MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, June 1997, p. 136–169 Vol. 61, No. 2
1092-2172/97/$04.00+0

Common Themes in Microbial Pathogenicity Revisited

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INTRODUCTION

In 1989 we published a review article entitled Common Themes in Microbial Pathogenicity (89). That article reflected the burgeoning interest in the field of microbial pathogenicity, as well as our own enthusiasm for the application of the methods of cell biology to study bacterial pathogens. In fact, when we wrote that article, there were only a few examples of "common themes." Our decision to revisit this subject reflects our belief that distinctly different pathogenic bacteria bring about infection and disease via a relatively small number of common pathways. The massive influx of DNA sequence data, the clever use of new techniques like PCR, the exploitation of cell biology and cellular immunology methods (beyond our wildest dreams in 1989), and the impact of crystallographic methods and other molecular structure-function studies have been applied to the study of a variety of microbial pathogens and the genes that encode pathogenic traits. Significant information has emerged from these data that suggests that there are a number of common strategies for a bacterium to become a pathogen. That is not to say that all virulence factors and mechanisms can be placed into neat, specific classes; like most things in biology, there are many exceptions. However, increasing numbers of virulence determinants appear to share similar mechanisms and traits. These general themes are the subject of this review.

There is at least an order of magnitude more literature to review now than there was in 1989. We have made no attempt to review everything, although this article is much longer and factual than the 1989 effort. As was true in 1989, "Our choice of topics and the detail with which we discuss any subject reflect our own interests and not necessarily the relative importance of the subject to understanding microbial virulence" (89). Despite this caveat, we hope the reader will find useful views into the evolution of microbial pathogenicity and will share our excitement about the marvelous strategies that pathogens utilize in their interactions with the host. At a time when there is so much interest in emerging pathogens, along with the failure of anti-infective therapy to control some of the most common disease-causing microbes, there is more interest than ever in the field that has become known as microbial pathogenesis.

The mistake in the past has been to seek the control of infectious diseases with a "quick fix" based on the empirical discovery of novel "wonder drugs" or new vaccines. This approach worked but not as well or for as long as one would have wished. Additionally, it has been at least two decades since any new class of antibiotic has been discovered. We now have the opportunity to understand the fundamental factors that microbes use to cause infection and disease. It is from this understanding that eventually we will devise better means for the control of microbial disease. However, we also must remember that human life has evolved to live in a constant sea of microorganisms, and it is doubtful (if not impossible) that a gnotobiotic life is human destiny; instead, by understanding virulence mechanisms, we can attempt to design strategies to control microbial infection and disease. Realizing that seemingly diverse pathogens share virulence traits increases the likelihood that novel compounds can be developed to inhibit such processes. It also allows one to build a framework with which to understand the evolution of microbial pathogenicity.

TOXINS

Pathogenic bacteria produce many substances that are directly or indirectly toxic to host cells. Secreted microbial proteins, usually enzymes, that kill host cells at exquisitely low concentrations are called exotoxins. Other nonproteinaceous toxic bacterial substances, like lipopolysaccharide (LPS) or endotoxin, do not use a direct enzymatic mechanism to injure host cells and are biologically active at much higher concentrations. We do not discuss endotoxic mechanisms here, although we recognize, of course, that endotoxin is a critical component of the gram-negative cell wall. All gram-negative pathogens make endotoxin, although its toxicity varies among species.

Exotoxins often play a central role in the pathogenesis of microbial disease. For example, purified toxin alone can cause most of the clinical features of cholera, tetanus, and diphtheria. However, like most other virulence factors, exotoxins rely on other bacterial components to fully potentiate their expression, delivery, and effects on the host. These other microbial components include regulatory mechanisms, which often are linked to other virulence traits, and specialized machinery, which secrete toxins out of the bacteria. Also, adherence factors bring the microbe and host cell into close contact to deliver the toxin correctly to its specific host cellular target. Hence, although cholera toxin alone can cause many of the symptoms of disease, a cholera adhesin is needed for full bacterial virulence (319). Toxins also can have other functions that facilitate virulence, in addition to toxigenicity. For example, pertussis toxin also appears to facilitate the adherence of *Bordetella pertussis* to mammalian cells (339).

A-B Toxins

Several toxins that were previously thought to be unrelated actually have common structural and functional features, which facilitates the grouping of many known toxins into a limited number of families based on structural homology and biochemical activity. Many exotoxins exhibit an A-B structure (213). A-B toxins have two components: the B subunit(s), which mediates binding to the host cell receptor and facilitates delivery of the toxin into the host cell; and the A subunit, which contains the enzymatic (ultimately toxic) activity that acts on the host cell. The enzymatic activity in the A subunit varies among toxins and ranges from ADP-ribosylating activity (e.g., cholera, pertussis, and diphtheria toxins) to proteolytic activity (e.g., tetanus and botulinum toxins). Although the B domain often varies (presumably to provide host and tissue binding specificity), parts of the A subunit often are conserved, especially the regions that are critical for enzymatic activity. For example, at least five ADP-ribosylating toxins (pertussis toxin, cholera toxin, diphtheria toxin, *Escherichia coli* heat-labile toxin, and *Pseudomonas* exotoxin A) have a common NAD binding site (73). Unexpected similarities also have been found with B oligomers. For example, although cholera and heat-labile toxins have five identical B subunits, pertussis toxin has four different B subunits associated with its A subunit, which mediate toxin binding and activity. Two of the B subunits of pertussis toxin are folded similarly to the B subunit pentamers of the cholera and Shiga toxin families, which bind carbohydrates (310, 321–323). Equally noteworthy is a finding that emerges from the examination of the crystal structure of *E. coli*

verotoxin 1, now known as Shiga-like toxin 1. This A-B toxin belongs to the Shiga toxin family and has no detectable sequence homology to cholera toxin. However, the molecular structures of these two diverse toxins are remarkably similar, which suggests a distant evolutionary link between Shiga- and cholera-like toxins (323).

Proteolytic Toxins

Strikingly different diseases can be caused by remarkably similar toxins delivered to different places in the body. For example, botulinum toxin is ingested orally and causes a flaccid paralysis in peripheral nerves, while tetanus toxin arises from deep wounds colonized by *Clostridium tetani* and results in a spastic paralysis through the central nervous system. It now has been shown that both toxins act by using the same mechanism, blocking neurotransmitter release by proteolytic cleavage of synaptobrevins, which are proteins that are components of synaptic vesicles (291). Moreover, both proteins are zinc metalloendoproteases and share conserved zinc binding domains and other homologies. Botulinum toxin from at least one serogroup has the same substrate profile as tetanus toxin in vitro (226). It appears that the mode and location of toxin delivery contribute to the different diseases caused by these two very similar neurotoxins.

Pore-Forming Toxins

Other bacterial toxins function by insertion into the host cell plasma membrane, which causes the formation of a pore (or channel) that leads to lysis of the host cell. A large family of such toxins, the RTX family (named because of a repeat found in each toxin), is found in many gram-negative pathogens (356, 357). The prototype member of this family is *E. coli* hemolysin (HlyA). Some other members of this large family of toxins include adenylate cyclase from *Bordetella pertussis* and the leukotoxin from *Pasteurella haemolytica*. Although the generalized mechanisms of pore formation and sequences appear to be conserved in this family, the target host cell specificities vary among RTX toxins (96), which may contribute to host tissue and disease specificity. This family of toxins also shares a conserved secretion mechanism (type I) that is responsible for transporting these toxins out of the bacteria. This mechanism is discussed in a later section.

