Basic Principles in the Diagnosis and Management of Infectious Diseases

A Microbial Pathogenesis

A Molecular Perspective of Microbial Pathogenicity

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DIVERSITY OF HUMAN-MICROBE RELATIONSHIPS

Beginning immediately at birth, humans are colonized by a myriad of microorganisms that assemble into complex stereotypic communities, creating a beneficial indigenous microbiota. The result is a “supra-organism” in which microbial symbionts outnumber human cells by 10-fold. Most currently available information about the human indigenous microbiota concerns the bacterial component, although they are by no means the only important members. Bacteria are the focus of the following discussion.

In contrast to the relatively rare harmful encounters with pathogens, indigenous human-microbe relationships in which either microbe or host benefits without causing harm (commensal relationships) and relationships in which both benefit (mutualistic relationships) are the dominant forms of interaction and are fundamentally important to human biology. Coevolution, co-adaptation, and co-dependency are features of our relationships with our indigenous microbiota. The human microbiota facilitates nutrient acquisition and energy extraction from food, promotes terminal (postnatal) differentiation of mucosal structure and function, and stimulates both the innate and adaptive immune systems. By so doing, it helps to maintain epithelial boundary function and integrity, as well as to “educate” our innate immune defenses. It also provides “colonization resistance” against pathogen invasion, regulates intermediary metabolism, processes ingested chemicals, and provides small amounts of human accessory growth factors. The rules and features of microbial community assembly are fundamentally important but, so far, are poorly understood.

In the neonatal period, the community assembly process is especially dynamic and is influenced by early environmental (in particular, maternal) exposures and stochastic effects. The composition and functional capabilities of the indigenous microbiota evolve in a generally orderly fashion, as diet, hormonal environment, other environmental factors, and occasional ecologic disturbances play out their effects on a distinct, albeit diverse, human genetic background.

Bacterial diversity in the indigenous communities of the human body is striking in its richness of distinct species and strains but also noteworthy for the limited number of phyla commonly found. Despite exposure to more than 100 bacterial phyla in the surrounding environment, members of the phyla Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria dominate human body sites, suggesting a role for strong selective forces and microbial diversification over hundreds of thousands of years of coevolution with their host. Within the domain Archaea, diversity in the human body is apparently limited to a handful of methanogen species: Methanobrevibacter smithii is commonly found in the healthy distal gut, and Methanobrevibacter-related species are found in the inflamed subgingival crevice in some patients with moderate or severe chronic periodontitis. Of interest, patterns of bacterial diversity in humans display individual-specific features. The distinctness of an individual’s microbiota is less evident when viewed in terms of the overall functional capabilities of the community, rather than in terms of the names and relatedness of the strains and species; this difference probably reflects the functional redundancy of strains and species within the human microbiota, which, in turn, may contribute to stability of this ecologic system. Yet, differences in the capability of strains may explain variation among individuals in the metabolism of drugs such as digoxin and other exogenous chemicals.

Infections (or colonization) is simply the establishment of a microorganism on or within a host; it may be short lived, as in our encounters with “transients” (Table 1-1), or be persistent and may result in only low gain or harm to either participant. The term infectious disease applies when an interaction with a microbe causes damage to the host and the associated damage or altered physiology results in clinical signs and symptoms of disease. A pathogen is usually defined as any microorganism that has the capacity to cause disease. It is a medical definition; it is not a biologic definition, and certainly, not all pathogens have an equal probability of causing clinically apparent disease. Virulence provides a quantitative measure of pathogenicity or the likelihood of causing disease. For example, encapsulated pneumococci are more virulent than nonencapsulated pneumococci, and Escherichia coli strains that express Shiga-like toxins are more virulent than those that do not express these toxins. Virulence factors refer to the properties

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KEYWORDS
bacteriophage; clonality; commensal; commensalism; diagnostics; ecology; evolution; genomics; horizontal gene transfer; infectious diseases; intracellular parasites; metagenomics; microbiota; opportunistic infection; pathogen; pathogenicity island; plasmid; population biology; regulation of virulence; virulence
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isms, such as intact disease in some proportion of susceptible individuals with apparently casual disease or in those who are otherwise compromised. Thus, it is useful to distinguish pathogens that cause well-defined, well-known human diseases. Immunization against them prevents, in an antigen-specific fashion, their ability to colonize the first two of these microbes not only protects against disease but to a new susceptible host.

The difficulty, therefore, is that the distinctions between a commensal, an opportunist, and a pathogen can be blurred at times, in part because some commensals cause disease, albeit usually in immunocompromised patients. Many microorganisms with a capacity for sustained multiplication in humans, including members of the indigenous microbiota, cause disease more readily in individuals with underlying chronic disease or in those who are otherwise compromised. The common term opportunist suits this category of pathogen well (see Table 1-1). An emerging concept of microbial disease causation, with origins in the field of ecology, is the notion of “community as pathogen,” in which a conserved broad feature of the microbial community contributes to pathology, rather than any one specific member or component. This concept is in contrast to a widely held view of virulence, host processes of skin and mucosa, including inflammatory bowel disease and chronic periodontitis. It suggests that studies of pathogenesis consider general properties of microbial communities, such as resilience, or conserved functional interactions, such as syntrophy (cross-feeding), rather than the role of single microbes in isolation, especially for development of novel approaches for maintaining or restoring health.

What are the distinguishing characteristics of microbes that live in humans? A successful pathogen or commensal must do the following: (1) enter the human host; (2) become established, which includes successful competition with indigenous microbes; (3) acquire nutrients; (4) avoid or circumvent the host’s innate defenses and a powerful immune system; (5) above all, replicate; (6) disseminate if necessary to a preferred site; and (7) eventually be transmitted to a new susceptible host.

Whether a pathogen or a commensal, a microorganism must also possess an active group of complementary genetic properties, sometimes coregulated, that promote its interaction with the human host. For a given microorganism, the genetic traits define unique attributes that enable it to follow a common sequence of steps used in establishing infection or, in some cases, subsequent disease.9,10,11

Elegant molecular and genetic techniques now permit the identification, isolation, and characterization of many of these genes and their products. We now also possess the complete genome sequences of virtually every major pathogenic bacterial species. This information provides important clues and insight into the potential of a microorganism for causing disease and facilitates new experimental strategies for understanding pathogens and commensals alike.12,13 The availability of the host (e.g., human) genome sequence also enables multiple synergistic approaches for understanding virulence, including the identification of host genes that are underrepresented in pathogenic microorganisms and host response, and clues about the mechanisms of host defense and pathogen counterdefense.14 It is important to recognize that pathogenicity can only be understood in the context of a specific host.

These genomic analyses have lent credence to the working hypothesis of almost a half-century of research—that the distinguishing characteristic of microorganisms that regularly cause disease is a set of special genetic traits that provide them with the capacity to breach intact host anatomic, cellular, or biochemical barriers that ordinarily prevent entry by other microorganisms into sterile tissue sites. Thus, pathogens “go where other microbes dare not.” In addition, many pathogens, such as Mycobacterium tuberculosis, Treponema pallidum, Chlamydia trachomatis, and Salmonella typhi, have the capacity to establish persistent (usually asymptomatic) infection in the human host and have evolved the extraordinary capacity to live in the inner sanctums of our innate and adaptive immune defenses or, in general, to compete well in the face of otherwise hostile host conditions. For example, Salmonella profits from the inflammatory response that it provokes in the gut by using the oxidized form of a locally produced host factor for a selective growth advantage against commensals.1 A distinction, then, between a primary pathogen and opportunist is that the pathogen has an inherent ability to breach the host barriers that ordinarily restrict other microbes, whereas the opportunist requires some underlying defect or alteration in the host’s defenses, whether it

