Pathogenesis of Bacterial Infections in Animals

Fourth Edition

1
Themes in Bacterial Pathogenic Mechanisms

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INTRODUCTION

The speed of progression of our understanding of pathogenic bacteria and their interactions with the host at the molecular level is providing novel insights and perspectives on pathogens and pathogenicity at an almost overwhelming rate. Such information and insights are of fundamental value in designing better and unprecedented ways to counter infectious diseases. For example, studies on the use of drugs that jam quorum sensing communication systems have shown promise that this approach may be an effective method of preventing virulence regulons from being activated (Hentzer et al. 2003; Rasko et al. 2008). In a recent study, Rasko et al. (2008) identified a novel compound (LED209) that blocks the bacterial histidine sensor kinase, QseC, which is found in several gram-negative bacterial pathogens and is required for expression of certain virulence genes. These authors have shown that LED209 is nontoxic to mice and protected mice against death due to *Salmonella* Typhimurium or *Francisella tularensis*. Rasko and coworkers (2008) have noted that, unlike antibiotics, this anti-virulence approach does not threaten the life of the bacteria and may therefore not exert a selective pressure that selects for resistant organisms. However, if this method is a threat to a critical niche for these bacteria, it could also have a selective effect.

Although an overview of the basic themes in bacterial pathogenic mechanisms provides a conceptual skeleton for the extensive details of individual pathogens given in later chapters, understanding of virulence and pathogenicity is changing rapidly. The fundamental concepts have withstood the test of time fairly well, but new knowledge has brought the complexities of host–pathogen interactions into sharper focus and has identified nuances that had not been recognized *previously* (Finlay and Falkow 1997; Bhavsar et al. 2007). Although more is understood about bacteria, especially through the application of genome sequencing and related technologies, bacterial infections seem to be increasing and changing, in particular those associated with increased antibiotic resistance, driven by exposure to more powerful antibiotics. Numerous anthropogenic activities including antibiotic use at both therapeutic and subtherapeutic concentrations may be driving bacterial evolution and the selection of pathogens adapted to changed circumstances (Chopra et al. 2003; Davies et al. 2006). Against the background of stunning advances in technologies, there is increasing recognition of the poor general application of well-established simple infection control techniques such as hand-washing to reduce the burden of infection in people and in animals in clinical settings. The fight against bacterial infections requires constant vigilance and disciplined use of hard-earned knowledge, not simply the application of new technology.

BASIC STEPS IN PATHOGENESIS CONTINUE TO PROVIDE A SOUND FOUNDATION

The basic steps in the establishment of infection by a bacterial pathogen are:

1. attachment or other means of entry into the body;

2. evasion of normal host defenses against infection;
3. multiplication to significant numbers at the site of infection and/or spread to other sites;
4. damage to the host, either directly or through the nonspecific or specific immune host response to the bacterium;
5. transmission from the infected animal to other susceptible animals, so that the infection cycle can continue.

As would be expected for carefully regulated systems, the infection process is a dynamic continuum rather than a clear series of steps, but breaking it down into progressive steps allows ease of understanding.

Pathogen Association with the Host

Successful colonization of the skin or a mucosal surface of the host is usually the first prerequisite of the infectious process. Some organisms need to employ motility and chemotaxis as well as resistance to acid and bile in order to reach their target host cells. Initial contact between bacterial pathogen and host cell is usually mediated by fimbrial or nonfimbrial adhesins on the bacterial surface (Kline et al. 2009). Binding may result either in extracellular colonization or in internalization of the pathogen. The adhesins bind to specific host cell surface receptors, and both host and organ specificity of infection may be determined by differences among animals in cellular, receptors for the bacterial adhesins. For example, the *Listeria monocytogenes* adhesion molecule internalin A (InlA) promotes uptake of the bacterium into intestinal epithelial cells by binding to E-cadherin. InlA binds to human and rabbit E-cadherin and causes disease in these species; however, it fails to bind to mouse E-cadherin and so does not cause disease in mice. Interestingly, Wollert et al. (2007) recently showed that by making two substitutions in InlA they could increase the binding affinity to mouse E-cadherin by 10,000-fold and thereby establish experimental infection in mice. The researchers noted that newly emerging diseases may arise by similar naturally occurring mutations.

As many receptors are developmentally regulated, age specificity may also be determined by the receptor to which a pathogen binds. This is seen in K99 (F5) pili of porcine and bovine enterotoxigenic *Escherichia coli* (ETEC), which bind to the intestinal epithelium of neonatal animals, and in F18 pili of porcine ETEC, which bind to the intestinal epithelium of recently weaned pigs.

