I never met a microbe I didn't like

Stanley Falkow

At the age of 11, I read Paul de Kruif's *Microbe Hunters*, which dramatized the discovery of bacteria and viruses and their roles in human disease. The heroes of *Microbe Hunters*—Louis Pasteur, Robert Koch and others—became my heroes, and I dreamed of becoming a bacteriologist, doing research on the bacteria that cause disease. I was lucky enough to fulfill my boyhood dream; however, I could never have imagined the path I eventually followed, or how much my views of microbes and disease would change (and continue to do so) in the process. Of course, I did not make this journey alone. During the five decades I worked as an active scientist, I helped train over 100 graduate students, postdoctoral fellows and clinical fellows, and collaborated with 75 other scientists (**Fig. 1**). Each of us, in our own way, wondered, "What is a pathogen?"

Figure 1 Lab alumni reunion in 2004, Falkow/Tompkins home, Hamilton, Montana, USA. Photo courtesy of Manuel Amieva.

Entering the genetic and molecular world

I was a hospital bacteriologist and an autopsy diener before I became a graduate student. Thus, I learned about the world of microbes from a practical standpoint before I learned the tools to perform research. The medical bacteriology of the 1950s focused on differentiating the 'good guys' from the 'bad guys', and a pathogen was simply defined as any organism that caused disease. In basic bacteriology I was taught that bacteria were Schizomycetes—'asexual primitive plants'. So, it was hard to think of them as being inherently virulent.

When I entered Brown University as a graduate student in 1957, I pestered my professors asking what they thought made pathogens different from non-pathogens. Professor C. A. ('Doc') Stuart encouraged me to learn genetics as a foundation for answering the question. Professor Herman Chase, a mouse geneticist, thought about my question and announced that he had just the book to start me on my voyage, the just-published compilation *The Chemical Basis of Heredity*[1]. In this volume, I learned for the first time about the structure of DNA and embarked on my research during the beginning of what Salvatore Luria called the "Golden Age of Molecular Biology." At the time, bacterial genetics was basically restricted to *Escherichia coli* K-12.

Shortly after I became a student, Lou Baron at the Walter Reed Army Institute of Research described for the first time the transfer of genetic information from *E. coli* to *Salmonella typhi*. I was anxious to use the tools of genetics and molecular biology to establish the specific genes that defined the difference between pathogens and non-pathogens. There were, after all, *E. coli* that were clearly part of the normal flora and *E. coli* that caused diarrhea in infants. So, I immediately contacted Baron, who supplied me with the necessary cultures to study this problem.

I performed conjugation experiments using *E. coli* K-12 donors and clinical isolates of *E. coli* and *Salmonella*. I was excited when I could actually detect *E. coli* surface antigens in *Salmonella*. However, my conjugation experiments revealed little about pathogenicity. I was unable to transfer any gene from *Salmonella* or *Shigella* that altered the measurable pathogenicity or host range of another *Salmonella* species, or that made *E. coli* K-12 pathogenic[2,3].

When I presented these results at a Cold Spring Harbor meeting in 1963, the opinion was almost unanimous that I was wasting my time and ought to be concentrating on more important biological questions. Indeed, at that time there was a growing consensus that infectious diseases were no longer of interest for Western society. So, at age 30, I put my dream of doing research on the meaning of bacterial pathogenicity to rest for a time and devoted my energy to examining the molecular nature of extrachromosomal elements—now called plasmids—with help from Julius Marmur (then at Brandeis University)[4]. I focused especially on R plasmids, as these mediators of antibiotic resistance were of clear medical significance[5].

Plasmids and pathogenicity

I discovered the joys of teaching in 1967 and moved to Georgetown University. The following year I met H. Williams Smith at a meeting in London. He was a veterinarian who had used basic bacterial mating experiments to show that diarrhea in pigs and calves depended on

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Figure 2 Photo taken on 25 May 1967 during my lecture at the Symposium on Infectious Multiple Drug Resistance, held at the Georgetown University School of Medicine with support from the US Food and Drug Administration. In the front row are, left to right, Arthur K. Saz (lighting his pipe), Piet A. Guinée, Naomi Datta, David H. Smith (a Lasker Award Winner) and Tsutomu (Tom) Watanabe. The last four were instrumental in discovering R plasmids and demonstrating their significance in clinical medicine.