TABLE 1-1 Microbe-Human Host Interactions

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Transient</td>
<td>A microorganism that we encounter in our food or exposed to our environment, if at all, is just “passing through” and of little consequence; however, regular encounters over extended periods of time might lead to host adaptation or even dependence.</td>
</tr>
<tr>
<td>Commensal (literally, those that “eat at the same table”)</td>
<td>A microorganism that is a normal inhabitant of the human body. In commensal relationships, either the microbe or host benefits, possibly through mutualistic relationships, both derive benefit.</td>
</tr>
<tr>
<td>Pathogen (derived from the Greek, pathos, meaning the “birth of suffering”)</td>
<td>A microbe that may or may not be a member of the indigenous microbiota, but it regularly causes disease in apparently normal individuals.</td>
</tr>
<tr>
<td>Opportunistic pathogen</td>
<td>A microbe that causes disease only in humans who are in some way compromised in their normal defense mechanisms.</td>
</tr>
<tr>
<td>Accidental pathogen</td>
<td>A microorganism that is encountered by accidental contact with animals, insects, or the environment. These microorganisms are often deadly in humans and sometimes cause a causative agent of disease in other animals. These microbes are often distinguished from human-specific pathogens because they are not directly or readily transmissible from human to human.</td>
</tr>
</tbody>
</table>

(e.g., gene products) that enable a microorganism to establish itself and replicate on or within a specific host species and that enhance the microbe’s potential to cause overt pathology. In many ways, what we refer to as virulence factors are in the biologic sense colonization factors that permit replication in the host and subsequent transmission to a new susceptible host.

Thus, the capacity of certain microorganisms to cause disease in healthy, uncompromised human hosts on a regular basis should reflect fundamental biologic differences in their virulence capabilities from those of opportunists and commensal species that rarely, if ever, cause disease. In the following sections, we address this issue and discuss how insight into pathogenesis has been applied to the practice of contemporary infectious disease medicine.
be genetic, ecologic (altered microbiota), or caused by underlying disease, to establish itself in a usually privileged host niche. Clearly, the nature of the host plays as important a role as the pathogen in determining outcome. An initial step required of a pathogen is to gain access to the host in sufficient numbers. Such access requires that the microorganism not only make contact with an appropriate surface but also then reach its unique niche or microenvironment on or within the host. This requirement is not trivial. Some pathogens must survive for varying periods in the external environment. Others have evolved an effective and efficient means of transmission. To accomplish this goal, the infecting microbe may make use of motility, chemotactic properties, and adhesive structures (or adhesins) that mediate binding to specific eukaryotic cell receptors or to other microorganisms. Pathogens that persist at the surface of skin or mucosa usually rely upon multiple redundant adhesins and adherence mechanisms. If the adhesin is immunogenic, expression is usually regulated; in addition, antigenic variants may arise (see “Regulation of Bacterial Pathogenicity”). Preexisting microorganisms, the indigenous microbiota, provide competition against establishment of the newcomer; furthermore, the latter must adapt, at least temporarily, to the particular nutrient environment in which it now finds itself.

Normal inherent host defense mechanisms pose the most difficult set of obstacles for pathogens and commensals in establishing themselves in a host. For any set of specific host defenses, an individual pathogen will have a unique and distinctive counterstrategy. Some of the best-known mechanisms that pathogenic microbes use for countering host defenses include the use of an antiphagocytic capsule and the elaboration of toxins and microbial enzymes that act on host immune cells and/or destroy anatomic barriers. Microorganisms also use subtle biochemical mechanisms to avoid, subvert, or, as we now increasingly understand, manipulate host defenses. These strategies include the elaboration of immunoglobulin-specific proteases, iron sequestration mechanisms, coating themselves with host proteins to confuse the immune surveillance system, or causing host cells to signal inappropriately, leading to dysregulation of host defenses or even host cell death. Examples of these mechanisms include the production of immunoglobulin A1 protease by the meningococcus, the use of receptors for iron-saturated human transferrin and lactoferrin by N. gonorrhoeae, and the coating of T. pallidum with human soluble fibronectin. Yersinia, Mycobacterium, and Bordetella induce host cell production of interleukin-10, which is a potent immunosuppressive cytokine, thereby downregulating important elements of the innate immune defense. Antigenic variation and intracellular invasion are other common strategies used by successful pathogens to avoid immune detection. The intimacy of the relationships between viral pathogens and host is reflected in the frequency with which these pathogens co-opt host molecules and/or use sophisticated host defenses (see “Subversion of Host Cellular Processes and Immune Defenses”).

The ability to multiply is a characteristic of all living organisms. Whether the pathogen’s habitat in the relevant host is intracellular or extracellular, mucosal or submucosal, within the bloodstream or within another privileged anatomic site, pathogens have evolved a distinct set of biochemical tactics to achieve this goal. The ultimate success of a pathogen, indeed, of any microorganism, is measured by the degree to which it can multiply. The pathogen must not only replicate sufficiently to establish itself in a host on reaching its specific niche, but it also must replicate sufficiently at some point in its life cycle to ensure its potential transmission to a new susceptible host. The rate of pathogen multiplication is appreciated by a clinician in terms of a characteristic incubation period spanning the time of exposure to the appearance of signs and symptoms of disease.

Infectious disease, in one sense, is simply a byproduct of the method and site chosen by pathogens for replication and persistence; disease per se is not a measure of microbial success. Disease, in part, reflects the status of the host as much as it does the virulence characteristics of the offending microorganism. Death of the host is fortunately a rare event and one that must be viewed with the dispersion of biology as being detrimental to both parties involved! The usual rules of host-pathogen engagement most often produce a tie: sufficient multiplication of the pathogen to ensure its establishment within the host (transient or long-term infection) and to ensure its successful transmission to a new susceptible host, while at the same time, no more than is tolerated by the host as it gains immunity from further incursion by the same and even related pathogens.

Why do some pathogens cause disease more readily than others? The strategy used for multiplication on or within the host (i.e., its ability to overcome host barriers) often defines fundamental differences between pathogens that commonly cause acute disease symptoms and those that do not. An organism that can reach and multiply in privileged anatomic sites away from the competitive environment of skin and mucosal surfaces is more likely to disrupt homeostasis in the host and cause disease than one that chooses a different strategy. If a microorganism has evolved a means to nullify or destroy phagocytic cells to multiply successfully, it is more likely to be found in deeper compartments and associated with acute disease. Commensal or mutualistic organisms are restrained so that they multiply just enough, in the midst of competing microbiota, to persist but not damage the host’s self-preserving homeostatic and innate immunity mechanisms. It is important to emphasize that a microorganism equipped to multiply efficiently in a human may be exceptional in the biologic sense but unexceptional as a pathogen in the medical sense and only infrequently, if ever, a cause of clinically manifested disease. Some organisms, such as P. aeruginosa, are “only” opportunists in humans despite their impressive array of virulence factors. These virulence factors work well in some plant hosts and in predators it encounters in the environment. However, for Pseudomonas, these same pathogenic determinants usually fail to overcome the average human’s defenses. For opportunistic pathogens, the state of the host is the major determinant of whether disease is the outcome of their interaction with the host. Commensals and mutualists, for example, which are the usual cause of opportunistic infections, may be very adept at colonization, but because of their preferred growth locale (e.g., at the mucosal surface) and preferred growth conditions (e.g., a microaerobic environment), they may have limited growth opportunities outside their restricted niche in an unimpaired individual. Innate immune factors are difficult to overcome for the vast majority of commensals. Little more than 50 years ago, there was a prevalent view that pathogens had undergone retrograde evolution and caused disease because they were little more than parasites. Pathogens were then viewed as organisms often adapted to their hosts and that elaborated potent toxins or other powerful aggressive factors causing the signs and symptoms of disease. However, bacterial pathogenicity has been redefined over the past quarter century by using the tools of molecular genetics, genomics, and cell biology. We can now directly address the question: why are some bacteria pathogenic for humans, whereas other (closely related) bacteria are not?