Several other families of pore-forming toxins have been discovered within the last few years. For example, there is a large family of sulfhydryl-activated cytolysins that are found in many gram-positive organisms. Included within this family is listeriolysin O, which mediates the escape of *Listeria monocytogenes* from a vacuole inside host cells by lysing the phagosome (see below) (13). Interestingly, listeriolysin O is the only member of the sulfhydryl-activated toxins whose activity is optimal at an acidic pH, a pH which is encountered in an intracellular vacuolar environment and acts as a trigger for vacuole lysis.

Other Toxins

A growing family of secreted bacterial products contains the immunoglobulin A (IgA) protease-type proteins that are found in several gram-negative pathogens, including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Haemophilus influenzae* (180, 325). Recent additions to this family are the vacuolating cytotoxin from *Helicobacter pylori* (61, 293), the serine protease from *Serratia marcescens* (365), a hemagglutinin from an *E. coli* avian pathogen (260), and secreted proteins from *Shigella flexneri* (20) and enteropathogenic *E. coli* (EPEC) (320). Members of this family encode their own secretion (autotransporters), which is discussed in a later section (180). The actual host substrates and mechanisms of action for most of the molecules in this family have yet to be identified.

Another family of conserved toxins contains the heat-stable toxins (290). Originally characterized in *E. coli*, these short cysteine-rich polypeptides are found in several gram-negative organisms, including *Yersinia* species, *Citrobacter freundii*, and *Vibrio mimicus*. These toxins activate host guanylate cyclase. Remarkably, they also share homology with guanylin, an endogenous activator of intestinal guanylate cyclase (66), which may indicate an evolutionary relationship between these molecules or, alternatively, a convergent evolution of a virulence factor that exploits an existing host cell function.

A growing number of bacterial toxins that affect the host cell cytoskeleton have been identified. Besides the antiphagocytic properties of several *Yersinia*-secreted proteins (Yops; see below), other molecules, such as the *E. coli* cytotoxic necrotizing factor type 1, cause necrosis by altering host cell actin organization, in this case by causing actin stress fiber formation and membrane ruffles in the host cell (83). Small GTP binding proteins (the Rho family) mediate actin polymerization states within host cells. Cytotoxic necrotizing factor type 1, as well as several other toxins like *Clostridium botulinum* exoenzyme C3 and *Clostridium difficile* toxins A and B, affects Rho or other specific members of the small GTP-binding protein Rho family (360). *C. difficile* toxins A and B both glucosylate RhoA and other specific members of the Rho family with UDP-glucose as the donor, thereby causing disaggregation of actin filaments (174). *E. coli* cytotoxic necrotizing factor type 2 and *C. botulinum* C3 exoenzyme alter Rho proteins but by a different mechanism, i.e., ADP-ribosylation of Rho proteins (236). Several microbes have developed specific toxins to neutralize the host cell central mechanisms that control actin polymerization levels, resulting in a cytotoxic effect. Given the critical role of small GTP binding proteins in cytoskeletal regulation (the Rho family), vesicle transport (the Rab family), and cell growth, division, and regulation (the Ras family), it is likely that other bacterial virulence factors that affect these central cellular processes will be found.

While we list a formidable "rogues' gallery" of bacterial toxins, they are not as diverse as originally thought. We now recognize a limited number of major toxin families that display common structural and biochemical motifs. Although membership in a particular family is determined most often by sequence homology to other family members, elucidation of tertiary structures also can provide important information regarding relatedness to other toxins. Moreover, the striking similarity of tetanus and botulinum toxins instructs us that differences in disease cannot necessarily be depended upon to reflect differences in toxin (or other virulence factor) structure. Since exotoxins are secreted, conservation of various secretion mechanisms also plays an important role in defining a toxin family.

ADHERENCE

A necessary step in the successful colonization and, ultimately, production of disease by microbial pathogens is the ability to adhere to host surfaces. This fundamental idea has led to an enormous amount of research over the last two decades that deals with understanding how bacterial pathogens adhere to host cells. Consequently, we have learned a great deal about adhesin biogenesis, the regulation of adhesin factors, and, to a lesser extent, the identity of host receptors that are the targets of microbial adhesin factors. Not all adhesins are essential virulence factors (185). The specific role of a

particular adhesin in disease has been surprisingly difficult to define, since a single pathogen expresses many adherence factors.

P Pili: Model Fimbriae

A wide array of bacterial adhesins have been found in a variety of pathogenic microbes. Some pathogens have been reported to possess no less than a dozen different adhesins expressed at one time or another or in different strains of the same species. Fortunately, some common features of adhesin molecules and their assembly are apparent. Bacterial adhesins can be divided into two major groups: pili (fimbriae) and nonpilus adhesins (afimbrial adhesins). One of the major features among diverse pili is conservation of the molecular machinery needed for pilus biogenesis and assembly onto the bacterial surface (157). One of the best-studied examples of pilus assembly is the family of P pili (pyelonephritis-associated pili), which are encoded by *pap* genes (153, 166, 186). *E. coli* strains that express P pili are associated with pyelonephritis, which arises from urinary tract colonization and subsequent infection of the kidney. It is thought that P pili are essential adhesins in this disease process.

The *pap* operon is a useful paradigm since it contains many conserved features that are found among various pilus operons. Two molecules guide newly synthesized pilus components to the bacterial surface. PapD, a conserved chaperone molecule, has an Ig-like fold (153), which is necessary to transport several pilus subunits from the cytoplasmic membrane to the outer membrane. PapC accepts molecules from PapD and has been proposed to serve as an outer membrane usher. The major subunit of the pilus rod is PapA, which is anchored in the outer membrane by PapH. At the distal end of the pilus rod is the tip fibrillum, composed of PapE and the actual tip adhesin, PapG. Two other proteins, PapF and PapK, are involved in tip fibrillum synthesis.

Although the host receptor varies for different bacterial pili, the general concepts provided by studying the P pilus operon are conserved in many other pilus systems, and the components are often interchangeable. For example, the PapD chaperone also can modulate the assembly of type 1 pili. There is a large (>13-member) family of such periplasmic PapD-like chaperones that are necessary for the assembly of several pili, including K88, K99, and *Haemophilus influenzae* pili (158). Additionally, homologous chaperones are needed for several afimbrial adhesins, including filamentous hemagglutinin (FHA) and the pH 6 antigen from *Yersinia pestis*. Homologous sequences to *pap* genes also have been found in genes involved in bacterial capsule and LPS biosynthesis. The gene organization among such pilus operons also is usually conserved. Thus, type I and P pili have very similar operons and functionally analogous sequences that can be aligned. However, they are structurally different pili (type I are flexible, rod-like fibers, while P pili are rigid structures) and bind to different receptors. As additional genomic sequences of pathogens become available, it should be possible to identify new pilus operons by alignment with existing pilus operons.

