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Perspective

Invasion and Intracellular Sorting of Bacteria: Searching for Bacterial Genes Expressed During Host/Pathogen Interactions

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Defining pathogenicity

A pathogen has the ability to access a privileged niche within a host. Pathogens often cause direct or indirect host cell damage to gain access to this niche, and most pathogenic species have characteristic attributes to interact with, or stay in very close proximity to, a precise cell type. Commensals and opportunists cause infections as well if they are introduced into a privileged site or if one or more of the normal host defense barriers are breached. The key distinction is that the pathogen, through evolution, has gained the inherent capacity to breach host cell barriers, while commensal species and opportunists ordinarily cannot do so (1).

If pathogenic bacteria have distinctive properties that separate them from commensal species, what is the origin of these differences? It has been known that many of the essential determinants of bacterial pathogenicity are found on mobile genetic elements like bacteriophages and plasmids. However, one of the most striking recent findings about the nature of bacterial virulence has been the discovery that many pathogens have large inserts of DNA called pathogenicity islands (Pais)[1] that are not present in nonpathogenic microorganisms (2–4; Table I). For example, uropathogenic *Escherichia coli* and enteropathogenic *E. coli* possess large DNA inserts ranging in size from 35 to 170 kb, which include a number of virulence genes that are absent from commensal *E. coli* strains. While these inserts are clearly different loci in size and in the precise determinants that they encode, there are several striking similarities. Some of the genes found on Pais and some virulence plasmids have a striking degree of homology (5). In particular, many of the sequences are part of a unique secretion system (type III) that is thought to be triggered directly or indirectly by contact with host cells to deliver effector molecules to the host cell surface where they act to influence host cell function and signaling (2, 5). The DNA composition of the Pais often differ from the majority of genes found on the rest of the bacterial chromosome. Pais often are bound by unique DNA sequences, such as direct repeats or insertion sequences (IS). In some species, this is reflected by the tendency of the Pais to delete with high frequency. The site of insertion of Pais often is associated with a t-RNA locus, which has been known to be a site of bacteriophage insertion. Similarly, Pais sequences often contain homologs to bacteriophage attachment sites, transposon sequences, plasmid replication origins, or IS elements. Such findings suggest that Pais originated as genetic elements that were able to spread among bacterial populations by horizontal gene transfer.

Why is pathogenicity so uncommon relative to the myriad of microorganisms that we encounter and harbor on or within us? The likely answer is that bacterial populations tend to be clonal in nature (1). Thus, distinct bacterial clones are often responsible for disease outbreaks and increases in infection frequency. Most of these clones possess distinct combinations of genes. Thus, the inheritance of a Pais containing even a number of potential virulence traits does not necessarily create a new pathogenic species. Rather, the analysis of pathogenic bacterial populations suggests that certain unique combinations of virulence genes may arise only once during evolution. Allelic variants of the same pathogenic species (like the plethora of *Salmonella* serotypes) will still appear; however, only rarely, if ever, will a pathogen bestow its particular constellation of virulence attributes horizontally to other lineages of related species or to other microorganisms.

All pathogens must enter a host; find their unique niche; avoid, circumvent, or subvert normal host defenses; multiply; and eventually be transmitted to a new susceptible host. Although certain common pathogenic tactics have come to be appreciated, each microbe has a unique "pathogenic signature" that permits survival and leads to their freedom to multiply. As already reviewed in this series on host/pathogen interactions, bacterial pathogens encounter a number of differing environments during infection reflecting both extracellular and intracellular environments they may encounter during the infectious process (6). The appropriate network of virulence genes are expressed in response to these environmental stimuli. Yet, pathogens probably do not respond any differently than other bacterial species to changes in oxygen, ion concentration, pH, etc. Many of the regulatory cascades that control bacterial virulence likewise regulate genes that are not directly associated with pathogenicity or at least are not required for pathogenicity. The coordination of virulence nonetheless is exquisitely coordinated; the expression of the same virulence genes at the wrong time during the infection cycle could have devastating consequences for the microorganism. Presumably, the microbe can assess the difference between housekeeping chores and the requirement for expression of pathogenicity by

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1. *Abbreviations used in this paper:* DFI, differential fluorescence induction; GFP, green fluorescent protein; IS, insertion sequence; IVET, in vivo expression technology; Pais, pathogenicity islands; STM, signature-tagged mutagenesis.