We understand now that pathogenic bacteria are indeed often exposed to adapted to their hosts and that these organisms use sophisticated biochemical properties to distinguish pathogens from their nonpathogenic brethren derive from specialized genes possessed by pathogens but absent from nonpathogens. The driving force for the inheritance of pathogenic traits is not slow adaptation to the host but, rather, a more dynamic process of horizontal (lateral) gene transfer via mobile genetic elements. Hence, the genes for many specialized “bacterial” products, such as toxins and adhesins, actually reside on transposons (“jumping genes”) and bacterial viruses (bacteriophages) (Table 1-2). Larger packets of information have also been shared among bacteria by genetic transfer. The lateral inheritance of large blocks of genes, called pathogenicity islands, is often the key to the expression of pathogenicity in bacteria. Many of these virulence determinants acquired by lateral gene transfer have several features that are apparent by simple inspection of genome sequences, including distinct chromosomal nucleotide composition and association with plasmids or phages, suggesting that their ancestry derives from an unrelated microbe. One surprising finding is that the amount of acquired DNA associated with virulence and antibiotic resistance in a single bacterial habitat in many bacteria can be substantial. For example, uropathogenic, enterohemorrhagic, and extraintestinal types of E. coli all display mosaic genome structure, with hundreds of
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BR, Katz ME. A role for bacteriophages in the evolution and transfer of bacterial virulence of bacteria to animals.

CFA, colonization factor antigen.

gene acquisition. every new genome sequence, a new set of approximately 300 unique for a species.

arises the concept of the “pan-genome” or the complete set of genes distinct from others as each is from a nonpathogenic laboratory strain or gene inactivation is often associated with the adaptation of a par- lifestyle. Indeed, the study of host adaptation suggests that gene loss dispense with some genes that are no longer useful for a pathogenic loss of genes. This is not to say that, over time, some pathogens do not the distribution of these genes suggests that bacteria evolve to become "closed" pan-genome and a much preference instead display a relatively "open" pan-genome and a much greater fraction of shared genes.

Hence, we can conclude that, in most cases, human-adapted pathogens have virulence genes not present in nonpathogenic relatives, and the distribution of these genes suggests that bacteria evolve to become pathogens by acquiring virulence determinants and not by the gradual loss of genes. This is not to say that, over time, some pathogens do not dispense with some genes that are no longer useful for a pathogenic lifestyle. Indeed, the study of host adaptation suggests that gene loss or gene inactivation is often associated with the adaptation of a particular pathogen to a particular host. For example, S. typhi, compared with Salmonella typhimurium, has lost or inactivated a large number of genes. Yet, it also has acquired by lateral transfer a unique surface determinant, Vi, and a unique toxin. Thus, the fundamental evolutionary push to pathogenicity results from gene acquisition. This is not simply a mechanism that microbes use to become pathogenic but, instead, a general strategy for microbial specialization and success in some environmental niches that are highly competitive. Why bacteria have adopted this tactic to maximize their diversity and to increase their opportunity for continuing evolution is most likely a reflection of their haploid state and their need to conserve fundamental characteristics, such as the ability to live on a mucosal surface, while still being able to try new combinations of genes. The sharing of genes among seemingly disparate microorganisms occupying the same niche in a sense provides these microbes with an endless number of combinations of genes for evolutionary experimentation, as it were, within a habitat such as the human intestinal tract. Overall, across the bacterial world, the number of such successful experiments resulting in the emergence of a pathogen appears to have been quite rare (see "Clonal Nature of Bacterial Pathogens"). Yet, when successful, these experiments are surprisingly efficient, at least from the perspective of the microbe, yet manageable, from the standpoint of the host.

Some infectious diseases occur predominantly in dramatic epidemiemic form, which argues against the establishment of a balanced host-parasite relationship; however, in many such epidemics, mitigating circumstances involving herd immunity and other underlying social, economic, and political issues impinge on this relationship. So-called emerging infectious diseases reflect various aspects of imbalance in the relationships between host, pathogen, and environment. Many of the most serious and feared infectious diseases occur when humans are infected by microorganisms (accidental pathogens) that prefer and are better adapted to another mammalian host. In fact, most emerging infectious diseases in humans are of zoonotic origin. As seen in many zoonotic diseases, the rules of engagement between the host and the pathogen are blurred, often to the detriment of both the host and the microbe. It is often an evolutionary dead end for both parties.

Given the increasingly frequent and unexpected emergence of previously unrecognized pathogens, it is appropriate to question how well we appreciate the true diversity and distribution of extant microorganisms capable of causing human disease. Although most emerging pathogens are zoonotic agents and already adapted to a different host, the question also concerns a more basic uncertainty about how often, in what phylogenetic backgrounds, and through what mechanisms virulence for humans among microbes can arise. Pathogenicity appears to have arisen on multiple occasions throughout the domain Bacteria but only in a small fraction of the overall phyla, that is, those whose members typically colonize humans (see "Diversity of Human-Microbe Relationships"). Although there are currently no known traditional pathogens within the domain Archaea, the methanogens, through a synergistic interaction with other microbes, known as syntrophy, may contribute to pathology in certain clinical settings. For example, in chronic periodontitis, methanogens in the subgingival crevice may enhance the growth of fermentative would-be pathogenic bacteria by consuming the hydrogen produced by the latter. Finally, before considering several facets of pathogenicity in more detail, three further points should be considered: (1) pathogen detection and identification remain suboptimal, in part because of continuing dependence on cultivation methods, and therefore a number of novel pathogens may have gone undetected; (2) some potential pathogens may not have had adequate contact with humans to have made themselves known (yet); and (3) dominant ideas of microbial disease causation (e.g., a single pathogenic agent in a susceptible host) may be too restrictive. As mentioned earlier, some microbial diseases may require a consortium of agents (e.g., intra-abdominal abscess), thereby posing challenges for pathogen identification. If we define success for a microbial pathogen as disease without a requirement for long-term survival, a much larger number of organisms may qualify, in being able to cause devastating human misery but only over a limited number of generations. These matters have obvious relevance to the troubling issue of bioterrorism and the potential malevolent use and genetic manipulation of microorganisms.

### Clonal Nature of Bacterial Pathogens

As noted previously, pathogenicity is not a microbial trait that has become fixed by chance. Instead, particular microbial strains and

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**TABLE 1-2 Examples of Plasmid- and Phage-Encoded Virulence Determinants**

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>VIRULENCE FACTOR</th>
<th>BIOLOGIC FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmid Encoded</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterotoxigenic Escherichia coli</td>
<td>Heat-labile, heat-stable enterotoxins</td>
<td>Activation of adenylate/ guanylate cyclase in the small bowel, which leads to diarrhea</td>
</tr>
<tr>
<td>CFAI and CFAII</td>
<td>Adherence/colonization factors</td>
<td></td>
</tr>
<tr>
<td>Extraintestinal E. coli</td>
<td>Hemolysin</td>
<td>Cytotoxin</td>
</tr>
<tr>
<td>Shigella spp. and enteroinvasive E. coli</td>
<td>Gene products involved in invasion</td>
<td>Induces internalization by intestinal epithelial cells</td>
</tr>
<tr>
<td>Yersinia spp.</td>
<td>Adherence factors and gene products involved in invasion</td>
<td>Attachment/invasion</td>
</tr>
<tr>
<td><strong>Phage Encoded</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus anthracis</td>
<td>Edema factor, lethal factor, and protective antigen</td>
<td>Edema factor has adenylate cyclase activity, lethal factor is a metalloprotease that acts on host signaling molecules</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Exfoliative toxin</td>
<td>Causes toxic epidermal necrolysis</td>
</tr>
<tr>
<td>Clostridium tetani</td>
<td>Tetanus neurotoxin</td>
<td>Blocks the release of inhibitory neurotransmitter, which leads to muscle spasms</td>
</tr>
<tr>
<td>Corynebacterium diphtheriae</td>
<td>Diphtheria toxin</td>
<td>Inhibition of eukaryotic protein synthesis</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>Erythrogenic toxin</td>
<td>Rash of scarlet fever</td>
</tr>
<tr>
<td>Clostridium botulinum</td>
<td>Botulinum neurotoxin</td>
<td>Blocks synaptic acetylcholine release, which leads to flaccid paralysis</td>
</tr>
<tr>
<td>Enterohemorrhagic E. coli</td>
<td>Shiga-like toxin</td>
<td>Inhibition of eukaryotic protein synthesis</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>Cholera toxin</td>
<td>Stimulates adenylate cyclase in host cells</td>
</tr>
</tbody>
</table>

species adapted to a particular host have evolved to carry very specific arrays of virulence-associated genes. By examining the genetic organization of pathogens, opportunists, and nonpathogenic bacteria, one can begin to understand the origins of pathogenicity and why some pathogens are more pathogenic or more successful than their peers.