E. coli strains that possessed two plasmids, one encoding one or two enterotoxins, and a second encoding an adherence factor that specifically recognized the epithelial cells of the small bowel[6]. Willie asked me if my students and I could use our molecular methods to examine these virulence genes. By now we had become adept at isolating plasmids from bacteria on the basis of their circularity, and I was actively engaged in using DNA hybridization to examine the relationships among plasmids.

Smith's work became the foundation for looking at whether certain *E. coli* from humans possessed a similar plasmid arsenal and caused traveler's diarrhea. Our laboratory showed that the plasmids encoding enterotoxins from pigs had closely related counterparts in humans. Indeed, they were related to the classic F factor of Joshua Lederberg and to certain R plasmids[7]. Working with Naomi Datta and Bob Hedges, we discovered that there were many distinct groups of R plasmids (**Fig. 2**)[8]. Among some of them, the antibiotic-resistance genes seemed to have a common source, but the replication machinery, the restriction-modification loci, and the proteins that permit transmission of DNA from a donor to a recipient were different. Yet plasmids from a single group could carry antibiotic resistance or have one or more enterotoxin or adherence genes. It was as if different gene cassettes could be inserted or taken out of the same plasmid. It seemed likely that these extrachromosomal elements were an important part of bacterial evolution, including the evolution of pathogenicity.

Recombinant DNA, gene transposition and a return to understanding pathogenicity

In June 1972 I moved to the University of Washington in Seattle, where I would have a more active role in teaching medical microbiology and directly participating in research on infectious diseases. Soon after moving, I attended a joint meeting of US and Japanese plasmid researchers in Hawaii (**Fig. 3**).

A major focus of the meeting was to gain an understanding of how R plasmids acquired resistance genes and whether R plasmids were natural co-integrates of distinct replicons. A concurrence of results on the origin of R plasmid resistance genes discussed one evening in the unlikely setting of a Waikiki kosher delicatessen led to the idea of joining and splicing DNA using restriction enzymes. My only contribution was as a witness, occasional commentator and donor of replicon RSF1010, known to have a single *Eco*R1 cleavage site, for the first pilot cloning experiments performed by Stanley Cohen, Herb Boyer and their co-workers[9]. However, I was aware of the implications of the work. Indeed, I extracted from Herb and Stan the promise that, if the experiment succeeded and gene isolation and amplification became a reality, I would send one of my graduate students to Herb's lab to pursue the idea. It worked, and we cloned the first virulence determinant of bacteria—the *E. coli* heat-stable enterotoxin—in my laboratory[10].

The implications of recombinant DNA technology were enormous, of course. Because of my training in medical microbiology, their impact on me was not just scientific. Indeed, I participated in the historic Asilomar meeting on the societal impact of recombinant DNA. I served on the first I served on the first US National Institutes of Health Recombinant DNA Advisory Committee, established in 1974 in response to public concerns regarding the public health and safety issues of manipulating genetic material using recombinant DNA techniques and the potential ethical and social implications of the research. The committee was initially charged with drafting guidelines governing the safe conduct of recombinant DNA research by outlining appropriate biosafety practices and containment measures. These guidelines, now known as the NIH Guidelines for Research Involving Recombinant DNA Molecules, were first published in 1976 and have evolved over time to include other aspects of gene manipulation, including genetic therapy. This was a time-consuming and difficult task, and it was not

Figure 3 The US–Japan plasmid meeting in Honolulu, Hawaii, November 1972. Close-up from a picture taken the day after the meeting in the kosher deli, where we discussed the experiment that led to gene splicing. Left to right: Stanley Falkow, Robert H. Rownd, Herbert W. Boyer, Stanley N. Cohen, Toshihiko Arai, Charles C. Brinton Jr., Richard P. Novick (partly hidden) and an unidentified Japanese scientist.

helpful to hear more than one scientist complain that they had always poured their *E. coli* cultures down the drain and "why is it now a big deal?" It annoyed many scientists that there were any restrictions concerning recombinant DNA experiments that they deemed to be harmless. Yet when scientists and physicians participate in experiments that may have an impact on society, society has the right of informed consent.