Bacterial pathogens, including those associated with wound infections, may bind to extracellular matrix molecules such as fibronectin, collagen, laminin, or other proteins possessing RGD (Arg-Gly-Asp) sequences for binding of eukaryotic cell membrane integrins. Bacteria may use "invasins" to mediate their uptake into nonprofessional phagocytic host cells after attaching to molecules on the cell surface and activating host cell signaling to facilitate their entry, often through host cell cytoskeletal rearrangement (Galán and Cossart 2005). An excellent example of this is found in the adherence to and invasion of M cells by *Yersinia enterocolitica* and *Y. pseudotuberculosis*. The outer membrane protein invasin produced by these bacteria binds to β1 integrin on the surface of M cells and triggers uptake of the bacteria in a zipper-like internalization process (Hauck 2002). This entry provides the bacteria with access to the lymphoid tissue below, and to draining lymph nodes, in which the bacteria are well equipped to multiply.

Facultative intracellular pathogens may deliberately target macrophages, for example by entering through complement- or other lectin-binding receptors and thus avoiding the oxidative burst that might otherwise kill them. Remarkably and, at first sight, paradoxically, the safest place in the body for these organisms, which subsequently interfere with normal macrophage phagosome maturation, is actually a macrophage.

Pathogen Multiplication and Evasion of Host Defenses

After initial association with the host, bacterial pathogens need to evade host defenses and to multiply to numbers sufficient for the infection to be self-sustaining rather than to be aborted by the host response. The "defensins" involved in the evasion–multiplication process can be divided into those involved in defense against innate immune mechanisms and those involved in defense against specific immune mechanisms.

Innate immunity can be overcome in a wide variety of ways (discussed throughout the book, in particular Chapter 2). The lack of available iron that restricts the growth of many bacteria within the body is an important defense mechanism as iron is critical for iron-containing cofactors for enzymes

required for primary and secondary bacterial metabolism. This limitation is often overcome by the iron-acquisition systems of pathogens. Recognition of the importance of iron acquisition by pathogens has led to a recent focus on inhibiting siderophores in the development of novel antibacterials (Miethke and Marahiel 2007). This is part of an approach that recognizes that inhibition of bacterial growth alone as a screening approach to antibacterial-drug discovery will result in numerous potential important pathogen targets being missed (Davies et al. 2006).

Many organisms, particularly those that cause septicemia and pneumonia, have prominent, usually carbohydrate, capsules that help the organism resist phagocytosis in the absence of antibodies. Some capsules mimic host matrices so that the organisms are unrecognized by phagocytes. The lipopolysaccharide molecules of some gram-negative bacteria can protect them from the membrane attack complex of complement or from the insertion of antimicrobial peptides. Some bacteria such as streptococci can break down complement components through C5a peptidase or other proteases. Other bacteria may destroy or impair phagocytic cells through their leukocidins such as the RTX (repeats in the structural toxin) toxins, or enable bacteria to survive inside phagocytes through enzymes such as superoxide dismutases or catalases.

Acquired immunity can be overcome in several ways. These include the ability to degrade immunoglobulins with enzymes such as the IgA proteases of *Histophilus somni*, or the ability to alter the antigenicity of cell surface components such as fimbriae or outer membrane proteins. Bacterial superantigens can dramatically up-regulate certain T cell subsets with specific V_β regions, which may result not only in a "cytokine storm," which confuses the immune system, but also in the deletion of these cells from the immune repertoire. In ways that are not well understood, some bacteria, such as *Rhodococcus equi*, may modulate the cytokine response to infection so that an ineffective Th2 rather than effective Th1-based immune response leads to development of disease. The role of "modulins" in diverting the host immune response is far less well understood for bacteria than for viral infections.

Pathogen Damage to the Host

Bacterial damage to the host is usually essential for immediate or long-term acquisition of the nutrients that the bacterium needs to thrive and to continue its pathogenic lifestyle. Infection does not always lead to disease, which is only one of the possible outcomes of bacteria–host interaction. Other outcomes include commensalism and latency.

Among the wide variety of "offensins" produced by bacteria are many different types of toxins. Toxins can be classified in different and not fully satisfactory ways, although that based on activity is the most logical (Wilson et al. 2002). Type I toxins, the membrane-acting toxins, bind to cell surface receptors to transduce a signal that results in the activation of host cell pathways, leading to aberrant cell metabolism. Examples in *E. coli* include the heat-stable enterotoxin a STa, which binds to the receptor for guanylyl cyclase, resulting in hypersecretion due to excessive levels of cyclic guanosine monophosphate (cGMP), and the cytotoxic necrotizing factor (CNF) toxins, which activate Rho guanosine triphosphatases (GTPases), resulting in cytoskeletal rearrangements. Other examples include the *Bacillus anthracis* edema factor (EF), the *Pasteurella multocida* toxin (PMT), and the exoenzyme S (ExoS) toxin of *Pseudomonas aeruginosa*. The superantigens fall into this class. Type II toxins, the membrane-damaging toxins, include the membrane channel-forming toxins using the β-barrel structure (e.g., *Staphylococcus aureus* α-toxin), channel-forming toxins involving α-helix formation, the large range of thiol-activated cholesterol-binding cytolysins, and the RTX toxins. Type II toxins that damage membranes enzymatically include the phospholipases of many bacteria. Type III toxins, the intracellular toxins, are toxins that enter and are active within the cell. These are often active-binding (AB) two-component toxin molecules. Examples include the adenosine diphosphate (ADP)-ribosyl transferasesf (e.g., the *E. coli* heat-labile enterotoxin [LT]), the N-glycosidases (e.g., the Shiga toxins), the adenylate cyclases (e.g., the *Bordetella bronchiseptica* adenylate cyclase toxin), and the metalloendoproteases of the clostridial neurotoxins.