Techniques used in the study of genetic relatedness include primary protein or nucleic acid sequence comparisons and DNA hybridization methods, including DNA microarray-based approaches. Some genetic sequences, such as those of the small- and large-subunit ribosomal RNAs, have been used as reliable evolutionary clocks. Comparative analysis of these sequences allows one to infer phylogenetic relationships among all known cellular life, but these sequences provide only limited resolution between strains and limited insight into organismal biology and function. The increasing ease with which primary genomic sequence information can be acquired, differences quantified, and these data shared has led to more precise methods of strain characterization, such as multilocus sequence typing and whole-genome sequencing. Today, full-genome sequencing and genome-wide single nucleotide polymorphism analysis are feasible on a large-scale basis and offer the greatest insight into evolutionary relationships and population biology of microbes. All of these sequence-based approaches avoid the pitfalls of classic comparisons of phenotypes (i.e., gross observable characteristics of a microbe), which can be unreliable. When these sequence-based techniques are used, a consistent finding emerges concerning the population structure of microorganisms: most natural populations of microorganisms consist of a number of discrete clonal lineages.

A clonal population structure implies that the rates of recombination of chromosomal genes between different strains of the same species and between different bacterial species are very low. Clonal organization has been substantiated by the concordance between evolutionary trees derived from unrelated chromosomal sequences. Even though bacteria possess well-established, naturally occurring genetic exchange mechanisms, they retain their individuality, just as the human- and other animal-associated bacterial communities demonstrate stable membership over long periods of time in an individual. We might have thought that in light of unmistakable gene shuffling among and between bacteria, we might see homogenization of bacterial species and little specialization. In fact, the opposite is true. Bacterial species have remained discrete and distinct taxonomic entities because the bacterial chromosome is a highly integrated and co-adapted entity that has, in general, resisted rearrangement. The same may be true of the overall structure of the indigenous bacterial communities of humans.

It is intriguing that analysis of natural populations of microorganisms with pathogenic potential has revealed the prominent representation of a relatively few clones. In fact, most cases of serious disease may be explained by a few of the many clones of pathogenic bacterial species. For example, one sees this in meningococcal disease, where there is a clear predominance of a particular clone in large areas worldwide. In contrast, in the case of the typhoid bacillus, there is only one major clone worldwide, although antibiotic resistance may be forcing diversity. Indeed, in some extreme cases, all members of a species, such as Shigella sonnei or Bordetella pertussis, belong to the same clonal type or small group of closely related types. Not all pathogenic bacterial species reveal this pattern of clonal organization. Two notable exceptions are N. gonorrhoeae and Helicobacter pylori, which appear to use chromosomal recombination to increase their genetic diversity. It may be that organisms such as N. gonorrhoeae and H. pylori, which are quite specialized in their preference for discrete human anatomic sites where they rarely encounter related species, must resort to constant recombination and genetic reassortment by using DNA transformation as a means of sharing the well-adapted alleles that accumulate within their populations. Thus, genetic variability among gonococcal and H. pylori isolates from discrete geographic locations suggests that these organisms are essentially and appropriately sexual.

Clonal analysis has generated other important conclusions concerning the evolution of bacterial species and pathogenic strains in particular. Study of E. coli populations in the human intestinal tract indicates that only a small number of clonal lineages persist, whereas numerous unrelated cell lines appear and disappear. E. coli urinary tract pathogens that cause symptomatic disease in humans may be even less genetically diverse than E. coli strains found in the intestinal microbiota or those that cause asymptomatic urinary tract colonization. Perhaps the evolution of these E. coli strains to live in a more specialized epithelial niche results in constraints on recombination that preserve their added degree of specialization. At the same time, specialization for one body site may not preclude fitness for another site: in some individuals with recurrent urinary tract infections, there can be a simultaneous and identical shift in the dominant E. coli population of the bladder and distal gut between one episode and the next. Pathogens have even taught us about human prehistory: sequence-based population analysis of human-restricted and human-adapted bacterial pathogens, such as H. pylori, has clarified important aspects of human migration and human population structure.

**GENOMICS AND THE EVOLUTION OF PATHOGENICITY**

The first complete genome sequence for a free-living organism, H. influenzae, was described in 1995. Since then, more than 2900 bacterial and archaeal complete genome sequences have been released to public databases (see www.ncbi.nlm.nih.gov/genome/browse/). Despite the obvious value of a primary genomic blueprint, it is increasingly clear that genetic, genomic, biochemical, and epidemiologic approaches provide complementary advantages. Each contributes to the search for new determinants of virulence.

As noted earlier, comparisons of pathogenic and nonpathogenic representatives of a single genus or species usually demonstrate the nonpathogens to be relatively devoid of functional genetic sequences encoding the pathogenic trait or traits. Inactive mutational variants or portions of virulence-associated genes are sometimes found in nonpathogenic strains of the same species. In general, as bacteria evolve from free-living organisms with multiple habitats to obligate pathogens, host-restricted organisms, endosymbionts, or obligate intracellular organisms, their genomes appear to become reduced in size, or they accumulate inactive or defective genes (pseudogenes), or both. For example, the evolution of B. pertussis from a host-specific, human-adapted pathogen from a Bordetella bronchiseptica-like ancestor has been accompanied by extensive gene loss and gene inactivation (3816 coding sequences vs. 5007 for B. bronchiseptica; 9.4% of coding sequences are pseudogenes vs. 0.4% for B. bronchiseptica). In this case, a highly restricted host range (for B. pertussis, humans only) has meant loss of genetic diversity. In contrast to B. bronchiseptica, which infects multiple animal hosts and can survive in the environment, B. pertussis varies little in gene content among different strains isolated over the past 50 years and across several continents. B. anthracis, which exists predominantly as an inactive spore, and Mycobacterium tuberculosis, which exists predominantly as an active bacillus in a latent phase in human granulomas, also exhibit limited genomic diversity. Mycobacterium leprae displays an extreme degree of gene decay.

Not uncommonly, virulence-specific sequences are bounded by repeated DNA segments, some of which represent known insertion elements, which suggests that these virulence genes were once associated with a mobile genetic element or that these genes formerly occupied another chromosomal locale in either the same species or another microorganism altogether. Acquisition of an adhesin, toxin, or serum-resistance factor might enable a previously nonpathogenic organism to cause disease in a host that had previously been nonsusceptible.

Pathogenicity islands provide support for this concept. These islands comprise clusters of virulence-associated genes that encode specialized secretion systems, secreted effector molecules, adhesins, and regulatory proteins. S. typhimurium is believed to have begun evolving as a pathogen from a common ancestor that it shares with E. coli, from approximately 130 million years ago, through the sequential acquisition of at least two pathogenicity islands, one of which mediates internalization within host cells, and the other, survival and replication within an intracellular vacuole. Yersinia pestis provides a dramatic example of evolution through both acquisition and loss of genes. It is estimated that Y. pestis has evolved from the enteropathogenic Yersinia pseudotuberculosis only 2000 to 20,000 years ago. All pathogenic yersinia species harbor a 70-kb virulence plasmid (pYV) needed for
toxins and to overcome host immune system; but there are two Y. pestis–specific plasmids that were recently acquired by horizontal gene transfer. One encodes a plasminogen activator, a surface molecule that provides proteolytic, adhesive, and invasive functions and facilitates dissemination from an intradermal site of infection. The other plasmid encodes a capsular antigen that blocks phagocytosis and a toxin needed for survival in the flea. Thus, this organism evolved to establish a distinct mammalian reservoir, to ensure its transmission by a flea, and to gain attributes that permitted it to spread to systemic sites in its preferred murine host and with obvious devastating effect in an accidental human host. In the process, it rearranged its chromosome and inactivated genes that were relics of its previous gastrointestinal life.