Type IV Pili

Although *pap*-like sequences are common throughout gram-negative adhesins, there are other families of pili that use alternative biogenesis and assembly machinery to form a pilus. One such group is type IV pili found in diverse gram-negative organisms. This family includes pili from *Pseudomonas aeruginosa*, *Neisseria* species, *Moraxella* species, EPEC, and *Vibrio cholerae* (161, 327). Type IV pili subunits contain specific features, including a conserved, unusual amino-terminal sequence that lacks a classic leader sequence and, instead, usually utilizes a specific leader peptidase that removes a short, basic peptide sequence. Several possess methylated amino termini on their pilin molecules and usually contain pairs of cysteines that are involved in intrachain, disulfide bond formation near their carboxy termini. The pilus assembly genes also share homology to the type II secretion system (discussed in a later section), which is involved in assembling this complex organelle on the bacterial surface. It has been proposed that the pilin molecules located at the tip have different sequences exposed from those packed into repeating structures within a pilus and that these exposed regions may function as the adhesins. However, analogous to the P pilus tip adhesin, a separate tip protein (PilC for *N. gonorrhoeae*) may function as a tip adhesin for these pili (280). Alterations in the pilus subunit can also affect adherence levels. For example, although *P. aeruginosa* strains usually express only one pilus subunit, this subunit can vary significantly among strains, which affects their adhesive capacities. Additionally, the pilin subunit of *N. gonorrhoeae* that is expressed is always changing due to genetic switching of the expressed pilin gene, which leads to antigenic variation (see below). Different pilin sequences may lead to tissue tropism for adherence and different invasion capacities into epithelial cells. The recent crystallographic structure of the *N. gonorrhoeae* pilin may begin to provide additional clues about how this molecule functions in adherence (241).

Host Receptors

The host receptor that a pathogenicity-associated adhesin recognizes probably determines the tissue specificity for that adhesin and bacterial colonization or persistence; of course, other factors also may make a contribution. The location of the adhesin at the distal tip of pili ensures adhesin exposure to potential host receptors. One example of receptor specificity is illustrated by studies with P pili. As mentioned above, PapG is the tip-located adhesin for this pilus. P pili bind to the α-D-galactopyranosyl-(1-4)-β-D-galactopyranoside moiety present in a globoseries of glycolipids (157), which are found on host cells lining the upper urinary tract. However, there are three adhesin variants of PapG, i.e., G-I, G-II, and G-III, which recognize three different but related Galα-(1-4)-Gal receptors. It is thought that different hosts and tissue may contain differences in the distribution of these receptors, and differential expression of the PapG adhesins at the pilus tip could enhance tissue and host specificity for adherence.

Helicobacter pylori is a human-specific pathogen that adheres specifically to epithelial cells from the gastrointestinal tract but not to cells from the nervous system or urogenital tract. Additionally, it shows preferential adherence to the gastric mucosa rather than the colon, which correlates with the site of *H. pylori* infections (stomach). This organism also preferentially adheres to surface mucous cells of the gastric mucosa and does not bind to host cells that are found deeper within the mucosal layer. Recent work has shown that *H. pylori* binds to the Lewis^b blood group antigen that is expressed on cells in the stomach epithelium, presumably explaining the tissue specificity of this adherence (31, 32). Interestingly, *H. pylori* preferentially infects people who are Lewis^b-positive, but other individuals can also be infected, indicating the presence of other adherence factors or host receptors.

Afimbrial Adhesins

Bordetella pertussis, the causative agent of whooping cough, is a respiratory mucosal pathogen that possesses several po-

tential adherence factors that exemplify the complexity of bacterial adherence (287). Potential *B. pertussis* adherence factors include at least four fimbrial genes and several afimbrial adhesins, including FHA, pertactin, pertussis toxin, and BrkA. FHA is a large (220-kDa) secreted molecule that has several domains that are homologous to those of other bacterial adherence molecules or to eukaryotic sequences that mediate cell-cell adhesion. In this vein, FHA is homologous to two high-molecular-weight, nonpilus adhesins from *Haemophilus influenzae* (16), which is another pathogen that adheres to respiratory surfaces. FHA also contains an RGD tripeptide sequence that is a characteristic eukaryotic recognition motif that binds to host cell surface integrins. This RGD sequence mediates FHA binding to the leukocyte integrin CR3, which mediates bacterial uptake into macrophages without triggering an oxidative burst (268). It has been proposed that by possessing this RGD sequence, FHA mimics host molecules (at a molecular level), such as native ligands for the integrin CR3 on endothelial cells (287). Additionally, this RGD sequence induces enhanced *B. pertussis* binding to monocytes by activating a host signal transduction complex that normally upregulates the CR3 binding activity (165). (It should be noted that most bacterial molecules that mediate adherence to integrins, such as *Yersinia* invasin, do not possess RGD sequences, although their receptor affinity is often greater than that of the native host ligand.) FHA and one of the binding subunits of pertussis toxin (S2) share homologous sequences that mediate binding to lactosylceramides, which suggests that these two proteins might use similar motifs to bind to host molecules. Pertussis toxin also mimics eukaryotic adhesive molecules (287). For example, the S2 and S3 subunits have several features in common with eukaryotic selectins. By mimicking host molecules, these bacterial adhesins can effect responses in the host that enhance "desired" (from the bacterial viewpoint) interactions with host cells. These examples also highlight the ability of bacteria to mimic host molecules and to use this mimicry to enhance their pathogenesis. At least two other *B. pertussis* molecules are involved in adherence to host cells. Both pertactin and BrkA, which are 29% identical, contain RGD motifs that may be involved in adherence (86, 191). Additionally, BrkA is involved in serum resistance.

Adherence to the Extracellular Matrix

After entry into a host, a bacterial pathogen can adhere either directly to host cell surfaces or to the extracellular matrix, which serves as the underlying foundation for mammalian cells. When tissue is damaged (possibly due to intoxication or inflammation), the underlying extracellular matrix becomes exposed. There are numerous examples of pathogens that bind to extracellular matrix molecules such as fibronectin, collagen, laminin, etc. (358). These matrix proteins often possess RGD sequences to which the cell membrane-localized integrins adhere, thereby mediating their attachment. *B. pertussis* synthesizes three distinct adhesins, FHA, pertactin, and BrkA, that contain RGD sequences, which presumably permit adherence to host cell integrins (190, 287). At what point during the infection cycle these adhesins come into play is not known, although their expression is at least in part regulated differently.

Alternatively, several mechanisms exist whereby pathogens can bind specifically to matrix molecules. For example, YadA of *Yersinia enterocolitica* mediates binding to cellular fibronectin but not to plasma fibronectin, which may provide a possible mechanism for the organism to adhere to tissue rather than to bind to circulating molecules (298, 333). YadA also mediates adherence to various collagens and laminins (299). The loss of YadA decreases virulence in mice by 100-fold, which suggests that adherence to these molecules may potentiate disease (250). Similarly, *E. coli* and *Salmonella enteritidis* make tightly regulated, thin, aggregative fiber polymers called curli in *E. coli* and SEF17 fimbriae in *S. enteritidis* (56, 235). The amino acid sequences and genetic organization of these two fimbriae are quite similar and mediate adherence to a variety of extracellular matrix and serum proteins, including fibronectin, laminin, and plasminogen (235, 311). The synthesis of curli requires molecular machinery that is unique (138) and represents a family of surface organelles in enterobacteria that mediate adherence to the extracellular matrix.