Much of our subsequent research was concerned with the application of genetic and molecular tools, which now included recombinant DNA, to the study of infectious diseases. Our laboratory and others discovered that the antibiotic resistance genes of R plasmids were transposable genetic elements, the 'jumping genes' envisioned by Barbara McClintock decades earlier[11].

As happens in science, there was a juxtaposition of what we learned about gene transposition and the sudden appearance of R plasmids in *Haemophilus influenzae* and the gonococcus[12,13]. We could say with reasonable confidence that the penicillin-resistance genes found in gonococci and *E. coli* isolated from patients had a common ancestor, and transposition of tetracycline, chloramphenicol and ampicillin antibiotic-resistance genes from enteric species into commensal *Haemophilus* was the harbinger of the appearance of the same resistance in pathogenic strains of *Haemophilus* that cause meningitis. The agarose gel electrophoresis methods that we applied to help construct the famous cloning vector pBR322 was applied to characterize plasmids from clinical specimens[14]: the first plasmid fingerprints—and what has come to be known as molecular epidemiology—was born[15,16]. A few years later we could also show that a DNA sequence from a specific or unique virulence gene could be used for epidemiological investigations and even for pathogen identification[17].

The work on plasmid enterotoxins was intriguing, and as I talked to those in the bacterial toxin field, I began to realize that the dinucleotide-ribosylating enzymes secreted by the *E. coli* enterotoxin-producing strains resembled the toxins secreted by *Vibrio cholerae*, the diphtheria bacillus and *Bordetella pertussis* (the agent of whooping cough), and were similar in function to the large heterotrimeric G proteins of mammals[18]. It seemed to me that microbes weren't poisoning us as much as they were undermining and subverting the normal function of animal cells for their own survival. Classical microbiologists often viewed toxins as potential protective antigens that might be used in vaccines. Clinicians viewed toxins as the causative factors of disease. I was interested in these facets, but thought that the toxin had to be understood both in terms of the biology of pathogenesis and of the utility of the toxin for bacterial survival, persistence and transmission: what's in it for the bug?

I decided that we now had the tools to revisit my initial dream of understanding the biology of pathogenicity. So, after a sabbatical leave in England, I returned to the University of Washington in 1978 and recruited a cadre of students with the idea to examine bacterial pathogenicity at the genetic and molecular level. Perhaps because of my early background in medical microbiology, I did not attempt to focus the laboratory on a particular pathogen, but worked on many: gonococcus[19], *Bordetella pertussis*[20], and the plague bacillus[21]. The research ranged from clinical studies to molecular epidemiology of nosocomial infection, but the major focus was on the basic structure and function of virulence genes and their regulation. A clinical investigation into the phenotypic differences between commensal *E. coli* and clinical isolates from urinary tract infection led to the cloning of suspected determinants of pathogenicity[22,23], although we argued a great deal about what exactly constituted a 'virulence gene'.

What is a pathogen?

I moved to Stanford University in the summer of 1981. I spent my first years there isolating and defining bacterial determinants that we believed to be associated with pathogenicity. I decided that one way to define virulence genes was to apply some sort of 'molecular Koch's postulates': the specific inactivation of the genes suspected to be associated with virulence should lead to a measurable loss in pathogenicity, and reversion or allelic replacement of those genes should lead to restoration of virulence[24]. It was an inadequate test, but if we were to employ it at all, we needed a quantitative way to measure virulence. We could do animal challenge experiments, but death is a harsh end point. Instead, we learned cell culture and adopted the methods of cell biologists. We could now use different aspects of cell injury and cell death instead of host death. Similarly, we examined microbial numbers at different sites in a host as a measure of invasiveness. Our focus shifted from looking at the microbe only to looking at the microbe–host interaction and at the consequences for both parties[25,26].