That a microorganism can accomplish this remarkable feat of evolution in what is a relative blink of the eye, in evolutionary terms, should be a cautionary lesson for what the future may hold in store for any living entity that is host to microbes.

Although genomic analyses provide us with fascinating stories of how pathogens evolved by genetic acquisition of specialized secretion systems and the role of these systems in exporting a variety of genes that provide the microbe with extraordinary properties to survive in a specific host, we still remain ignorant of the precise origins of these and other virulence-associated systems. It seems likely, however, that pathogenicity is an old and honorable bacterial trait that can be traced to a pathogen’s need for avoiding predation as more sophisticated organisms evolved, such as free-living amebae, nematodes, and a host of other equally invisible creatures that exploit microbes for food.

REGULATION OF BACTERIAL PATHOGENICITY

Part I Basic Principles in the Diagnosis and Management of Infectious Diseases

If an organism possesses specialized gene products for its virulence, it must be able to use them when needed but not squander its metabolic energy producing them aimlessly or risk having them detected by host defenses and prematurely neutralized. Consequently, regulating the expression of virulence factors is an additional, yet essential complication of a pathogenic microbe’s life. The host presents an array of conditions strikingly distinct from those of the outside environment, conditions that are not easily reproduced in the laboratory. In fact, laboratory culture conditions bias our understanding of microbial adaptation to natural environments. This bias is reflected in the concept of a “viable but nonculturable state” for bacteria in their natural external environment. Vibrio cholerae, for example, is thought to persist in this state in brackish estuaries and other saline aquatic environments, sometimes associated with the chitinous exoskeleton of various marine organisms. Transition from this milieu to the contrasting environment of the human small intestinal lumen must be accompanied by substantial genetic regulatory events.

The microbial cell is relatively simple, yet it possesses the means to adapt to its changing environment, for example, via changes in gene expression in response to factors such as the amount of calcium, amino-acid concentration, temperature, and pH. ToxR directs expression of these genes indirectly by activating transcription of a specific plasmid, to which it binds, and the regulatory activities of this protein are regulated by changes in the cell density and regulation of its behavior accordingly.

Reversible regulation of the expression of virulence genes by temperature is a feature common to many pathogens, including enteropathogenic and uropathogenic E. coli (K-88 and K-99 fimbriae, pyelonephritis–associated pilus fimbriae, and K-1 capsular antigen), Shigella spp. (pyridoxine- and methylmalonic acid–resistant, and Yersinia spp. (virulence-associated determinants, including a low-calcium response and outer membrane proteins). Changes in DNA topology, messenger RNA conformation, and protein conformation and stability mediate thermal regulation of these diverse virulence determinants.

The number of well-characterized virulence regulatory systems is impressive. One common mechanism for bacterial transduction of environmental signals involves two-component regulatory systems that act on gene expression, usually at the transcriptional level. Such systems make use of similar pairs of proteins; one protein of the pair spans the cytoplasmic membrane, contains a transmitter domain, and may act as a sensor of environmental stimuli, whereas the other is a cytoplasmic protein (response regulator) with a receiver domain that regulates responsive genes or proteins. Sensor proteins are often kinases that phosphorylate themselves at a conserved histidine residue. These high-energy intermediates then transfer their phosphate groups to a conserved aspartate residue within the receiver domain of the response regulator proteins. Competing dephosphorylases determine an overall phosphorylation state of these response regulators and hence their level of activity. Many of these regulators are DNA-binding proteins that regulate transcription of multiple gene targets. Systems of this type control, for example, the permeability properties of the E. coli cell envelope in response to osmotic stimuli (EnvZ/OmpR), motor activity involved in E. coli chemotaxis (CheA/CheY, CheB), expression of numerous virulence factors in Streptococcus pyogenes (CovR/CovS), the switch from vegetative growth to sporulation by Bacillus subtilis (KinA/SpoOF, SpoOA), and even the ability of the soil bacterium Agrobacterium tumefaciens to induce tumors in susceptible plant cells in response to phenols found within plant wound exudates (VirA/VirG).

The coordinated control of pathogenicity incorporates the important concept of a regulator, a group of operons or individual genes controlled by a common regulator, usually a protein activator or repressor. This regulator may, in some cases, be the second component of a two-component system. A regulator provides a means by which many genes can respond in concert to a particular stimulus. At other times, the same genes may respond independently to other signals. Global regulatory networks are a common feature of microbial virulence as well as basic microbial physiology (Table 1–3). The complexity of virulence regulation in a single microbial pathogen is magnified by the coexistence of multiple interacting (cross-talking) systems and by regulons within regulons. P. aeruginosa, for example, contains genes for 55 sensors and 89 response regulators, whereas H. pylori contains genes for 4 and 7, respectively. Perhaps the most restricted numbers and types of microenvironments occupied by the latter organism limit the number, while increasing the relative importance, of cues that it must recognize. It appears that some, but not all, regulatory systems are essential for virulence.

Regulation of the expression of virulence determinants by V. cholerae involves a global regulatory protein that, in this case, serves a dual function. The toxR gene product is a transmembrane, DNA-binding protein that regulates expression of cholera toxin (stx), pilus, fimbriae, and other virulence factors in Vibrio cholerae. The ToxR protein is thought to sense a variety of environmental regulatory signals, including osmolarity, amino-acid concentration, temperature, and pH. ToxR directly activates transcription of the genes it binds, whereas a repressor blocks transcription of other genes indirectly by activating transcription of toxT, its own repressor. A common mechanism for bacterial transduction of environmental signals involves two-component regulatory systems that act on gene expression, usually at the transcriptional level. Such systems make use of similar pairs of proteins; one protein of the pair spans the cytoplasmic membrane, contains a transmitter domain, and may act as a sensor of environmental stimuli, whereas the other is a cytoplasmic protein (response regulator) with a receiver domain that regulates responsive genes or proteins. Sensor proteins are often kinases that phosphorylate themselves at a conserved histidine residue. These high-energy intermediates then transfer their phosphate groups to a conserved aspartate residue within the receiver domain of the response regulator proteins. Competing dephosphorylases determine an overall phosphorylation state of these response regulators and hence their level of activity. Many of these regulators are DNA-binding proteins that regulate transcription of multiple gene targets. Systems of this type control, for example, the permeability properties of the E. coli cell envelope in response to osmotic stimuli (EnvZ/OmpR), motor activity involved in E. coli chemotaxis (CheA/CheY, CheB), expression of numerous virulence factors in Streptococcus pyogenes (CovR/CovS), the switch from vegetative growth to sporulation by Bacillus subtilis (KinA/SpoOF, SpoOA), and even the ability of the soil bacterium Agrobacterium tumefaciens to induce tumors in susceptible plant cells in response to phenols found within plant wound exudates (VirA/VirG).