Tissue damage and impairment of host function is often due to the inflammatory response mounted by the host in response to infection with a bacterial pathogen. Sepsis represents an extreme case in which hyperresponsiveness to lipopolysaccharide (LPS) and/or other host signaling molecules unleashes an excessively strong inflammatory response resulting in vascular damage, hypotension, and multiple organ damage. The inflammatory response

mounted by the host may also provide a point of entry for certain invasive enteric pathogens, such as *Shigella dysenteriae*. This organism carries a virulence plasmid-encoded homologue of the *msbB* gene in addition to the chromosomal copy, and it has been suggested that this may ensure complete acylation of lipid A and production of highly stimulatory LPS. The massive leukocyte infiltration between epithelial cells promotes invasion by the pathogens (D'Hauteville et al. 2002). A similar arrangement for the *msbB* gene exists in *E. coli* O157:H7.

Pathogen Transmission from the Host

Although not often considered in a discussion of bacterial pathogenesis, a crucial feature of bacterial pathogens is their ability to use their pathogenic nature to ensure their further transmission from the host, either back into their environmental reservoir or directly to other susceptible hosts. Depending on the infection, further transmission to animals may be immediate or may even involve decades.

An important aspect of transmission involves bacterial infections of animals that are important primarily because of the transmission of organisms from animals to humans. In some cases, as with Shiga toxin-producing *E. coli* (STEC) O157:H7, the bacteria are normal flora in the intestine of animals, where they do not cause disease; however, they do induce severe disease following transmission to humans. A similar situation exists for *Campylobacter jejuni and* most serotypes of *Salmonella* in poultry. Efficient transfer from their reservoir hosts to their accidental host occurs directly through contamination of foods of animal origin and indirectly through fecal contamination of water and the environment.

CONCEPTS OF VIRULENCE ARE BEING REFINED

Bacteria cause disease by a variety of virulence mechanisms in a highly complex process that usually involves penetrating the host's protective barriers, evading deeper host defenses, multiplying to significant numbers, and damaging the host, leading to escape from the host to continue the cycle. Although this concept of virulence is well established, the resurgence or emergence of infectious diseases in humans in recent years because of changes in host susceptibility (AIDS, immunosuppressive drugs, hospital-acquired infections) emphasizes the importance of host factors in determining the outcome of encounters with microbes. Many people now die in hospitals from infectious agents that are not pathogens in healthy people. A parallel situation exists in many small animal hospitals, especially in intensive care units. Similarly, the ability of some bacteria rapidly to develop or acquire antimicrobial resistance and then to emerge as significant problems in hospital or community settings emphasizes the importance of environmental factors in determining the outcome of infection. Virulence does not occur in a vacuum; it is contextually dependent. In this case, antibiotic use in hospitals may remove the inhibitory effects of the normal microbial flora in reducing colonization by exogenous, resistant, bacteria. Furthermore, bacterial pathogens themselves may carry genes for bacteriocins that are sometimes linked to virulence genes or bacteriophages that possess virulence genes, which are an important part of their success as pathogens. Selective pressures other than just interaction with the host may exert profound influence on the evolution of pathogens (Brown et al. 2006).

The impact of infection on the evolution of animal hosts can generally only be speculated upon but may be profound. The evolution of hosts and the pathogens that exploit them are inexorably linked (Brown et al. 2006). For example, the target of the *Vibrio cholerae* toxin (CT) and *E. coli* heat-labile enterotoxin (LT) is the cystic fibrosis transmembrane conductance regulator (CFTR) protein, whose response to CT leads to fluid outpouring in the intestine. The CFTR protein is necessary for fluid secretion in the intestine and in airways, and intestinal tissue from patients with cystic fibrosis fails to respond to CT. It has been suggested that the defects in the CFTR gene that provide resistance to cholera may have led to the maintenance of defective genes in the human population and the high frequency of the delta F508 mutation (1 in 25); individuals who are homozygous for this mutation develop cystic fibrosis. Interestingly, recent evidence suggests that this mutation may also provide protection against infection with *Salmonella* Typhi (Pier et al. 1998). The historical association of pathogens and their hosts, and the coevolutionary nature of this relationship, are also part of the host–pathogen–environment triad that determines the outcome of an infection.