In 1987, we began to define what Catherina Svanborg, one of our collaborators, called a "pathogenic personality"[27] (**Box 1**). The more we studied pathogens, the more it became apparent that there were common themes of bacterial pathogenicity[28]. As we studied these themes, we started learning as much about the microbe as about the host. In 1987, we knew that mobile genetic elements—plasmids, bacteriophages and transposons—had been central factors in the evolution of pathogenic traits. Today, genomic analysis has revealed that a wide range of bacteria, including plant pathogens and obligate intracellular parasites such as *Chlamydia* and *Rickettsia*, have related blocks of genes that distinguish them from their related commensal and nonpathogenic brethren. These blocks of genes are ordinarily found as contiguous, large DNA chromosomal insertions called pathogenicity islands[29]. They seem to have been transmitted by horizontal gene transfer, as if the island DNA once resided in a microbe distantly related to that in which it now is found. In many cases, these gene blocks encode a specialized secretory pathway designed to dispense specific effector virulence proteins to the bacterial surface or through a protein structure into the host membrane and cytoplasm. For pathogens such as *Salmonella*, the proteinaceous delivery appendage viewed in the microscope looks quite like a hypoder-

Box 1 Attributes shared by bacterial pathogens

- Entry into the host. Entry is not a random event but has selectively evolved to exploit the host's needs to breathe, eat, see, hear, eliminate waste and reproduce.
- Attainment of a unique niche. All pathogens have evolved a specific means of association with at least one unique host cellular target shortly after entry. The specificity of this molecular interaction may dictate the host–pathogen interaction for hours or even days afterwards.
- The pathogenic signature. Pathogens avoid, circumvent, destroy or manipulate one or more essential host defenses.
- Multiplication. The definitive goal of the pathogenic strategy is to produce sufficient number of cells to persist in the host or to be transmitted to a new host.
- Exit from the host. It is likely that microbes have specialized determinants for leaving their host, preparing for subsequent entry in a new host.
- Limited host range and the inherent ability to cross anatomical barriers and/or breach other host defenses to establish themselves in areas usually devoid of other microorganisms. This property is essential for their survival in nature.

mic syringe and needle. Pathogens know their cell biology! Depending on the pathogen, the bacterial effector proteins delivered to the host cell can lead to actin rearrangement (**Fig. 4**), to the covalent modification of signaling molecules, or to the induction of apoptosis[30].

If there is satisfaction with the relatively rapid discovery of these common themes, we must also be cautious, as we seem perilously close to a situation in which molecular sequencing directs the biologist, instead of the biologist directing the sequencing[31]. Yet the molecular fossil record in the DNA of contemporary microorganisms has revealed extraordinary information. It suggests to me that pathogenicity is an ancient, honorable microbial profession that has, at its roots, the requirement for microbes to defeat their predators—protozoa, nematodes and other organisms that use microbes as their main food source. The microbes that infect us inherited these principles, and evolution has finely honed them not just to help bacteria avoid predation, but to take advantage of larger organisms for their own survival.

Still … what is a pathogen?

The complete genome sequence of virtually every important human and animal pathogen is at hand. The complete sequence of their hosts is also at hand. Complete gene arrays allow us to look at gene transcription and genetic variability in both the host and the pathogen. Yet, I still struggle with the question, "What is a pathogen?" Those who are concerned with infectious diseases must adhere to the concept that any microbe that causes disease is a pathogen. It matters not whether an accidental or deliberate exposure to bacteria leads to disease. Moreover, I have been fond of invoking Walt Kelley's *Pogo* and declaring, "We have met the enemy and he is us!" Human behavior has led to health crises such as Legionnaire's disease, toxic shock syndrome and an increase in food-borne disease caused by international food-distribution networks[32]. It seems to me that a better term for many emerging infectious diseases would be 'diseases of human progress'.

Humans live with hundreds of commensal species that reside in every inhabitable nook and cranny and are present for our entire lives but cause no harm. These commensal species have become a focus of increasing interest. In sheer number of cells they predominate in a human by a factor of 10 (ref. 33). Microbiologists are uncomfortably aware that most species that inhabit us remain unknown, except for a snippet of sequence that reveals their presence. To what extent these members of our bacterial flora influence our health and disease is still an open question[34].