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Table I. Characteristics of Several Pathogenicity Islands Found in Enteric Bacteria

Organism	Name	Location	Borders	Stable?	Evidence of foreign origin G+C: % island/ % chromosome	Functions	Size
Uropathogenic *E. coli* J96	Pai I	near *pheV**, 64′			•Absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin I •pap (fimbriae: adherence to host cells) •IS element sequences •R plasmid sequences •P4 phage sequences	> 170 kb
	Pai II	*pheR**, 94′	135-bp imperfect direct repeats	No	•Direct repeats •Absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin II •prs (fimbriae: adherence to host cells) •cytotoxic necrotizing factor type 1 •IS element sequences •P4 phage sequences •OmpR homolog	106 kb
Enteropathogenic *E. coli* (EPEC)	Locus of enterocyte effacement, LEE	*selC**, 82′	No repeats or IS elements found	Yes‡	•G+C: 39%/51% •Not present in closely related, non–AE-producing bacteria	•Mediates formation of AE lesions •Type III secretion system	35 kb
S. typhimurium	*Salmonella* pathogenicity island 1, SPI 1	Between *fhl* and *mutS,* 63′	No repeats or IS elements found in *S. typhimurium*; IS3 on one border in certain *Salmonella* serotypes	Yes‡; unstable in certain serotypes	•G+C: 42%/52% •Absent from *E. coli*	•Invasion into cultured epithelial cells •Type III secretion system	40 kb
	SPI 2	Between *ydhE* and *pykF*, 31′		Yes‡	•G+C: 45%/52% •Absent from *E. coli*, conserved among *Salmonella*	•Type III secretion system	40 kb

*tRNA gene; ‡apparently.

integrating a number of host signals simultaneously. For example, for *Salmonella* invasion to occur, the organism must simultaneously sense the proper levels of oxygen, pH, the right osmolarity, and the appropriate level of Mg^{2+} to the PhoP/Q regulon (6, 7). If even a single condition is unfavorable, microbial entry into cells and their subsequent replication is repressed.

Understanding this complex interplay between pathogen and host will help us determine the biological foundation of pathogenicity and the differences between pathogens and other bacteria. One formidable experimental barrier to understanding this interplay is that we have been dependent largely on in vitro methods or, at best, cell culture models to identify and measure genes that are important in pathogenicity. Not all genes are expressed in culture, nor does the tissue culture flask act as a surrogate for an entire animal. How can we identify microbial factors that are essential for pathogenicity that are temporally expressed during infection or are confined to a particular host cell or organ system?

Probing the intracellular environment

A number of pathogens like *Salmonella*, *Mycobacterium spp.*, *Legionella pneumophila*, and *Francisella tularensis* survive and replicate within phagocytic cells, which are part of the host's antimicrobial defense system (8, 9). Determining how these microorganisms avoid the phagolysosomal environment has been the object of considerable study. Organisms like *Francisella* actually enter the dangerous phagolysosomal environment and seem to dismiss the antimicrobial elements normally found in this cellular compartment. Other pathogens appear to selectively redirect or exploit the host cell's intracellular trafficking to avoid its killing mechanisms. *Salmonella typhimurium* provides a useful example of the information that has been gained through the investigation of bacterial invasion of cultured cells (10). *S. typhimurium* entry into both epithelial cells and macrophages, presumably through a host cell pathway that activates CDC-42, is associated with marked cellular ruffling and macropinocytosis (11–13). Invading *Salmonella* do not behave identically in epithelial cells and macrophages. The

most notable difference is that *Salmonella* induces apoptosis in macrophages, particularly those macrophages that have been activated (14, 15). The bacterial genes essential for bacterial entry and their capacity to induce apoptosis are encoded within a single pathogenicity island called SpiI. Most intracellular bacteria are subsequently found in an acidic (pH 4.5) compartment (16); this low pH appears to be essential for subsequent *Salmonella* survival and persistence in the intracellular environment. Confocal microscopy studies on intracellular *Salmonella*, both in epithelial cells and in macrophages, suggest that this organism directs the selective fusion with components of the exocytic pathway, as well as the endosomal pathway (17–19); however, there is no apparent fusion with cellular compartments containing mature lysosomes.

We are beginning to comprehend the broad outline of the cell biology of infection. Yet, most bacterial genes that are operative during the intracellular phase of growth remain unknown. We can expect that intracellular life leads to the transcription of a wide spectrum of genes necessary for metabolic adaptation of the bacterium to the host vacuole environment, protection from host cell killing mechanisms, and fusion with selected alternative host cellular pathways usually devoid of overt antimicrobial factors. We expect that many of these genes will act cooperatively, even synergistically, and other genes may have redundant functions. Thus, as we investigate the intracellular environment and begin to follow microorganisms within infected animals, we need to be able to identify genes that are induced, even transiently, in the intracellular environment. It is essential to devise ways to measure gene activity and their regulation outside of the laboratory flask. Finally, we must be able to determine the importance of gene sequences that are active in the host environment to the pathogenesis of infection.