Earlier definitions of virulence often derived from older studies of classic bacterial pathogens ("Koch's postulates"), many of which have been controlled by immunization, hygiene, or antimicrobial drugs. These definitions were markedly updated (Falkow's [1988] "molecular Koch's postulates") but were still largely pathogen-centered and focused on a narrow

range of virulence determinants such as exotoxins of *Corynebacterium diphtheriae* and A more recent theme, even ignoring host and environmental interactions with the pathogen as determinants of disease, has emphasized that bacterial virulence is multifactorial, involving not only "true" or "essential virulence genes" that are directly responsible for host damage, but also "virulence lifestyle genes" that regulate essential virulence genes or are otherwise required for their expression, secretion, or processing, as well as " virulence lifestyle genes" that allow bacteria to colonize the host, evade host defenses, use host factors for survival, or survive intracellularly (Wassenaar and Gaastra 2001). An analogy is to a gun: the bullets can be considered the true virulence genes, the gun can be considered the virulence-associated genes, and the criminal can be considered the virulence lifestyle genes. Clearly, inactivation of any of these three elements will stop the bullets killing a victim, but ultimately it is the bullets that kill. Recognition of the distinction of these different elements will prevent some of the potential confusion that faulty interpretations of modern experimental methods produce. Bacterial virulence is thus more clearly than ever recognized as the truly complex, dynamic, changeable, and often surprising phenomenon that it is.

This view of bacterial virulence highlights the survival and successful further spread of bacteria under potentially adverse conditions in the ecological niche(s) into which they have been introduced or to which they have adapted, and all the complexity that successful survival implies. From this perspective, antimicrobial resistance genes may contribute to virulence as they are virulence lifestyle genes that contribute to survival in antibiotic-containing environments.

HOST–BACTERIA COMMUNICATION IS CRITICAL

It has long been recognized that the outcome of infection is dependent on complex multistep processes involving host, pathogen, environment, and their interactions. Nonetheless, the tendency has been for researchers to tackle problems of pathogenesis primarily by investigation of virulence attributes of the pathogen. One of the outcomes of this approach is that we now have an impressive catalog of virulence genes of bacterial pathogens, but we have a long way to go in understanding issues of regulation, timing, cross talk, and interplay with host structures and physiology. In recent years, researchers have sought to redress this imbalance, and we have seen numerous investigations of pathogens in either their natural host environments or in *in vitro* settings that seek to simulate aspects of the *in vivo* environment. It is therefore not surprising that a major theme in pathogenesis research is that communication among bacteria, host, and environment is a critical aspect of pathogenesis. Studies in this field have led to a new branch of microbiology, namely cellular microbiology, which investigates bacterial signal transduction as a tool for characterizing host signaling pathways.

Bacteria have an astounding ability to sense their environment and rapidly to respond to it. Bacteria–host–environment communication systems that are important in pathogenesis may involve combinations of bacterial type III secretion systems (TTSS), type IV secretion systems, host cell cytoskeletal rearrangement, quorum sensing, two-component regulatory systems, and stress responses. Studies of TTSSs have identified a conservation of the secretion apparatus and a remarkable diversity in the effector functions mediated by the systems in extensively investigated bacterial pathogens such as *Salmonella*, *Shigella*, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *Yersinia*. The effectors of the TTSSs are virulence factors that interact with specific host cell structures and factors that set off complex host cell pathways (Gruenheid and Finlay 2003).

Bacteria need to be aware of their environment so as to know when to deploy their virulence genes. Cues to bacterial location are as diverse as temperature, pH, growth phase, nutrient availability, oxygen levels, ion concentrations, and quorum sensing molecules, or combinations of these cues. Depending on the environment, some virulence genes may be upregulated while others are downregulated only to reverse when the environment changes. The regulation of virulence is complex, with several regulators often controlling the expression of a particular virulence gene and with coordinate regulation of genes whose products are required under the same circumstances. The practical application of knowing how virulence genes are regulated is that regulation is a potential and underexploited target for novel synthetic or natural inhibitory molecules.

An interesting study by Hentzer and colleagues (2003) showed in a dramatic fashion how sabotage of the bacteria–host communication system might be used to attenuate bacterial virulence. These

researchers targeted the quorum sensing circuits of *P. aeruginosa*, which are known to regulate critical virulence factors in this organism. The researchers demonstrated that a synthetic furanone compound, C-30, was antagonistic toward the quorum sensing systems of *P. aeruginosa.* The researchers then used transcriptome analysis to identify 93 genes that were more than fivefold affected by application of C-30 to a culture of *P. aeruginosa*, and noted that 30 of 85 C-30-repressed genes were quorum sensing-controlled major virulence factors. Additional experiments showed that growth of *P. aeruginosa* biofilms in the presence of C-30 resulted in biofilms that had lost their resistance to sodium dodecyl sulfate (SDS) and to tobramycin. Subsequent experiments demonstrated that C-30 was effective against *P. aeruginosa* in the lungs of infected mice: those mice that were treated had lung bacterial contents that were, on average, 1/1000th those of untreated mice. Encouraging data obtained during the study included observations that the effect of C-30 was highly specific as it targeted only the *las* and *rhl* quorum sensors, and was effective against both planktonic and biofilm cultures. The authors suggested that an attack on expression of virulence was unlikely to create pressures for resistant mutants to develop.

Further studies that pursue this "antipathogenic drug principle" have used computer-assisted structure-based virtual screening to identify other compounds that inhibit quorum sensing-regulated gene expression (Yang et al. 2009).