The distinction between commensals and pathogens can be blurred at times because some commensals cause disease, albeit usually in immunocompromised hosts. Some microbes could indeed be called 'commensal pathogens'[35]. For example, pneumococci and meningococci regularly inhabit the human nasopharynx and are mostly carried asymptomatically, although they can cause life-threatening diseases. Immunization against these microbes not only protects against disease, but also prevents their ability to colonize the host. From the bacterial viewpoint, the production of a toxic protein might be seen more precisely as colonization factors than as virulence factors. Are these organisms simply normal flora that evolved to live in a perilous location, where they face less competition but pay for it by coming into contact with deadly elements of the immune system? A number of the most frightening human pathogens, such as *Mycobacterium tuberculosis*, the typhoid bacillus and *Helicobacter pylori*, cause disease only in a relatively small number of people and can often persist asymptomatically for a lifetime[36]. Might we say that organisms such as *Mycobacterium* and *H. pylori*, which have been with humans from the beginning, can be considered indigenous flora?

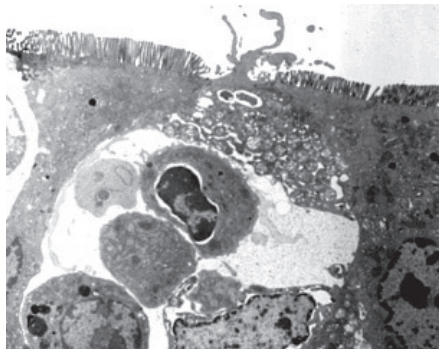

Figure 4 Electron micrograph of *Salmonella typhimurium* entering into an M cell in the mouse intestine. The organism causes 'ruffling' of the cell surface to gain entry. Underneath the breached epithelial cell are cellular elements of the immune system, including macrophages and dendritic cells, which can take up the invading *Salmonella*. This particular photograph, which I took in 1988, was important because it showed that the phenomena we saw in cultured cells had their counterparts during natural infection.

After 50 years of study, I concede that there is no simple definition that accounts for what a pathogen is. It is important to have a medical definition of a pathogen and its relation to disease. On the other hand, I would argue that disease does not encompass all of the biological aspects of pathogenicity and of the evolution of the host–parasite relationship. For example, CagA, a protein of *H. pylori* delivered by the microorganism to host cells probably as a means to loosen epithelial tight junctions and gain nutrients, can cause gastric cancer over decades in the right setting of diet and host genetic determinants[37,38]. Good riddance to *H. pylori* by antibiotic therapy, immunization or increased sanitation! But the disappearance of *H. pylori* from human flora may have the equally important effect of predisposing humans to esophageal cancer, asthma and other diseases[39]. The biology is more complex than we realized.

It really doesn't matter how we define a pathogen! To underestimate the evolutionary potential of microorganisms and their ability to survive, even in the face of enormous pressure to eradicate them, would be a mistake. Infectious agents will emerge as long as there are microorganisms. Humans help the evolutionary process, sometimes unwittingly, and sometimes by arrogance or ignorance. Fortunately, humans have evolved in wondrous ways to avoid and repel microbial incursion. Our immune system, both innate and adaptive, is a tribute to Nature's skill in doing so, but I confess the belief that microbes will always have the last laugh (**Box 2**).

Box 2 Microbes may be smarter than you think

- They understand mathematics. They have mastered exponential equations and understand biostatistics.
- They understand physics. They know that a small amount of energy applied to the right point can 'move' a large object.
- They understand military tactics. They strike quickly with overwhelming numbers, cut the lines of communication and wear camouflage.
- They are expert biologists. They have studied biology longer than any other living thing, understood Darwin before he did, and also invented neo-Darwinism. They have mastered genetics, cell biology and immunology.
- They always have the last laugh. They are generally the first living things we encounter after birth and, when we die, they are the last living cells on our bodies. Then, they devour us.

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I wish to thank S. Fisher and L.S. Tompkins for reading and commenting on this manuscript.

I was asked to write a commentary in conjunction with my selection to receive the Lasker~Koshland award. In science, the operative word is more often 'we' instead of 'I'. Indeed, I was fortunate to have worked with so many talented and genuinely nice people during my career. The publication of this article happens to coincide with the publication of an autobiographical sketch *The Fortunate Professor*[40], which I dedicated to my mentors, former students and colleagues. In that article, I wrote that my professional life could be summarized simply by a statement from the Talmud:

> I learned much from my parents.
> I learned more from my teachers.
> I learned even more from my colleagues.
> But I learned the most from my students.

Upon further reflection, I should add that all of us can learn a lot from the microbes as well.

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