Several new approaches are available to detect gene sequences that are differentially expressed within the host cell environment. In an age of bacterial genomics, these methods have the advantage of providing both sequence and functional information about genes of interest.

Molecular methods of detection. Bacterial gene expression within host cells can be monitored directly by identifying differentially expressed mRNA transcripts. In its simplest form, RNA is isolated from intracellularly grown bacteria and a reverse transcriptase is used to synthesize bacterial cDNA, which is used as a template for PCR with sets of primers specific for gene(s) of interest. The PCR products are compared with similar reactions prepared from laboratory grown cells (or cells grown under any desired, defined conditions). In this way it has been possible to identify differentially expressed genes in macrophage-grown *L. pneumophila* and in uropathogenic *E. coli* after attachment to cells (20, 21). Since the complete DNA sequence of many microbial pathogens is or will be available shortly, it will be possible to screen directly mass arrays of genes, together with the primers known for every open reading frame, to learn if a particular gene is expressed under a given condition. However, the identification of a particular gene of interest, even under defined conditions, does not necessarily reveal gene function, nor does it reveal the temporal or spacial timing of expression. Also, we cannot expect that the costly reagents needed to perform such heroic kinds of research will be widely available to most experimentalists in the near future.

Genetic methods to search for microbial genes important in pathogenicity. In vivo expression technology (IVET) was the first practical strategy described for selecting bacterial genes expressed preferentially during infection of an animal host (22–24). Using IVET, the genes are detected because they exhibit relatively elevated levels of expression in host tissues or in surrogate cell culture infection models, but they are poorly expressed on laboratory media. A recent variant of IVET used random DNA fragments of *Salmonella* fused to a tandem-reporter system of β-galactosidase (*lac*Z) and chloramphenicol acetyltransferase (Cat) (25). In this system, bacteria are isolated that are Lac^- under laboratory conditions but are chloramphenicol resistant when present inside macrophages. The *in vivo–induced* (*ivi*) genes identified by this method are similar to adhesins and invasins from prokaryotic and eukaryotic pathogens. The examination of the *ivi* genes suggests that many encode regulatory functions, that the host ecology can be inferred from the biochemical functions of the genes (for example Mg^{2+} and Cu^{2+} uptake), and that nutrient limitation plays a dual role in inducing functions to correct the nutritional deficiency and to signal bacterial pathways needed for bacterial survival and transmission. One current limitation of the IVET method is that it is relatively limited to bacterial pathogens with tractable genetic systems because of the requirement for high frequencies of homologous recombination and extensive strain manipulation before strain selection.

A novel mutagenesis system called signature-tagged mutagenesis (STM) has been used recently to good advantage to identify *Salmonella* genes essential for growth in the spleen of infected mice (26–28). It also has been reported to be applicable to the identification of in vivo–expressed staphylococcal genes and to study *Legionella*, *Candida glaubrata,* and *Helicobacter pylori.* This system differs from IVET in that it uses a negative-selection strategy to identify avirulent strains created by transposon mutagenesis. Transposon mutagenesis has been used for some time to prepare random insertions of an antibiotic resistance gene throughout the genome of a microorganism to generate mutants. STM "tags" each transposon with a unique oligonucleotide sequence flanked by unique PCR primers that allows for individual clones to be identified from a large pool of mutant strains. In the end, one can identify individual mutants with attenuated virulence from among a much larger population of mutagenized bacteria.

In its first application, pools of tagged mutants were inoculated into mice. After 3 d, the bacterial population was isolated from the spleen of moribund animals. DNA was extracted from the bacteria and PCR was performed to amplify the individual tags from the isolated bacteria. The PCR product was then used to detect the presence or absence of distinct tags from the bacterial population isolated from the spleen. Tags that are missing represent those mutants that failed to reach the spleen and, therefore, should have an insertion within a genetic sequence that is directly or indirectly essential for virulence.

This simple, but powerful, method permitted the identification of a previously unsuspected pathogenicity island in the *S. typhimurium* chromosome. To apply STM to an experimental system, it is necessary only to have a transposon delivery system in a microbe of interest that gives some degree of broad mutagenesis or to have any method that gives a distinct molecular "tattoo" that can be used to discriminate a single clone from other members of a large population of microbes. STM is a marvelous new approach for the identification of genetic sequences that are operative during infection. It takes into account the competitive aspects of virulent and avirulent clones

of the same species. STM does not relieve the investigator from cloning and understanding the precise nature of the genes identified as essential for pathogenesis, but it does provide the direct identification of the sequences and is not some deduced function from a sterile nucleic acid sequence.