New regulatory signals that are critical for virulence expression are being identified. For example, EHEC O157:H7 expresses the colonization genes encoded by the locus for enterocyte effacement (LEE) in response to a quorum sensing regulatory molecule that was initially considered to be autoinducer 2 (AI-2; Sperandio et al. 2001) but has now been shown to be a new autoinducer called AI-3 (Sperandio et al. 2003). Both AI-2 and AI-3 require LuxS for their synthesis. Interestingly, the mammalian hormones epinephrine and norepinephrine had the same effect as AI-3 in activating the LEE-encoded genes (Sperandio et al. 2003). Furthermore, either exogenous AI-3 or epinephrine could activate the LEE genes in a *luxS* mutant, and epinephrine antagonists could block this activation. These data suggest that AI-3 and epinephrine may use the same bacterial signaling pathway in cross-talk between host and pathogen. Epinephrine and AI-3 act synergistically, and it has been proposed that EHEC in the intestinal lumen respond to AIs produced by commensal and pathogenic bacteria, resulting in activation of motility. Once the bacteria arrive at the host epithelium, epinephrine stimulates expression of the LEE genes, thereby allowing EHEC to attach to the intestine (Kendall et al. 2007). Evidently, factors that influence the epinephrine/norepinephrine content in the intestine may play a significant role in expression of LEE and the virulence genes in EHEC. In *P. aeruginosa*, the quorum sensing signal can act directly on host cells to stimulate production of the pro-inflammatory cytokine IL-8 (Smith and Iglewski 2003). It will be interesting to see whether in an analogous fashion AI-3 has a direct effect on host intestinal epithelial cells.

Signaling that affects host apoptosis pathways is a common aspect of pathogenesis of bacterial diseases. Bacterial products that have been shown to induce apoptosis include outer membrane proteins, LPS, lipomannans, lipoarabinomaannans, lipoproteins, porins, and certain protein toxins. Macrophages, by their possession of receptors for conserved bacterial surface components such as LPS and lipoprotein, are highly vulnerable, as apoptosis may be triggered by reactions with these receptors.

Apoptosis is a common feature of pathogenesis in a wide range of pathogens including *Campylobacter*, *Chlamydia, Escherichia*, *Listeria*, *Mycobacterium*, *Pseudomonas*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, and *Yersinia.* Apoptosis may provide benefits to the host by curtailing the primary immune response, thereby limiting damage due to excessive release of cytokines and destructive neutrophil enzymes. On the other hand, it may be of value to the parasite by destroying host defense cells such as macrophages, thereby promoting bacterial invasion of tissues and prolonging infection.

Bacteria are able to induce apoptosis by stimulating proapoptotic molecules or inhibiting anti-apoptotic molecules (Grassme et al. 2001). Direct activation of pro-aptototic signals is seen in infection by *Salmonella*, *Shigella flexneri*, and *Staphylococcus aureus*. *Shigella flexneri* uses its TTSS to inject M cells with IpaB, which binds to caspase 1, and activates its proteolytic function, thereby inducing apoptosis. Simultaneous release of IL-1 by the M cells attracts neutrophils and widens the junction between epithelial cells, thus facilitating access to the basolateral surface of epithelial cells, a site that is vulnerable to invasion by *S. flexneri*. *Salmonella*

Typhimurium also uses its TTSS to inject SipB, which activates caspase 1. The mechanisms by which *S. aureus* induces apoptosis are less well understood, but they involve activation of caspases, JUN-N-terminal kinases (JNK), and acid sphingomyelinase. Alpha toxin has been identified as the staphylococcal protein that initiates apoptosis.

A TTSS also features in induction of apoptosis in macrophages by *Yersinia*. These bacteria both activate pro-apoptotic pathways and inhibit anti-apoptotic pathways. Injection of YopP (*Y. enterocolitica*) or YopJ (*Y. pseudotuberculosis* and *Y. pestis*) into macrophages activates caspases and inhibits activation of mitogen-activated protein (MAP) kinase and MAP kinase kinases as well as nuclear factor κB (NfκB). Inhibition of the translocation of NfκB into the nucleus causes suppression of the production of TNF-alpha, a stimulator of apoptosis.

The translocated intimin receptor (Tir) of EPEC has been identified as a TTSS-delivered molecule that can trigger apoptosis in cultured epithelial cells (Malish et al. 2003). Tir was localized to the mitochondria, but its mechanism of induction of apoptosis is unknown. Shiga toxin injures host vascular endothelial cells by an apoptotic mechanism (Erwert et al. 2003). This activity is dependent on the A subunit of the toxin and is effected by inhibition of McI-1, a member of the Bcl-2 family of antiapoptotic proteins. *P. aeruginosa* depends on its TTSS to upregulate expression on the surface of epithelial cells of CD95/CD95 ligand, a receptor/ligand pair that stimulates apoptosis through signaling of caspases.