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Quorum sensing is an approach by which bacteria keep track of their cell density and regulate their behavior accordingly. It is inextricably involved in the formation of complex community structures, called biofilms, by bacteria on environmental surfaces for long-term persistence and resistance to host defenses. Gram-negative organisms secrete and respond to acylated homoserine lactones as a means of quorum sensing and cell-cell communication. Production of light by marine vibrios and tissue-degrading enzymes by P. aeruginosa is activated by these autoinducing compounds when they reach sufficient numbers.
concentration. Gram-positive organisms such as *Staphylococcus aureus* use peptide autoinducers and repressors to sense cell density and regulate toxin expression. The ability of bacterial pathogens to take their own census enables precise choreography of virulence factor production during the course of growth in a vigilant host. For example, in the early stages of a developing soft tissue abscess, *S. aureus* turns on antiphagocytic toxins just as the bacteria reach numbers sufficient to draw the attention of neutrophils.\(^6\) *V. cholerae* relies on quorum sensing to regulate biofilm formation on marine plankton and mediates release from these biofilms upon entry into a human host.\(^7\) Quorum factors sometimes exhibit strain specificity and might serve as targets for novel therapeutic approaches.\(^8\)

Some microbial pathogens (e.g., *N. gonorrhoeae, Borrelia recurrentis,* and *Trypanosoma brucei*) periodically vary prominent antigenic components of their surface and, by so doing, avoid the host immune response. Antigenic variation in *S. typhimurium* and *N. gonorrhoeae* provides examples of alternative molecular mechanisms (i.e., DNA rearrangements) that mediate regulation of the expression of virulence factors. *S. typhimurium* varies an immunodominant antigen by alternating between the expression of two different flagellin genes, *H1* and *H2*. The mechanism for this form of variation involves inversion of a 995-bp chromosomal DNA sequence containing a promoter.\(^9\) By altering expression of flagellin, *S. typhimurium* may avoid a host antibody response directed against it.

Pili are essential for virulence of gonococci in the human host, probably as a result of their role in adherence to the mucosal target surface. They also elicit a specific local and systemic host antibody response. Intermittent production of pili, as well as variation in the antigenic type of a pilus, may be strategies used by the gonococcus to avoid a humoral host response. The molecular mechanisms behind these strategies are complex. In general terms, phase and antigenic variation result from DNA rearrangements that move pilin-related sequences scattered around the gonococcal chromosome (in silent pilS loci) to the expression site (pilE locus). Numerous different pilus types may be expressed by derivatives of a single *N. gonorrhoeae* strain. Among other microbial pathogens, DNA rearrangements account for the antigenic variation of variant surface glycoproteins of *T. brucei* and the antigenic variation of variable major proteins in *Borreli* spp.\(^10\)

Proper presentation of certain virulence-associated gene products on the microbial surface is now recognized to be as important to pathogenicity as the initial expression of these genes. Presentation entails export pathways, association with other periplasmic or surface factors, and sometimes macromolecular assembly at the surface and is also subject to regulation.\(^11\) Among bacterial pathogens, shared homology is apparent among families of proteins involved in these processes. One family consists of proteins that are known as chaperones and usherers, concepts first proposed in a model for the assembly of uropathelial-adherent *E. coli* P pili. Periplasmic chaperones, such as PapD, escort protein subunits from the cytosome to the outer membrane and assist in their proper folding. Outer membrane usherers, such as PapC, target these complexes to a surface assembly site. Folding, transport, and assembly enable a microorganism to present a specific array of surface molecules necessary for eukaryotic cell tropism, intoxication, or entry. A precise configuration of microbial surface molecules might be viewed as an “attack complex” with properties not found in any of the individual components.

**MICROBIAL PATHOGENS AS INTRACELLULAR PARASITES**

Despite their capacity for an extracellular existence, a wide variety of bacterial and protozoal pathogens have evolved the means to enter, survive, multiply, and even persist within host eukaryotic cells. By so doing, a microorganism avoids host immune defenses and gains access to what are otherwise restricted nutrients. These advantages impose a strong selective evolutionary pressure that is dramatically reflected in the refined strategies developed by microbial pathogens for life within a host cell. These strategies include molecular mimicry, coercion, and intimate adaptation to eukaryotic cellular processes, and they are accompanied by genome reduction.

To a large degree, the mechanisms used by a microorganism to adhere to a eukaryotic cell dictate whether and how it enters the cell and its subsequent intracellular fate.\(^12\) Most, if not all, intracellular pathogens have multiple means for attachment to a eukaryotic cell surface; the particular combination of microbial attachment factors and cognate host receptors favors selection of one of several entry pathways and predetermines basic features of the intracellular vacuole. However, in a general sense, it is unclear to what extent microbial pathogens accept preprogrammed pathways dictated by phagocytic (e.g., complement and Fc receptors) and nonphagocytic receptors and to what extent they may be able to modify or exploit these pathways. *Toxoplasma gondii* invades and replicates within all types of nucleated mammalian cells. After entry and through unidentified receptors, *T. gondii* resides within a vacuole that is permanently incapable of fusion with other intracellular organelles, including lyso- somes. Parasite survival within this vacuole depends on the accompanying lack of acidification, exclusion of lysosomal contents, and specific mechanisms for nutrient acquisition and environmental sensing. However, when this organism is directed to enter eukaryotic cells by means of an alternative pathway (e.g., mediated by receptors for the constant region of immunoglobulin G, Fc), this vacuole fusion block is overcome. Presumably, parasite-directed modifications of the surrounding vacuolar membrane and exclusion of certain host proteins during the earliest stages of entry help create conditions necessary for growth and development of the pathogen.\(^13\)

Some pathogenic microorganisms seem to regulate when and where they interact with host cells by using preexistent host signaling pathways.\(^14\) Among the receptors that recognize pathogens and, in some cases, mediate entry are integrins (*Vesirinia* spp.), tight junction apparatus cadherins (*Listeria monocytogenes*) and scaffolding protein ZO-1 (*H. pylori*), dystroglycans (arenaviruses), and growth factor receptors (*S. typhimurium*). In most of these cases, the pathogens do not depend on only one receptor family for cellular entry. In addition, cell or organ tropism may be determined by recognition of different members of the same family.

Signaling events at the surface of the host cell, between pathogens of the same type, and between pathogen and host cell indicate a

**TABLE 1-3 Examples of Bacterial Virulence Regulatory Systems**

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>REGULATORY GENE(S)</th>
<th>ENVIRONMENTAL STIMULI</th>
<th>REGULATED FUNCTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>drdX</td>
<td>Temperature</td>
<td>Pyelonephritis-associated pili</td>
</tr>
<tr>
<td></td>
<td>fur</td>
<td>Iron concentration</td>
<td>Shiga-like toxin, siderophores</td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>bggAS</td>
<td>Temperature, ionic conditions, nicotinic acid</td>
<td>Pertussis toxin, filamentous hemagglutinin, adenylate cyclase, others</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>toxR</td>
<td>Temperature, osmolarity, pH, amino acids</td>
<td>Cholera toxin, pilE, outer membrane proteins</td>
</tr>
<tr>
<td><em>Yersinia spp.</em></td>
<td>lcr loci</td>
<td>Temperature, calcium</td>
<td>Secretion of effector proteins</td>
</tr>
<tr>
<td><em>Shigella spp.</em></td>
<td>mxi</td>
<td>Temperature</td>
<td>Adherence, invasiveness</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>pag genes</td>
<td>pH</td>
<td>Invasiveness</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>agr genes</td>
<td>Cell density</td>
<td>Virulence, macrophage survival</td>
</tr>
</tbody>
</table>

complex, highly evolved process of co-adaptation and co-optation.\textsuperscript{34,35,36} Many of these signals induce rearrangement of host cell cytoskeleton to the advantage of the pathogen. In a particularly dramatic example, enteropathogenic \textit{E. coli} induces the effacement of normal epithelial cell surface architecture and the formation of a specialized structure containing reorganized actin that protrudes from the host cell surface and is called a “pedestal,” or pseudopod (Fig. 1-1).\textsuperscript{37} These events require a specialized secretion system (see “Subversion of Host Cellular Processes and Immune Defenses”) that delivers not only Tir but also effector proteins that direct host cell signaling events. Disruption of normal absorptive function results in diarrhea. Other bacterial pathogens are also capable of inducing pedestal formation on intestinal epithelial cells. (From Rosenshine I, Ruszkowski S, Stein M, et al. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. EMBO J. 1996;15:2615-2624. Courtesy B. B. Finlay.)