Mycobacterium species also bind fibronectin by using three related bacterial molecules (called the BCG85 complex) (358). At least two other fibronectin binding molecules also have been described for *Mycobacterium* species. One of these has been described for *Mycobacterium avium* and *M. intracellulare* (265) as a 120-kDa protein recognized by antibodies against $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins, which indicates that these bacteria may express integrin-like molecules that may mediate matrix adherence. Recently, a fibronectin attachment protein from *M. avium* has been characterized and shown to be highly conserved in *M. leprae*, *M. tuberculosis*, and other mycobacteria (294, 295).

Gram-positive pathogens often adhere to matrix proteins. Species of *Staphylococcus*, *Streptococcus*, and other Gram-positive pathogens that localize at mucosal surfaces have molecules that mediate fibronectin and other cell matrix adherence (140), and there is a large family of microbial surface components recognizing adhesive matrix molecules (246, 247). These matrix binding proteins contain repeated sequences that form domains that mediate binding and are assembled in cassette-like forms (237, 304). These sequences form an acidic core of highly conserved residues, which suggests that staphylococci and streptococci bind extracellular matrix by similar mechanisms. Mutants defective for fibronectin binding are significantly decreased for virulence in several animal models, which indicates that these adherence mechanisms participate in colonization and disease.

Host Cell Complicity in Bacterial Adherence

The interaction of a pathogen with a host cell often causes activation of host cell signaling pathways, either directly by bacterial components or by stimulating host activating factors, such as inflammatory cytokines. Such activations could alter the host cell surface, thereby providing the pathogen with alternate adhesin receptors. Two recent findings indicate that our previous concept that pathogens adhere to constitutively expressed host cell receptors may have been naive. EPEC, which is discussed in more detail in a later section, secretes proteins that activate host cell signaling pathways, including tyrosine phosphorylation of a host protein, Hp90 (272). Intimate adherence of this pathogen occurs only after EPEC-mediated activation of a host cell signaling factor. Remarkably, the tyrosine-phosphorylated form of Hp90 is a host cell receptor for the EPEC adhesin intimin, yet intimin does not bind to inactivated Hp90 (276). Thus, this pathogen must activate host cell signals before intimate adherence to the host cell surface can occur, a concept that has not been documented previously for bacterial adherence.

In an analogous case, a pathogen relies upon a host response to infection to activate a target receptor. *Streptococcus pneumoniae* adherence to and invasion into human umbilical vein endothelial cells is markedly increased by thrombin or tumor

necrosis factor alpha stimulation of endothelial cells (65). A cell wall component of *S. pneumoniae*, phosphorylcholine, appears to bind to a G-protein-coupled platelet-activating factor (PAF) receptor on activated endothelial cells. Endothelial cell activation results in PAF receptor surface expression and PAF binding to the receptor, which leads to its internalization. Enter the pneumococcal cell wall component, which, like the platelet binding factor, has a phosphorylcholine residue that binds to the PAF receptor and subsequently mediates bacterial uptake into the endothelial cell. It was hypothesized that although the PAF receptor binding by platelet binding factor normally triggers signals in the cell via its G-protein linkage, the pneumococcus does not activate these signaling pathways. These events do not occur with unactivated epithelial cells, indicating that the pneumococcus, at some point in its evolution, was selected to capitalize on a host response to its presence. Finally, *Borrelia burgdorferi*, the causative agent of Lyme disease, binds to the integrin $\alpha_{IIb}\beta_3$ (also known as glycoprotein IIb-IIIa) on human platelets but only if the platelets are activated (53).

The preceding examples and that discussed above for FHA (165) illustrate the intimate relationships that adherent organisms establish with host cells. As the role of the host cell is examined more closely instead of being treated as an inactive, static matrix, several additional examples of induced binding, adherence, and invasion will be identified. Perhaps the most common theme to emerge from the investigation of bacterial adherence is the degree of redundancy of distinct adherence mechanisms that bacteria possess with which to interact with their host.

INVASION

Many pathogenic microbes are capable of entering into and surviving within eukaryotic cells. Some organisms direct their uptake into host cells that are not normally phagocytic, including epithelial cells lining mucosal surfaces and endothelial cells lining blood vessels. Invasion may provide a means for a microorganism to breach host epithelial barriers. Presumably, this invasion tactic ensures a protected cellular niche for the microbe to replicate or persist. Alternatively, organisms may be internalized actively by phagocytic cells, such as macrophages, by several mechanisms. Pathogens that survive and replicate within phagocytic cells possess additional mechanisms that enhance their survival. Even quite different organisms can use mechanistically similar invasion strategies.

Cultured eukaryotic cell lines are commonly used to study bacterial invasion since they provide reproducible and uncomplicated infection models (332). Of course, few cultured cell lines closely mimic the original tissue from which they were isolated. Polarized epithelial cell lines have been used to better approximate epithelial barriers since they have apical and basolateral cell surfaces separated by tight junctions and well-defined microvilli (261). However, these cell systems have their limitations. Polarized cell cultures still lack other host factors that could affect invasion, including a mucus barrier and host immune factors such as antibodies and cytokines. Moreover, by their very nature, cell culture models usually are devoid of other host cells, such as inflammatory cells, that normally would be present in vivo. Despite these limitations, we have learned a good deal about the molecular nature of bacterial uptake into host cells.

Generally, invasive organisms adhere to host cells by using a class of adhesin molecules, generally called invasins, that also direct bacterial entry into cells. These adherence mechanisms usually trigger or activate signals in the host cell that directly or indirectly mediate and facilitate bacterial entry (28, 274). Thus, bacterial invasion is an active event that relies upon underlying normal host cell functions. Ultimately, host cell cytoskeletal components provide the machinery for bacterial uptake. Rearrangements in actin filament structure often can be visualized in the vicinity of the invading organism. Microtubules also may be required for invasion of some pathogens (see below), although pathogens that enter through this pathway usually do not replicate intracellularly after cell entry, nor do they cause diseases associated with cell-to-cell spread. Following cell entry, the invading bacterium immediately is localized within a membrane-bound vacuole inside the host cell. The organism may or may not escape this vacuole, depending on the pathogen and its strategy for survival (discussed below). A small number of bacterial species appear to forcibly enter directly into host cells by a local enzymatic digestion of the host cell membrane following adherence to the cell surface. One such pathogen, *Rickettsia prowazeckii*, produces phospholipases that appear to degrade the host wall localized beneath the adherent organisms, thereby enabling the pathogen to enter the cytoplasm directly (348). How the bacterium controls the enzymatic degradation to prevent host cell lysis and how the host cell reseals its membrane after invasion remain uncharacterized.