The end result of apoptotic changes varies in the different infections. For example, apoptosis triggered by *P. aeruginosa* appears to be critical for the host defense against lung infection, presumably by promoting phagocytosis of bacteria that are packaged in apoptotic bodies (Cannon et al. 2003). The muted inflammatory response involved in apoptosis may also be beneficial to the infected tissue and the host. In the intestine, the elimination of invading bacterial pathogens by an increased rate of apoptosis of intestinal epithelial cells may be an important mechanism for the elimination of pathogens such as EPEC (Malish et al. 2003). At the same time, deeper tissues may become vulnerable to invasion by intestinal bacteria. In contrast, apoptosis by *S. flexneri* is a critical aspect of infection by the bacterium. It is needed for invasion of the intestinal epithelium.

Host cells also have elaborate mechanisms for identifying conserved bacterial structures and relaying this information to pathways that respond to the presence of bacteria. Pattern recognition receptors (PRRs) on the surface of innate immune cells permit the recognition of infectious agents through their possession of pathogen-associated molecular patterns (PAMPs) such as LPS, lipoproteins, peptidoglycans, and DNA with unmethylated CpG motifs. Included among the PRRs are the Toll-like receptors (TLRs), which are signal transduction proteins that, among other actions, trigger the secretion of cytokines through activation of the transcription factor NfκB. Signaling by TLRs occurs primarily through the adaptor molecule MyD88. Recently, another adaptor molecule (Trif) was shown to be required for signals leading to the production of interferon-β following activation of TLR3 or TLR4. TLR3 detects double-stranded RNA; TLR4 recognizes LPS; and TLR2 recognizes lipoproteins, peptidoglycans, and lipoteichoic acid. Flagellin binds to TLR5 and causes the release of IL-8 from intestinal epithelial cells. Interestingly, TLR5 is expressed on the basolateral and not on the apical surface of intestinal epithelial cells so that the alarm is sounded only when bacterial invasion has occurred or bacterial products have reached this site. CpG–DNA interacts with TLR9, which is located intracellularly rather than at the cell surface. Internalization of CpG–DNA and endosomal maturation are necessary for activation of TLR9 (Ahmad-Nejad et al. 2002).

The TLRs help to link the innate immune response with the acquired immune response, as macrophages and dendritic cells that contact pathogens become activated, causing the upregulation of co-stimulatory cell surface molecules as well as class I and II major histocompatibility complex (MHC) molecules. Differential expression of TLRs on the various types of cells of the innate immune system and differences in the signals that are generated allow for a system in which the type of pathogen that is encountered is met with the appropriate Th1 or Th2 response. (Wagner 2002). Innate immune responses that occur following binding of the pathogen to a TLR include killing of the pathogen through antimicrobial compounds such as nitric oxide in macrophages and antimicrobial peptides at the surface of epithelial cells (Sieling and Modlin 2002). Adaptive immune responses are influenced through the activation of B cell proliferation, release of chemokines, and adjuvant effects of the PAMPs.

PATHOGENESIS IN THE POST-GENOMIC ERA

The enormous influx of information from genome sequencing is revolutionizing the science of pathogenesis, ranging from understanding the most basic aspects of gene content to elucidating the regulatory networks of virulence gene expression, to investigating the global patterns of host response to infection (Medini et al. 2008). Examining differences in specific genes between a pathogen and a closely related nonpathogen, or between the parent and its offspring with a specific null mutation, has been a valuable approach for identifying virulence genes. The rate of recognition of potential virulence genes is increasing dramatically as complete or almost complete genome sequences are now available for thousands of bacteria, and genomic data and microarray analysis are now frequently combined rapidly to identify hundreds of potential virulence factors simultaneously. However, these potential virulence factors will need to be tested individually to assess their roles in virulence.

The immensity and complexity of data that need to be analyzed are almost overwhelming (Medini et al. 2008). Initially, genomics brought us the concepts of the core genome (encoding the basic aspects of the bacterial biology) plus the dispensable and strain-specific genes for an isolate. We have now moved beyond this and the pan-genome (the genetic information of a bacterial species) and the metagenome (the genetic information of a community of bacteria in a specific environmental niche) have moved from theoretical constructs to reality.

Functional genomics can be used to investigate the transcriptome under specific conditions. Data from transcriptome studies are beginning to lead to a better understanding of memberships in virulence regulons and to the identification of the complex environmental cues that modulate virulence expression. Ideally, bacterial mRNA collected from infected tissues would be examined. However, relatively low numbers of bacteria in most infected tissues, relatively small amounts of bacterial compared with host RNA, and instability of bacterial mRNA make this approach impractical for most infections. Hence, it has been necessary to use amplification methodologies such as selected capture of transcribed sequences (SCOTS) or *in vitro* conditions to simulate the *in vivo* setting. One of the challenges in these studies is to accurately simulate the host microenvironment. Currently, it is common for only one or two aspects of that environment to be examined in simulations, for example temperature, iron concentration, and pH. It is likely that much more complex simulations will be attempted in the future.