Early escape from the vacuole is essential for the growth and virulence of some intracellular pathogens. \textit{Listeria monocytogenes} relies on several molecules for lysis of the early phagosome, including a pore-forming hemolysin (listeriolysin O) and two forms of phospholipase C. Once in the cytoplasm, \textit{Listeria} replicates and induces its own movement through a remarkable process of host cell actin polymerization and formation of microfilaments within a comet-like tail. \textit{Shigella} also lyases the phagosomal vacuole and induces the formation of similar structures for the purpose of intracytoplasmic movement and cell-cell spread. In both cases, bacterial and host factors involved in actin polymerization have been identified.\textsuperscript{38} In the same way that microbial pathogens fare differently in their interactions with phagocytic cells, the outcome of intracellular parasitism for the host cell also varies considerably, depending on the specific host cell and pathogen involved.

**FIGURE 1-1** Scanning electron micrograph depicting pseudopod, or “pedestal,” formation by enteropathogenic \textit{Escherichia coli} (EPEC) as it interacts with the surface of an epithelial cell. This form of intimate adherence requires a bacterial adselin, intimin; a receptor of bacterial origin, Tir, that is injected into the host cell; and a series of EPEC-initiated signaling events. Disruption of normal absorptive function results in diarrhea. Other bacterial pathogens are also capable of inducing pedestal formation on intestinal epithelial cells. (From Rosenshine I, Ruszkowski S, Stein M, et al. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. EMBO J. 1996;15:2615-2624. Courtesy B. B. Finlay.)

**SUBVERSION OF HOST CELLULAR PROCESSES AND IMMUNE DEFENSES**

Pathogens can be distinguished from typical commensal microorganisms by the degree to which they subvert host cellular processes to their own advantage.\textsuperscript{39,40} Enhanced adherence or internalization of the pathogen, inhibition of host cell antimicrobial activity, altered inflammatory responses, enhanced pathogen multiplication, and host cell death are potential outcomes. As mentioned earlier, one common mechanism by which bacterial pathogens alter or subvert the host cell involves specialized secretion systems, such as the type III and type VI or contact-dependent secretion pathways and the type IV secretion pathway.\textsuperscript{41,42} Type III secretion systems from diverse bacterial pathogens share structural and functional features that suggest an evolutionary relationship with the bacterial flagellar apparatus.\textsuperscript{43} These systems are encoded by blocks of genes that are usually located within pathogenicity islands. Using a supramolecular structure that spans the entire cell wall and resembles a hypodermic syringe,\textsuperscript{44} pathogens secrete effector molecules directly across host cell membranes. Whereas Salmonella and \textit{Shigella} use type III secretion systems (Salmonella pathogenicity island 1 [SPI-1] and invasion plasmid systems, respectively) to mediate entry into host cells, Salmonella relies on a second type III system (SPI-2) for successful replication within an intracellular vacuole; this second system is expressed only when the organism occupies this privileged niche.

Type III and type VI secreted effector molecules mediate diverse tasks. \textit{Salmonella} SopE is secreted by the SPI-1 system and binds directly to members of the Rho small-molecular-weight guanosine triphosphatase protein family in the host cell cytoplasm; this action activates membrane ruffling.\textsuperscript{45} The \textit{Yersinia} YopH effector protein is a potent protein tyrosine phosphatase, virulence factor, and antiphagocytic factor; YopH inactivates inactivated tyrosine phosphatases, promotes tumor necrosis factor-\textit{z} production, and is critical for \textit{Yersinia} translocation from Peyer’s patches to lymphoid tissue and replication in the spleen. A number of pathogens, including \textit{Shigella} and \textit{Salmonella}, are capable of inducing cell death in macrophages and dendritic cells but not epithelial cells. Although the induction of cell death and frank apoptosis is a common shared strategy of many pathogens, each accomplishes this outcome through different mechanisms and with a different precise temporal program.\textsuperscript{46-49} Contact-dependent type VI secretion has been associated with bactericidal activity in a variety of gram-negative bacteria and a form of “dueling” behavior between heterologous bacterial species.\textsuperscript{50}

Manipulation of host cell fate and orchestrated choreography of inflammatory responses are recurrent themes in the strategies of microbial pathogens. For example, mycobacteria shut down host-beneficial myeloid differentiation primary response protein 88 (MyD88)-dependent recruitment of macrophages and activate an alternate recruitment program to gain residence in more permissive macrophages. Hence, the granuloma, for 100 years assigned a central role in “walling off” \textit{M. tuberculosis} infection, is as much a structure built by mycobacteria to promote their expansion and dissemination during early infection.\textsuperscript{51} Preclinical studies likewise suggest that long-term \textit{Salmonella} carriage is a reflection of the “selection” of a particular class of phagocytic cells to achieve long-term survival.\textsuperscript{52}
Because they establish dependent relationships with host cells, viruses often manipulate host cells in dramatic fashion. Human papillomaviruses and other animal viruses induce expansion of their preferred host niche by interfering with critical cell-cycle controls. In an interesting analogy, Rickettsia rickettsii blocks a host cell apoptosis defensive strategy to prolong the life of the infected cell, facilitate rickettsial replication, and then spread to other host cells. Mollusca contagiosum virus protects its host cell from oxidative or ultraviolet-induced damage by expressing a glutathione peroxidase–like selenoprotein that acts as a scavenger of toxic oxygen metabolites. Other opportunistic strategies of viral pathogens include suppression of viral antigen presentation by host cells and interference with host cytokine, complement, and interferon activities.

A wide variety of intracellular pathogens and their patterned molecular components are recognized by members of the NOD (nucleotide oligomerization domain)-like family of receptors, which then leads to activation of a cytosolic protein complex known as the inflammasome and activation of caspase-1, a protease that, in turn, promotes activation of proinflammatory cytokines and host cell death. The inflammasome can be viewed as a kind of ancient record of the long-standing interplay between phagocytes and microbes. The scope and sophistication of the means by which pathogens overcome the host barrier to their establishment, replication, persistence, and subsequent exit and transmission to a new host must be seen as one of the most impressive examples of evolutionary diversity and adaptation. We cannot hope to do justice to the topic here, but we do hope to inspire appreciation and respect among clinicians for their microscopic adversaries.

**IDENTIFICATION AND CHARACTERIZATION OF VIRULENCE GENES**

Characterization of microbial pathogenicity at the molecular level has traditionally begun with the identification of a virulence-associated phenotype. Such identification may come from clinical observation, epidemiologic investigation, or the use of a model system that reliably reproduces the microbial phenotype in a manner similar to that seen in natural infection. Traditionally, a virulent strain was compared with a naturally occurring avirulent variant. Such variants, however, may have complex genotypic alterations involving multiple genetic loci. Today, increasingly sophisticated computational tools and ever more easily acquired complete genome sequences have led to predictions of phenotype and identification of candidate virulence genes from the sequence. The inspection of genome sequences from multiple strains, each with variant virulence phenotypes, provides a powerful starting point for subsequent hypothesis testing by using specific engineered mutant strains, when the microbial species is genetically tractable. Some pathogens and many commensals, however, remain genetically intractable or resistant to laboratory cultivation. For these organisms, emerging methods offer the possibility of examining their behavior and putative properties directly from clinical specimens without the need for laboratory propagation (see "Molecular Microbiology at the Bedside: Pathogen Detection, Pathogen Discovery, and Genomic Profiling").

Some of the now-standard approaches for identifying virulence-associated genes include the use of insertional elements (e.g., transposons) as mutational agents for generating isogenic mutant strains. Transposons have the advantage of marking the mutagenized genetic locus with a new selectable phenotype, typically antibiotic resistance, but the disadvantage of possible pleiotropic effects on cotranscribed genes or on overall microbial fitness. The development of broad-host-range plasmid vectors carrying well-defined transposons has extended this method of analysis to a number of pathogenic species for which a method of genetic manipulation was not previously available. By using transposable elements with unique genetic tags, negative selection can be applied to a pool of random mutants in a relevant model of pathogenesis. This approach, known as signature-tagged mutagenesis, has identified a number of genes in gram-negative and gram-positive bacteria that are essential for virulence.