The interaction between bacterial pathogens and their host cells has been studied using fluorescence-based techniques like confocal microscopy and fluorimetry. In particular, these methods have been applied to follow adherence and internalization of pathogenic bacteria and their intracellular trafficking. Recently, a simple fluorescence system called differential fluorescence induction (DFI), which, like IVET, is dependent on the identification of bacterial promoter sequences, has been developed that can be applied to the rapid identification of bacterial genes differentially expressed in virtually any environment including the intracellular environment of macrophages and the tissues of an infected animal (29, 30). The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* is used as a selectable marker in a fluorescence activated sorter (FACS®). A pool of bacteria bearing plasmids with random DNA fragments that contain possible promoters of differentially expressed virulence genes is inserted upstream of a promoterless *gfp*. This bacterial population is used to infect cultured cells or to inoculate animals. Single bacteria that become fluorescent, even transiently, in the new environment can be "selected" by FACS® sorting. For example, this method was applied to *S. typhimurium* used to infect cultured macrophages. After 6 h of infection, macrophages containing bacteria with a transcriptionally active *gfp* gene were collected in a cell sorter. The bacteria present in these macrophages are pooled, grown ex vivo, and analyzed by FACS®. The least fluorescent bacteria (i.e., those not transcriptionally active in vitro) are collected and used to reinfect macrophages. Infected macrophages that become fluorescent are then isolated. The individual bacteria are collected by FACS® and analyzed to confirm that they display intracellular-dependent induction of the *gfp* gene fusion (Fig. 1). Several macrophage-induced genes (*mig*) have been isolated in this way. The methodology can be used as well to find regulatory genes that affect the expression of such *gfp*-induced gene fusions. Thus, it has been possible to show that while many of the *mig* genes are regulated by the level of intracellular Mg^{2+}, others respond to acid regulation or still unknown signals that trigger transcription.

DFI provides a tool to dissect the genetic basis of the interactions between two or more organisms. The only genetic requirements are that the organism of interest be able to maintain a plasmid or episomal element and to express a functional GFP molecule. Thus far, the technology has been applied to bacterial, fungal, and protozoan pathogens and can be used to study symbiosis and gene regulation in complex microbial populations.

Figure 1. RAW 264.7 macrophage cells infected with *S. typhimurium* bearing a *gfp* gene fused to a promoter region of a macrophage inducible chromosomal sequence. A merged image of an infected cell visualized by fluorescent and differential interference contrast (DIC) microscopy. Synthesis of the *gfp* fusion is seen as a bright green fluorescence. Extracellular microbes are stained with an antibody conjugated to phycoerythrin (red). Extracellular bacteria that do not synthesize *gfp* stain red, while those that synthesize *gfp* extracellularly are visualized as bright yellow. In this case, the bacteria display preferential synthesis of the *gfp* fusion in the intracellular environment.

Conclusions

Our knowledge of the molecular basis of bacterial pathogenesis has increased substantially over the past decade. However, our ability to understand the dynamics of bacterial gene expression in response to a host cell has been hampered by the lack of experimental tools to probe the intracellular life of invasive bacteria and their activities when hidden from view in infected tissue. Consequently, there has been a simplistic portrait of the infectious process. It seems likely that the examination of the initial interactions between the invading microorganism and the innate elements of the immune system will provide the key to understanding the pathogenesis of infection, as well as provide the basis for designing new antiinfective agents and preventative vaccines.

IVET, STM, and DFI represent a new generation of experimental probes to detect and follow specific virulence factors as discrete stages of host/pathogen interaction. The development of cell culture methods, the explosion of microbial genomics, and the development of experimental approaches, such as those described here, place us at the threshold of understanding the precise nature of the interplay of microbial life and our own. It is not just the pathogens with which we need be concerned. It is extraordinary we know so little about the complex communities of microbes that inhabit our bodies, how they establish themselves in unique niches within us, or how they are transferred from us or acquired by us. To ignore these factors is to ignore the very foundation for understanding many of the infections that confront modern medicine; nosocomial pathogens are biologically far more than a nuisance originating "simply" from the selection of drug-resistant bacteria. Surely the methods designed to detect the genes of pathogenicity will serve us equally well for the study of the many classes of diverse microbes that share our bodies.

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