Comparative genomics involving comparison of open reading frames (ORFs) of genomes have been a valuable starting point in the identification of virulence genes. For example, a comparison of the genomes of pathogenic *L. monocytogenes* and the closely related nonpathogenic *L. innocua* identified 270 genes that were specific to *L. monocytogenes* and 149 that were specific to *L. inocua* (Buchrieser et al. 2003). These genes were present in coding regions that were scattered over the genomes but typically different in guanosine plus cytosine (G+C) content than the 34% for Listeria-specific genes. The analysis has not so far identified new virulence genes. There are also exciting studies under way that exploit knowledge of the *E. coli* O157:H7 genome and the comparative virulence of other EHEC to identify genes that are associated with the extreme virulence of O157 EHEC compared with other Shiga toxin-producing *E. coli* (STEC), as well as variants within O157 in terms of host adaptation and virulence (M. A. Karmali, pers. comm.).

Genomic data, in combination with microarray technologies, have sometimes been used to probe not only bacterial metabolism in the host but also host changes in response to the presence of the bacteria. The enormous amount of data generated in these studies is often quite a challenge for analysis and interpretation. Typically, a large number of genes is up-regulated and down-regulated, and it is difficult to differentiate primary from secondary responses. The time at which a readout of mRNA is made is also critical as too long a delay may reveal only the steady state after much of the series of responses by bacteria and host have been completed. Like *in vivo* expression technology (IVET), these analyses identify genes expressed under certain conditions, and subsequent testing is needed to determine the subset of these genes that are essential for infection of the host and for disease. One meta-analysis of changes in gene expression by *Mycobacterium tuberculosis* in response to growth in macrophages showed poor concordance between the numerous studies (Kendall et al. 1994), suggesting that what may be apparently minor differences in experimental design, methodologies, and analysis

can have a dramatic effect on the results, which can essentially be useless.

Data mining of complete and incomplete genome sequences has been used to generate valuable information on virulence-related genes in bacteria. For example, 21 novel sequences that might encode ADP-ribosyl transferase activity were identified by this method in bacteria as diverse as *Streptococcus pyogenes*, *Mycobacterium avium*, *S.* Typhi, and *Mycoplasma pneumoniae* (Pallen et al. 2001). There is, however, a large gap between genomic analyses and functional genomics. This is exemplified by the fact that only about 60% of the genes of *E. coli* and 56% of the genes of *P. aeruginosa* have known functions. In addition, the presence of gene sequences does not necessarily mean that functional proteins are produced.

Newer rapid genome sequencing ability has permitted studies of metagenomics, thereby adding a new dimension to our capability to investigate pathogens in their natural environments. This will be particularly valuable in host niches such as the intestine, which have rich microbial communities whose interactions with pathogens are critical to health and disease.

EVOLUTION OF PATHOGENS—THE PATH TRAVELED MAY PROVIDE INSIGHTS INTO THE ROAD AHEAD

Evolutionary studies of pathogenic bacteria have shown that many have arisen by "quantum leaps" from nonpathogens as a result of acquisition of blocks of genetic material (that are sometimes very large) rather than by progressive mutations of existing genes. A large number of essential virulence genes are found on a variety of mobile genetic elements (bacteriophage, plasmids, transposons) that have been spread from other microbial sources through transformation, transduction, or conjugation, or combinations of these processes. The discovery of blocks of virulence genes on pathogenicity islands in phage insertion chromosomal hot spots has been one of the major surprises in recent years, reinforcing the concept that the evolution of virulence can be characterized in many cases as a dramatic process of "evolution by jerks" rather than as a slow, long-term, progressive refinement of "evolution by creeps" (point mutations, rearrangements) of existing genes to improve survival in different niches (although this is also important). Genomic islands, including pathogenicity islands, are dynamic and ancient integrative elements in the evolution of bacteria, including pathogens (Boyd et al. 2008). Clonal analysis of bacterial populations has been used to characterize the different times and populations at which these dramatic changes occurred.

For example, *Salmonella* evolved as a pathogen over the last 100 million years in three distinct phases and continues to evolve. Its infection by bacteriophages may have played a vital role in this process (Figueroa-Bossi et al. 2001). The first phase in this evolution involved acquisition of *Salmonella* pathogenicity island I (SPI 1). *Salmonella enterica* then diverged from *S. bongori* by acquisition of a second pathogenicity island (SPI 2). The final major phase was the process of branching into distinct phylogenetic groups, with a dramatic expansion of *S. enterica* subsp. I into warm-blooded animals (Bäumler et al. 1998). It may have thus evolved from a dinosaur into a mammalian pathogen. Some subsp. I serotypes further acquired the *Salmonella* virulence plasmid, which is characteristic of the major host-adapted serotypes, as well as the most virulent of the nonhost-adapted serotypes Enteritidis and Typhimurium. Possession of the virulence plasmid and its *spv* operon makes these serotypes particularly pathogenic (Bäumler et al. 2000).