Other methods for the identification of virulence-associated genes are based on the regulation of such genes by the transitions between external and internal host and cellular environments. Two approaches allow investigators to select for genes and promoters that are preferentially expressed by a microbial pathogen within a host cell or within a host organ. These approaches rely on specially designed vectors into which a complete library of chromosomal genes are cloned such that when the promoters for these genes are activated, they turn on the expression of factors that can be easily selected, either by expression of antibiotics, by complementation of an engineered growth-attenuating mutation in the pathogen, or by expression of a fluorescent protein. In the first approach, the application of *in vivo expression technology* to *V. cholerae* and *S. typhimurium* has clarified the conditions encountered by pathogens in vivo as well as their regulatory responses. By using a second approach, termed *differential fluorescence induction*, researchers identify promoters that are selectively induced within host cells or tissues by fusing random fragments of a pathogen's genome to the gene encoding green fluorescent protein and then applying fluorescence-activated flow cytometry to a pool of recombinant organisms bearing these reporter gene fusions.

Broad-based, nonselective approaches for screening an entire genome and its complement of expressed genes are now quite feasible with DNA microarray and DNA sequencing technologies. DNA microarrays are high-density grids of probes displayed on a solid surface; tens or hundreds of thousands of probes can be arrayed in an area of 1 cm². By displaying probes for every gene and intergenic feature of a given pathogen on a microarray and hybridizing labeled genomic DNA or complementary DNA (cDNA), one can obtain a complete gene content or expression profile, respectively, for the pathogen under any desired condition. Comparative genomic hybridization of analysis of multiple strains of pathogens reveals localized regions of frequent gene gain and loss ("regions of difference," equivalent in many cases to pathogenicity islands) and can provide insight into pathogen evolution as well as novel mechanisms of virulence. More recently, the ease with which complex pools of DNA or RNA molecules can be sequenced simultaneously in parallel has facilitated other approaches for screening large numbers of transposon mutant strains and identifying genes associated with microbial fitness. Today, quantitative measurements of gene transcripts and comparisons of transcript abundance are greatly facilitated by high-throughput random sequencing of cDNA and the generation of millions of expressed sequence tags that are then mapped back to genes and genomes with a method called "RNAseq." With these approaches, genes and their products are incriminated by their relationship with a disease-associated process. Final proof, however, that a gene is associated with pathogenicity requires that certain criteria be met. In a manner analogous to Koch's original postulates, these criteria must include return of the putative causal agent (the cloned virulence-associated gene mutated or intact) to the host of origin. Unless one can demonstrate an effect on pathogenicity by this kind of manipulation, causality with respect to virulence has not been proved. Just as the original Denle-Koch postulates have provided a reference point for later revised criteria of microbial causality, a molecular form of Koch's postulates can serve as a guideline for an experimental approach to the molecular genetic basis of pathogenicity. These postulates continue to coevolve in conjunction with emerging insights into microbial virulence and rapidly improving experimental approaches and technologies. For example, alternative approaches for proof of causation are necessary for pathogens that cannot be isolated and for disease in which a "pathogenic community" is believed to be the cause.

**MOLECULAR MICROBIOLOGY AT THE BEDSIDE: PATHOGEN DETECTION, PATHOGEN DISCOVERY, AND GENOMIC PROFILING**

As mechanisms of microbial pathogenicity, acquisition of virulence, and drug resistance are revealed, pathogen detection, strain identification, and pathogen discovery assume increasing importance in the practice of clinical infectious diseases. It is already apparent that studies of microbial pathogenicity at the molecular level have made substantial contributions to our understanding of the epidemiology, clinical manifestations, diagnosis, treatment, and prevention of infectious diseases. Even the fundamental issue of disease causation and the
possible role of microorganisms and microbial communities in chronic diseases of uncertain etiology must be reexamined in light of newer experimental methods and insight.

Infectious disease epidemiology hinges on a clear definition of the clinical problem under study and, moreover, precise identification of the etiologic agent. Molecular techniques provide for the sensitive and specific detection of putative pathogens and supply a means for establishing relationships among multiple isolates of the same species. As a result, seemingly unrelated cases occurring during an outbreak have been connected; similarly, geographically or temporally distinct outbreaks have been linked to the same pathogenic clone. Molecular techniques have been used in other epidemiologic investigations to study transmission mechanisms and the role of virulent microbial variants in the spread of disease. Whole-genome sequencing sometimes provides the only clue that a group of cases are related, that is, that an outbreak of disease has occurred, as well as the relationships of the outbreak strain to other strains.78 Morphologic and general metabolic features often fail to indicate the important genetic diversity found within strains.

Nucleic acid amplification techniques have had a far-reaching impact on our study of microbial pathogenesis and the diagnosis of infectious diseases. For a variety of reasons, some of them economic (reimbursement structure for clinical testing) and some of them technical (suboptimal sample preparation methods, and because of insufficient attention to the complexity of specimen matrix), specific polymerase chain reaction assays for microbial pathogens have failed to penetrate the clinical workplace to a thorough degree. Current methods for the identification of microbial pathogens have failed to cultivate or propagate in the laboratory. Molecular pathogen discovery methods avoid these dependencies and have spawned new search algorithms that might play important causal roles in a wide variety of poorly explained acute and chronic diseases.10,19,100 By providing a speedy and specific microbiologic diagnosis, these methods will encourage the development and deployment of targeted therapeutics, and damper the empirical use of broad-spectrum antimicrobials, thereby reducing selection for resistant organisms. The principle behind these methods is reliance on molecular signatures to identify or classify a previously unrecognized pathogen; the most commonly used signature is the genomic sequence, but other small molecules may prove useful. One of these methods targets highly conserved regions of ribosomal RNA gene sequences by amplifying them directly from digested, infected human tissue.99 Reliable evolutionary relationships of a putative organism can then be established from these amplified sequences. A number of organisms resistant to cultivation or propagation have been identified with non–culture-based methods.100,102 Some of the same molecular methods are used to explore diversity within the indigenous microbial communities that populate the skin and mucosal surfaces of the human body.

Conceptual advances in our understanding of microbial virulence, revolutionary developments in our technical means, and emerging challenges from a rapidly changing environment around us suggest a number of future scenarios and goals. First, we should focus our efforts on the identification and characterization of pathogens directly from clinical specimens and infected hosts, using cultivation-independent approaches. Manipulation and genome-wide characterization of single bacterial cells is now entirely feasible.103 Deep sequencing–based pathogen identification from clinical specimens is also a reality.104,105 We should expect to be able to measure genome-wide microbial transcript abundance and metabolic activity directly from human specimens as well. Second, the composition and function of the indigenous microbial communities can be assessed using metagenomic and other community-wide postgenomic technologies.8,106 By combining assessments of community and human response, we stand to gain novel insights into the nature of chronic inflammatory disorders of skin and mucosa.107 Third, it is now time to fully embrace the importance of host genetic variation in differential susceptibility to infection and subsequent disease.100 Fourth, genomic and postgenomic technologies enable us to measure and interpret patterns of human gene and protein expression associated with the response to infectious disease; these patterns may serve as the basis for signatures, enabling early recognition and classification of patients on the basis of agent or future disease course.109-112 As virulence factors for essential steps in pathogenesis are identified, it should be possible to interfere with their function. As they become better characterized, manipulation of virulence regulatory systems may have therapeutic value. New acellular or recombinant live-attenuated vaccines and vaccine candidates have already resulted from the identification of immunoprotective antigens through molecular and genomic approaches.113 The result of these efforts should be a more informed and effective approach to the detection, treatment, and prevention of infectious diseases.

Key References

The complete reference list is available online at Expert Consult.


References