Yersinia Invasion

Perhaps the best-studied invasion system is that found in *Y. pseudotuberculosis* and *Y. enterocolitica* (summarized in Table 1) (81), which possesses a chromosomal gene encoding an outer membrane protein, invasin, that mediates attachment and entry into nonphagocytic cells (163). Work by Isberg and colleagues has shown that invasin binds tightly to a family of β_1 integrins, which are host molecules that bind to extracellular matrix proteins such as fibronectin on the basolateral surface of epithelial cells (162). By binding tightly to integrins, invasin mediates bacterial uptake via a "zipper"-like mechanism, zippering the host cell membrane around the bacterium as it enters (162). Invasin, by itself, is sufficient to mediate this uptake; invasin cloned into noninvasive *E. coli*, or purified invasin coupled to inert particles such as beads, mediates particle uptake. Host actin is needed for bacterial uptake since cytochalasins inhibit particle uptake (90). However, cytoskeletal rearrangements are not dramatic and disappear within a few minutes of bacterial entry (366). Host signal transduction mechanisms appear to be necessary for invasin-mediated bacterial entry, since host tyrosine kinase inhibitors that block host cell signaling prevent bacterial uptake but adhere to cultured cells (273).

Enteropathogenic *Yersinia* species also contain two other invasins: Ail and YadA. Ail mediates high levels of adherence to epithelial cells and low levels of bacterial invasion (220). It also provides serum resistance in *Y. enterocolitica* and other *Yersinia* species (27). It belongs to a family of bacterial outer membrane proteins that mediate several different virulence functions, which include *S. typhimurium* PagC (a plasmid gene product that is needed for intracellular survival in macrophages) (263) and Rck (a plasmid gene product that confers serum resistance) (142); a *Bartonella* invasion protein, IalB (222); an *Enterobacter cloacae* invasion protein, OmpX (69); and a bacteriophage lambda outer membrane protein, Lom (263).

Pathogenic *Yersinia* species harbor a plasmid that is essential for pathogenicity (see below) (59). *Yersinia* YadA is a virulence plasmid-encoded protein that, like invasin, binds to host β_1 integrins (298). Ironically, *Yersinia* species that express both

TABLE 1. Comparison of various invasion mechanisms

Bacterium	Bacterial invasin	Host receptor(s)	Features
Yersinia species	Invasin	β_1 integrins	"Zipper phagocytosis," actin mediated but not membrane ruffling, tyrosine kinases activated, basolateral
	YadA	β_1 integrins	?, less efficient than invasin
	Ail	?	Inefficient invasion, high adherence
Listeria monocytogenes	Internalin (InlA)	E-cadherin	"Zipper phagocytosis," actin mediated but not membrane ruffling, tyrosine kinases activated, basolateral
Shigella flexneri	IpaB-D	$\alpha_5\beta_1$ integrin?	Membrane ruffling, major cytoskeletal rearrangements, no calcium flux, basolateral
Salmonella typhimurium	SipB-D	?	Membrane ruffling, major cytoskeletal rearrangements, macropinocytosis, calcium flux and several other signals (not inhibited by kinase inhibitors), apical, formation of surface appendages (invasome)
Enteropathogenic *E. coli*	Invasin	Hp90	Pedestal formation and intimate adherence, poor invasion (microtubule and actin dependent), cytoskeletal accumulation under bacteria, tyrosine kinase mediated

invasin and a full complement of virulence plasmid gene products actually paralyze normal phagocytic mechanisms (discussed below). Thus, during active infection, wild-type *Yersinia* species remain extracellular but firmly affixed to the host cell surface (82). While invasin alone is a potent mediator of bacterial attachment and entry into cells, in concert with other *Yersinia* virulence genes it acts primarily as a highly specific and avid adhesin that attaches to epithelial cells and macrophages. This attachment to β_1 integrins is the key step in the contact-dependent secretion of plasmid virulence factors and the subsequent translocation of several of these bacterial proteins into the host cell cytoplasm (see below) (97). YadA can substitute for invasin as an adhesin, but it shows a much higher avidity for activated macrophages than for epithelial cells or nonactivated phagocytes. Thus, in fully virulent *Yersinia* species, invasin and YadA probably function mainly as adhesins, despite their invasive potential when studied as isolated virulence determinants.

Salmonella Invasion

S. typhimurium triggers a dramatic response from the epithelial cell surface shortly after it contacts the host cell. The host cell surface extrudes outward (from the point of bacterial adherence) with attendant localized membrane ruffling and macropinocytosis (11, 91, 101). Additionally, actin filaments are rearranged and host surface proteins are capped (93). Several signal transduction pathways, including calcium and inositol phosphate fluxes, are activated within the host cell (238, 281). *Salmonella* invasion is rapid, and bacteria appear within membrane-bound vacuoles within a few minutes of initiating host cell contact. The host actin and cell surface return to normal after the bacteria are internalized.

S. typhimurium invasion initiates a cascade of host cell signaling events. Defining the sequence of events accompanying bacterial invasion has been difficult. It is not clear which changes seen in infected host cells are primary, rather than secondary, responses to bacterial invasion. Clearly, *S. typhimurium* triggers a Ca^{2+} flux in cultured epithelial cells; strains that contain mutations in bacterial invasion genes no longer cause the intracellular Ca^{2+} flux and actin rearrangement (238). Additionally, chelators of intracellular Ca^{2+}, but not extracellular Ca^{2+}, block *S. typhimurium* entry into cultured epithelial cells (281). Release of intracellular Ca^{2+} is often mediated by a transient increase in the inositol triphosphate level. *S. typhimurium* also triggers fluxes in inositol phosphates that correlate with bacterial invasion. Further information about the signal(s) used by *S. typhimurium* to enter cultured cells comes from work that describes the activation of the epidermal growth factor receptor in response to *S. typhimurium* in Henle-407 cells (112). However, despite the initial appeal of this model (238), there were several paradoxical results. At this point, it is probably fair to say that epidermal growth factor receptor is not directly involved in *S. typhimurium* uptake into cells (101, 172, 207, 275) and that the mechanism involves a host cellular pathway regulating cytoskeletal elements. Central to this regulation are members of the *ras*-related superfamilies of small GTPases, *rac*, *rho*, and CDC42 (231), that coordinate the formation of actin-based structures that play a pivotal role in cell motility, cytokinesis, phagocytosis, and intracellular transport processes. *Salmonella* entry appears to be *rac* and *rho* independent (172). Recent studies provide evidence that *Salmonella* entry is CDC42 dependent (45a).

S. typhimurium has multiple genetic loci involved in invasion that are clustered in one region on the *Salmonella* chromosome (58 to 60 min) called pathogenicity island SPI1 (also described below) (88, 109). Within this region, there are several different invasion operons, including regulatory loci. Many of the proteins encoded in one gene cluster, *inv/spa*, share considerable homology with those seen in other pathogenic bacteria. The first indication of such homology came from *invA*, which encodes a predicted protein that shared homology with LcrD from *Yersinia*, MxiA from *Shigella flexneri*, and other proteins (111, 343). LcrD is a membrane-bound calcium regulator involved in *Yersinia* pathogenesis, while MxiA is involved in transporting *Shigella* invasion antigens to the bacterial surface. Several plant pathogens have similar secretory proteins, including HrpO from *Pseudomonas solanacearum* and HrpC2 from *Xanthomonas campestris* (343). It is clear that

most of the *inv*/*spa* locus of *S. typhimurium* encodes products that share significant homology with other pathogenic species. Indeed, several *Shigella* virulence genes can complement noninvasive mutations of *S. typhimurium* (131).

