that can be drawn from these studies is that the ingestion by phagocytic cells inhabiting the Peyer's patch is the key to escaping oral infection in the caspase-1 knockout mice. Even so, it seems remarkable that the invading *Salmonella* does not simply avoid phagocytosis by entering the more numerous cell populations within the Peyer's patches, such as B and T lymphocytes or the adjacent epithelium. The alternative explanation is that it is not only the capacity of the macrophages to escape apoptosis that is key for the host to prevail in the face of a bacterial onslaught but that the inflammatory cascade induced by *Salmonella* is a key determinant for the invading microbes to reach an intracellular haven in another host cell component. In the absence of this inflammation, *Salmonella* is limited to the gastrointestinal tract. It appears that the bacteria actually use the inflammatory response of the host for spreading to adjacent lymph nodes and eventually to the spleen and liver. *Salmonella* also possesses a strategy to gain access to the blood stream and disseminate to the liver and spleen of mice even in the absence of a Peyer's patch or a functional caspase-1 gene. Here again the bacterium undermines a specific host cell type, those bearing the cell surface marker CD-18 (34). While it seems likely that gastrointestinal infection is the usual portal of entry, this alternative pathway is also operative and it is noteworthy that CD-18 knockout mice are resistant to salmonellosis as well. Thus, the cells of a host's innate immune system play a major role in disseminating *Salmonella* in a host, and it seems that this bac-

terium not only survives an inflammatory host response but, indeed, requires it to establish a successful infection.

Similarly, *Shigella* is thought to induce inflammation through the release of proinflammatory cytokines, which orchestrate the migration of neutrophils from the circulation into the lamina propria (26, 35). It is thought that the neutrophils cause the breakdown in epithelial integrity, allowing for greater numbers of bacteria in the lumen to enter the tissue. The *Shigella* gene IpaB, which also induces apoptosis in macrophages, and the *Salmonella* gene SipB are very close homologs and have the same activity on caspase-1 (35). Yet *Salmonella* has at least one further layer of complexity in its host cell interaction. It induces another complete set of virulence genes in a separate pathogenicity island, SpiII (2, 16, 30), which provides the bacteria with the capacity to invade the adjacent lymph nodes and reside in the liver and spleen, which often, at least in adult animals, leads to long-term, chronic infection and bacterial shedding.

Anti-infective strategies have usually been aimed at killing the invading microbe. Initial attempts at altering the immune response of infected patients using anticytokine therapy to alter the course of bacterial sepsis have been disappointing if not counterproductive to the patient's well-being. However, further, more precise dissection of arms of the host defense cascades, as well as our more precise understanding of the biology of bacterial pathogenesis, might indeed someday lead to the development of immune modulators that do influence the outcome of the infectious process in favor of the infected host.

Bacterial pathogenicity can be dissected by a sort of molecular Koch's postulates (6), and we have discovered that redundant or not, certain virulence factors are essential for pathogenicity. Of course, we are still faced with the problem of just what exactly pathogenicity means. To some individuals, if an organism devoid of a particular virulence trait does not kill a susceptible host, the trait is an essential virulence trait. Other investigators would take the view that if a mutant derivative cannot effectively compete with wild-type pathogens of the same strain, then the mutated trait is essential. In bygone days, I recall that the definition of an essential virulence trait was whether or not antibodies that were induced against a particular bacterial factor were protective. So long as an investigator clearly defines the parameters by which pathogenicity or virulence is measured and defined, a universal definition probably doesn't even matter at this point in time. The critical issue is only whether further experiments can be defined to illuminate the role of a factor in the biology of the microbe under study.

The way we work with and think about pathogenic bacteria is about to change completely with the advent of genomics and the attendant informatics (33). Bacteriologists are the fortunate first beneficiaries of this new technology. Most of the genomes of the most important pathogenic bacteria will be available over the next few years. The chromosomes are small enough so that a complete representation of the full genome can be accommodated on a single glass slide. The Southern blots of tomorrow will be the global comparison of entire genomes at the nucleotide level. With time, and not too long a time at that, we will be able to examine global bacterial gene expression in infected animals and even in infected-patient material. We won't have to guess what gene cascades are being expressed over time in the bacterial pursuit of multiplication and transmissibility. What is even more exciting, in a way, the same samples we extract from infected animals and tissue can be used to probe for the expression of at least a representative group of host genes. Perhaps the most powerful tool we shall initially employ is the comparison of wild-type and mutant bacterial infection in a susceptible host and the host response pattern to each. We will be inundated with data, and it will be impossible to analyze all of it for a very long time. Web sites will be posted with raw experimental data for the taking. It will be a bonanza for investigators around the world in big and small institutions, who can quietly, maybe even leisurely, mine information from their computers and test experimental ideas "in silico." I find it the most exciting time in my scientific career, although I confess to having said this at other times in my life as well. I hope that this continues to be the case for me (as well as you) in the new millennium.

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