The basis of the remarkably specific host adaptation of certain serotypes of *Salmonella* is unclear but may relate to the relative plasticity of the *Salmonella* genome afforded by phage-mediated transfer of a small number of host-specific virulence factors (Rabsch et al. 2002). It is in part a function of the presence of different types of specific fimbrial adhesins that recognize intestinal surfaces. For example, *S.* Typhimurium definitive phage type (DT) 49 and DT 104 appear to have a broad host range. However, in contrast, in Rock doves, *S.* Typhimurium var. Copenhagen is considered a specifically adapted subtype, with DT 2 and DT 99 being isolated almost exclusively from this species in Europe and North America (Rabsch et al. 2002). Certain strains of *S.* Typhimurium, particularly DT 40 and DT 160, may have become adapted to certain species of songbirds.

Not only has horizontal gene transfer through mobile genetic elements played a key role in the evolution of virulence, but many bacterial species are naturally competent for DNA molecules, so that DNA taken up from lysed bacteria within

microcolonies can lead to homologous recombination with mosaic genes that may give an advantage to their host. This may have both long-and short-term benefits to the organism. A classic example of immediate benefit is the formation of antigenically distinct variants of fimbrial adhesins by *Neisseria gonorrhoeae* selected out by the immune response of the host to the older major antigenic type.

In other cases, there has been an orderly buildup of virulence-related genes by horizontal transfer. This has been shown for *V. cholerae*, EHEC, and EPEC. In EPEC and EHEC, there has been clear evidence of selection for increasing virulence over time (Reid et al. 2000). The main advantages of the ability to induce diarrhea in the host are presumed to be an increase in the opportunity for acquisition of DNA in the intestine as colonization results in large numbers of pathogenic *E. coli* and enhanced transmission of bacteria in fluid stool. It is possible that there is coevolution to greater fitness, but this aspect has not been explored.

Bacteriophage encode many virulence, notably toxin, genes. Classic examples include botulinum toxin, cholera toxin, diphtheria toxin, Shiga toxin, and the superantigen genes of *S. aureus* and *S. pyogenes*. Phage may also transfer pathogenicity islands. The extensive recombination that is characteristic of bacteriophages may explain the variety of related toxin genes that they may encode. Plasmids may carry virulence genes that can be transferred through conjugation; in addition, plasmids commonly carry insertion sequences or transposons that can further mobilize virulence genes to the chromosome or to other plasmids. Plasmids, transposons, and integrons may carry antimicrobial resistance genes, some of which may be linked to virulence genes, suggesting that the use of antimicrobial drugs in animals may drive not only the evolution of resistance but possibly also the evolution of pathogens. The mechanisms of bacterial change are the same.

The emergence of pathogens has been associated not only with gain of genes, but also with their loss. Insertion sequences have had a dramatic impact on bacterial pathogen evolution, usually in the direction of reducing genome size and increasing host specificity. This is well illustrated in the evolution of *Bordetella pertussis* from the broad host range pathogen *B. bronchiseptica* to become an exclusively human pathogen (Preston et al. 2004).

The wide dissemination of families of virulence genes in unrelated bacterial populations may be explained by horizontal transfer. One example is the thiol-activated cholesterol binding cytolysins found particularly among gram-positive bacteria (e.g., listeriolysin, perfringolysin, pyolysin, streptolysin).

There is inherent competition between the ability of a bacterium to evolve through acquisition of virulence genes horizontally and fitness genes through mutation and rearrangement, and the need to maintain the integrity of the genome through the stabilizing mechanisms of DNA mutation repair, DNA restriction or modification, and other genetic barriers. As characterized by their diversity of hosts, their ability to cause quite diverse diseases, their ability to colonize different ecological niches, or their ability to acquire antimicrobial resistance genes, successful pathogens such as *E. coli* and *S. enterica* may be concluded to have an inherently greater ability to evolve, for example through a greater tendency to develop mutator mutants defective in DNA repair, than some perhaps more classical but certainly now unimportant pathogens such as *Erysipelothrix rhusiopathiae*. The low rate of evolution of some pathogens, such as *B. anthracis*, can be related to their lifestyle and environments.

Sometimes there are trade-offs between virulence and survival outside the host, as in the case of *Shigella* and the *cadA* gene. The *cadA* gene in each of the *Shigella* species has been independently inactivated (Day et al. 2001). *cadA* encodes lysine decarboxylase whose activity results in the production of cadaverine, which blocks the action of enterotoxins of *Shigella*, inhibits *Shigella*-induced polymorphonuclear leukocyte migration, and interferes with escape from the phagolysosome. This is considered a pathoadaptive mutation (a mutation that enhances survival by modification of traits that interfere with factors that are needed for survival in host tissues). The gene is retained by *E. coli* and is likely valuable in environments outside the host.

There are also instances in the evolution of pathogens where infection of animals may be regarded as almost accidental. For example, the ability of bacteria to survive and sometimes even thrive in the environment of macrophages as facultative intracellular pathogens may have first been developed by selection for their survival in amoebae; *Legionella pneumophila* is a classic example. Other pathogens, of which *P. aeruginosa* is the best example, likely evolved initially as plant pathogens but use the same virulence factors in causing disease in animals.

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