The role of this secretory system in *S. typhimurium* invasion, which is common to so many pathogens, is still being defined. It represents a distinct secretory complex classified as a type III secretion system (see below). Recent results indicate that *S. typhimurium* assembles visible surface appendages upon contact with polarized epithelial cells (126). These structures are not seen on broth-grown organisms and appear to be induced by contact with host cell surfaces, although bacterial protein synthesis inhibitors do not block their formation. Immediately preceding bacterial entry, these structures disappear. Noninvasive mutants (with mutations in various *inv* loci) have one of two phenotypes: either surface appendages are never lost (*invA* and *invE*) or they are never formed (*invC* and *invG*), which suggests that the *inv*/*spa* locus mediates the formation of a surface organelle that mediates bacterial invasion. In this vein, some of the *inv*/*spa* proteins share homology with flagellar assembly proteins, another bacterial surface organelle.

Shigella Invasion

Shigella invasion into epithelial cells closely resembles *Salmonella* invasion (209). There is a dramatic localized polymerization of actin and myosin seen as membrane ruffling in the vicinity of invading bacteria (3). This localized actin polymerization into filaments causes pseudopod extension and engulfment of the organisms, which results in bacterial internalization within a vacuole (3). Several host proteins are implicated in *Shigella* invasion. For example, T-plastin, an actin-bundling protein, plays a functional role in invasion. *S. flexneri* invasion also causes tyrosine phosphorylation of a host cytoskeletal protein, cortactin, which is phosphorylated by a host protein kinase, $pp60^{c\text{-}src}$ (68). In contrast to *Salmonella* invasion (172), the small GTPase Rho is required for actin polymerization associated with *Shigella* invasion of HeLa cells (4). Presumably, these molecules are recruited to the site of bacterial invasion and exploited by the bacterium to facilitate bacterial uptake.

Although the bacterial components needed for *Shigella* invasion comprise a complex system, most of these proteins appear to be involved in transporting and delivering three surface or secreted effector proteins, IpaB, IpaC, and IpaD. These proteins are essential for invasion and, as an assembled complex, even can mediate latex bead uptake (210). In addition to a conserved secretion system, several groups have recently shown that the effectors of invasion for both *Shigella* (the Ipa proteins) and *Salmonella* (Sip/Sic/Ssp) are quite similar and even interchangeable (125, 147, 175, 176, 277), which indicates that they may have a common mechanism for invasion. It is not known if *S. flexneri* assembles surface appendages that resemble those seen with *S. typhimurium*, although macromolecular structures are secreted by *S. flexneri* when the type III secretion system is overexpressed (244). Remarkably, *Shigella* IpaB mediates escape from the endocytic vacuole; however, *Salmonella*, which has an IpaB homolog, remains within a membrane-bound compartment throughout its intracellular life. Bacterial contact with host cell components of the extracellular matrix causes the release of *Shigella* Ipa proteins (211, 350) and secretion of *Salmonella* InvJ (368), which indicates that these type III secretion systems are activated by host cells and/or serum. In addition to invasion, it has been proposed that the type III Mxi-Spa secretion system may play a role in the spread of *Shigella* between epithelial cells (7).

The receptor(s) to which the *Shigella* and *Salmonella* secreted proteins bind has been elusive. One hypothesis is that these secreted proteins do not have a specific host receptor but are pumped into the host cell via a type III secretion system analogous to the *Yersinia* system. Once in the host cell, they activate signal transduction pathways that lead to membrane ruffling and bacterial uptake. Alternatively, they may bind in small numbers to a host receptor that then multiplies the intracellular signal, much like hormone receptor kinases. In any event, activation of host small G-proteins would lead to increased ruffling and uptake of bacteria on the host cell surface. However, a recent report indicates that Ipa proteins from *S. flexneri* bind to $\alpha_5\beta_1$ integrins in CHO fibroblast cells, which activates signals that are normally associated with host cell focal contact formation (349). Such signals include activation of the focal adhesion kinase (FAK^{125}) and phosphorylation of paxillin. Genistein, which inhibits focal contact formation, inhibits *Shigella* invasion (349) but not *Salmonella* invasion (273). Thus, *Salmonella* and *Shigella* show remarkable similarities on the one hand but retain their distinctive host cell interaction that best serves their pathogenic strategy.

Yersinia Antiphagocytic Activity

Although the type III secretion system of *Salmonella* and *Shigella* species is necessary for invasion, a similar system in *Yersinia* species neutralizes host cell uptake by phagocytosis. Yersinae have several secreted proteins, collectively known as Yops, that mediate effects on host cells and are transported out of the bacteria by a type III secretion system (277, 317). In addition to cytotoxicity, several bacterial factors contribute to paralyzing phagocytic cells, thereby preventing bacterial uptake and invasion. It recently has been shown that YopE (an actin toxin) and YopH (a tyrosine phosphatase that rapidly dephosphorylates host proteins) are transferred directly into the host cell cytoplasm following contact with mammalian cells (2, 53, 317, 318). Additionally, another bacterial product, YpkA, shares homology with host serine/threonine kinases and presumably disrupts host signaling pathways in phagocytic cells, which is needed for *Yersinia* virulence (113). It is thought that bacterial contact with the epithelial cell surface triggers the virulence plasmid type III secretion system, which mediates the translocation of Yops from the bacterium through the eukaryotic plasma membrane into the host cell cytoplasm (277). As noted above, the productive delivery of Yops into the host cell must be mediated by either invasin or YadA by binding to host cell integrins.

Collectively, these results suggest that the complex machinery needed to transport virulence proteins to bacterial surfaces is conserved in *Yersinia*, *Shigella*, *Salmonella*, and EPEC (see below). A similar pathway also is conserved in flagellar export machinery and in various plant pathogens. However, the actual bacterial proteins that are transported and the regulatory mechanisms that control these systems vary, as do the targets in the host cell. At present, injection of bacterial proteins has been shown only for the *Yersinia* system, although given the signals that are mediated by *Shigella* and *Salmonella* invasion and EPEC adherence, it seems almost certain that these pathogens utilize a similar strategy to some degree (277).

Enteropathogenic *E. coli* Adherence

Although not considered an invasive pathogen, EPEC also causes host cell alterations when it interacts with epithelial cell surfaces; therefore, it is included in this section. The intimate attachment of this microbe to the host cell surface results in microvillus effacement, cytoskeletal rearrangement, and, ultimately, a pedestal or protrusion forming on the epithelial cell

surface beneath the adherent bacteria. This process is known as attachment and effacement.

Upon binding to apical epithelial surfaces via a type IV bundle-forming pilus, EPEC directly stimulates several host cell signals. These bacterial signals elicit calcium fluxes, inositol phosphate fluxes, and the tyrosine phosphorylation of a 90-kDa membrane protein and serine/threonine phosphorylation of two other proteins, including myosin light chain (15, 78, 100, 200, 272). Several bacterial mutants of EPEC are defective for triggering signals in host epithelial cells. One locus, *espB*, encodes a secreted protein and associates with the Triton-soluble (membrane and cytosol) fraction of epithelial cells (177). Bacteria with mutations in *espB* are unable to activate host cell signaling (99). Another EPEC secreted protein, EspA, is needed for activating epithelial signal transduction (178). Several other mutants, including *sep* and class 5 mutants, no longer trigger host cell signaling (272). These EPEC mutants are defective in the secretion of four proteins, including EspB and EspA (167, 177). Once again, we see a common theme—mutants that contain insertions in sequences that have homology to the type III secretion pathways are found in *Yersinia*, *Salmonella*, *Shigella*, and plant pathogens, while EspB shares homology with *Salmonella* InvJ, a secreted invasion protein (55). Thus, it appears that a similar secretion system is needed in EPEC to transport molecules that mediate signals in host cells, although the end result (pedestal formation instead of host cell invasion) is quite different.

As noted above, a 90-kDa protein accumulates beneath the adherent bacteria, as do several cytoskeletal components, including actin, α-actinin, talin, myosin, and ezrin (92). The product of the EPEC *eaeA* locus, intimin, is needed for effective organization of these cytoskeletal components beneath the organisms; however, *eaeA* mutants still trigger signaling to the host cell but fail to colocalize the cytoskeletal components (272, 276). Several microorganisms use a strategy similar to that of EPEC to form attaching/effacing lesions; these include enterohemorrhagic *E. coli* (EHEC) O157:H7, RDEC-1 (a rabbit pathogen that causes disease similar to EPEC infection in rabbits), *Citrobacter rodentium*, and, most recently, *Helicobacter pylori* (302).

Several similarities among EPEC, *Salmonella*, and *Shigella* with respect to their initial interactions with epithelial cells are apparent. All three cause localized changes in the host cell surface, rearrangement of host actin and related cytoskeletal components, and activation of host signal transduction pathways. The bacterial molecules responsible for mediating these events utilize a conserved secretion pathway. However, despite the similarities, differences between the three pathogens exist. For example, *Shigella* species invade the basolateral surfaces of polarized epithelial cells, while both EPEC and *Salmonella* species interact with apical surfaces. Both *S. typhimurium* and EPEC trigger calcium fluxes in epithelial cells, while *S. flexneri* does not. *Shigella* uses Rho to trigger actin polymerization, while *Salmonella* and EPEC do not. The cytoskeletal rearrangements also are different. For example, *S. typhimurium* and *Shigella* cause transient rearrangements in actin whereas EPEC causes the formation of a stable actin-based pedestal. Finally, both *Salmonella* and *Shigella* species are invasive whereas EPEC remains mainly adherent. The differences between these systems presumably are mediated by the types of signals that are transmitted to the host cell. The molecular dissection of the differences should provide us with information about pathogenic strategies and, as an added bonus, should tell us a good deal about normal mammalian cell cytoskeleton regulation and function.

Microtubule-Dependent Invasion

Although most invasive bacteria require host actin to enter nonphagocytic cells, there is a class of pathogenic organisms that also appear to require microtubules, which are cytoskeletal structures made of polymerized tubulin. Examples include EPEC, EHEC, *N. gonorrhoeae*, *Citrobacter freundii*, *Campylobacter jejuni*, and *Klebsiella pneumonia* (74, 130, 233). Although microtubules are not involved with normal host cell internalization processes such as phagocytosis, the addition of microtubule inhibitors to epithelial cells decreases the number of internalized bacteria of these species. The role of the invasion process in the virulence of these organisms remains undefined. As a general rule, organisms that use microtubules to invade also are unable to replicate or persist within host cells, which is in contrast to organisms that are thought to use invasion as an essential virulence mechanism (such as *Salmonella* and *Shigella* species).

Listeria monocytogenes Invasion

Some gram-positive organisms also actively invade host cells as an essential step in their pathogenesis of infection. *Listeria monocytogenes* invasion is mediated by a family of related bacterial molecules epitomized by internalin (InlA). InlA is a surface-secreted protein that is composed of several regions of repeats, with a C terminus similar to that of a family of surface proteins found in noninvasive, gram-positive cocci (106). Mutations in *inlA* negate *L. monocytogenes* invasion. When *inlA* is expressed in the noninvasive species *Listeria innocua*, this organism becomes capable of invading cultured epithelial cells. Like the Ipa proteins of *Shigella*, InlA mediates the infection of polarized epithelial cells (107) at the basolateral membrane (rather than the apical surface of cells). Recently, the host cell receptor for InlA has been identified as E-cadherin, a calcium-dependent, cell-cell adhesion molecule that is found on the basolateral surface of epithelial cells (212). There are several homologs of InlA in *L. monocytogenes*, including InlB, which appears to mediate uptake into host hepatocytes but not into intestinal epithelial cells (76). Both *inlA* and *inlB* are needed for virulence in a mouse infection model (194), and it has been proposed that each internalin may mediate invasion in different tissue types (InlA for epithelial cells, InlB for hepatocytes), thereby providing tissue tropism at different time points in invasion (108). Thus, *inlA* and *inlB* also are regulated differently, despite being adjacent to each other on the chromosome (306). The roles of the other internalin homologs and their receptors have not been established.

Pathogen Binding of Host Molecules To Mediate Invasion

Several pathogens defy human logic by deliberately parasitizing phagocytic cells, and some even utilize preexisting phagocytic mechanisms for their internalization. *Legionella pneumophila* and *Mycobacterium* species bind the complement fragments C3b and C3bi, which facilitate their uptake into phagocytic cells (19, 292). Uptake via this pathway decreases their exposure to toxic oxygen compounds. Several organisms possess the ability to bind fibronectin, which then functions as a bridge between the bacterium and the host cell fibronectin receptor to mediate invasion. For example, *Mycobacterium leprae* produces a fibronectin binding protein (fibronectin attachment protein) that mediates uptake into epithelial and Schwann cell lines, perhaps by functioning as an opsonic ligand (295).

Invasion mechanisms used to enter nonphagocytic cells also enhance or mediate novel entry into phagocytic cells. For ex-

TABLE 2. Comparison of the various lifestyles of intracellular pathogens that reside within vacuoles

Bacterium	Vacuole type	Features
Coxiella burnetii	Classic phagolysosome	Low pH required to initiate intracellular replication
Mycobacterium species	Specialized vacuole, blocked at early endosome	No proton pump, no M6P receptor-mediated delivery, contains lysosomal glycoproteins
Salmonella typhimurium	Specialized vacuole not linked to main endocytic route	Devoid of M6PR-delivered molecules, contains lysosomal glycoproteins, often a spacious vacuole, later development of *Salmonella*-induced filaments
Legionella pneumophila	Rough endoplasmic reticulum	Devoid of any lysosomal markers, vacuole surrounded initially by mitochondria and then ribosomes
Chlamydia trachomatis	Specialized vacuole devoid of host proteins	Intersection with anterograde vesicles from Golgi

ample, *S. typhimurium* mutants with a decreased capacity for epithelial cell invasion also have a decreased capacity for uptake into phagocytic cells (105). Similarly, *Yersinia* binds preferentially to β_1 integrins of phagocytic cells rather than binding to the normal host cell receptors of phagocytosis. It is possible that by utilizing active invasion pathways, these bacteria bypass the traditional phagosome target pathways, thereby avoiding the antibacterial activities associated with phagocytosis (see below).

INTRACELLULAR LIFESTYLES OF BACTERIAL PATHOGENS

One of several possible outcomes occurs once a pathogen has entered a host cell. The majority of bacteria (including many bacterial pathogens) that are phagocytosed by macrophages and polymorphonuclear leukocytes (PMNs) are killed. However, several pathogens have devised successful strategies that enable them to survive and replicate within potentially lethal, phagocytic host cells. *S. flexneri*, *L. monocytogenes*, and the spotted-fever rickettsiae dissolve the initial membrane-bound vacuole following entry and thereby gain direct access to the nutrient-rich cytoplasm. Alternatively, some organisms like *Coxiella burnetii* have mechanisms (undefined) that allow them to survive the bactericidal agents that the host cell delivers to the phagolysosome and actually thrive in the lysosomal environment. Finally, organisms like *Salmonella*, *Mycobacterium*, and *Legionella* use mechanisms that avoid or inhibit targeting of the internalized bacteria to the phagosome-lysosome fusion pathway, although it has not been established whether common mechanisms are used by these organisms.

Successful bacterial multiplication plays the pivotal role in causation of infection and disease. Thus, many intracellular bacterial pathogens have devised strategies to grow within host cells (intracellular multiplication). Not only can intracellular organisms replicate, but also some devise specific mechanisms to escape from the host cell and spread to neighboring host cells, often without leaving their intracellular niche. For the few studies that exist, there is a direct correlation between successful intracellular growth and virulence.

Life within a Vacuole

The majority of pathogens that are internalized or phagocytosed into membrane-bound inclusions within host cells remain enclosed within such structures for the duration of their intracellular life. Pathogens have evolved several different strategies to circumvent host cell mechanisms designed to take up and kill invading microorganisms (Table 2) (116). Some pathogens synthesize new molecules or modify existing cellular structures to resist antibacterial agents. Virulence factors that resist phagocytosis and that enhance intracellular survival include bacterial capsules and LPS, which provide a protective envelope around the bacteria. Bacterial enzymes that neutralize oxygen radicals and secreted proteolytic enzymes that degrade host lysosomal proteins also can enhance intracellular survival. Some organisms actually depend on factors that are found in phagolysosomes as signals to trigger intracellular multiplication. For example, *C. burnetii* and *S. typhimurium* require an acidic pH as a cue to initiate intracellular replication and to synthesize factors to allow them to persist in the intracellular environment (137, 266). In contrast, pathogenic mycobacteria and *L. pneumophila* seem to either modify the primary phagosome to prevent acidification or exploit a normal host cellular pathway to become enclosed at the outset in a cellular vesicle of neutral pH (155, 328). Presumably other pathogens use similar signals, such as the presence of lysosomal glycoproteins or a lysosomal enzyme, to selectively express gene products or genetic cascades that potentiate their survival and growth upon arrival in a phagolysosome.

It now seems clear that most pathogens that survive or replicate within membrane-bound inclusions share the theme of avoiding living within true phagolysosomes. Instead, pathogens that persist inside vacuoles occupy a privileged niche. Host cell invasion is not a random event. The specific receptor that a pathogen utilizes to invade an epithelial cell or to be taken up by a phagocyte can affect significantly the ultimate intracellular localization of the vacuole that surrounds the pathogen. Invasion mechanisms often target the invading bacteria to a different vesicular pathway than that normally used during phagocytosis. *Salmonella* provides an instructive example, as it is one of the better studied intracellular pathogens.

There is general agreement that *Salmonella* species reside within a membrane-bound vacuole within both phagocytic and nonphagocytic cells. Colocalization of several host cell markers with *Salmonella*-containing vacuoles (SCV) has been examined (115). In cultured epithelial cells, *S. typhimurium* invasion triggers the capping of several host cell surface proteins, but only one of several surface proteins is present in SCV at early times postinfection (119). This finding implies that *Salmonella* entry entails a sorting mechanism at the host cell surface that excludes some cytoskeleton-associated host proteins from entering the SCV or causes rapid recycling of host cell surface components back to the plasma membrane. No such apparent selectivity in host surface markers is observed in vacuoles that contain modified *Salmonella*, which invades by using the *Yersinia* invasin pathway. These findings suggest that from the outset, *S. typhimurium* directs the components of the SCV for its own needs. Once the *Salmonella* vacuole is formed, its composition changes over time with the appearance of lysosomal glycoproteins and the disappearance of surface markers. However, in both epithelial cells and macrophages, this pathway is not connected with the main endocytic pathway and lacks the vacuole in several lysosomal markers normally deliv-

ered by a mannose-6-phosphate receptor pathway. One report indicates that *S. typhimurium* resides within phagosomes that have fused with lysosomes (42), while others conclude that *S. typhimurium* inhibits phagosome-lysosome fusion within several types of mouse-derived macrophages (36, 164). Perhaps these discrepancies are due to the incomplete inhibition of phagosome-lysosome fusion or occur because only part of the intracellular population is capable of blocking this event or are due to the presence of two populations of bacteria inside the phagocytes (see below). Although one report indicates that phagosomes that contain *S. typhimurium* acidify slowly and it takes 4 to 5 h before the pH drops below 5.0 (10), a more recent investigation found that acidification of the *Salmonella* vacuole to pH 4.5 usually is complete within 30 min of entry into macrophages, and this acidification appears necessary for survival and replication (266).

S. typhimurium entry into cells is associated with membrane ruffling, macropinocytosis, and the formation of "spacious phagosomes" where the organisms initially reside. Formation of spacious phagosomes facilitates the survival of *Salmonella* in macrophages, perhaps by diluting the host antibacterial enzymes or affecting their intracellular delivery (10). There is a direct correlation with the ability to form spacious phagosomes and the susceptibility of the host to infection with various *Salmonella* serotypes (10). *S. typhimurium* also triggers the formation of spacious phagosomes in epithelial cells (114). Initially, it seemed logical to assume that *Salmonella* entered directly into a vacuole that potentiated its survival. However, *Salmonella* mutants that are selected for their inability to enter epithelial cells appear to enter macrophages by an alternative entry mechanism (phagocytosis?), and eventually reside in the same privileged niche as do fully virulent wild-type strains.

It appears that the distinctive entry of *Salmonella* is more closely associated with subsequent cytotoxicity for host cells than in directing subsequent intracellular trafficking. Recent findings (46, 193, 225) document that entry of *Salmonella* into macrophages by ruffling leads to a significant proportion of these host cells undergoing programmed cell death (apoptosis) (225). Mutants unable to enter epithelial cells fail to induce apoptosis in macrophages. It seems noteworthy that entry into epithelial cells always is associated with membrane ruffling but does not lead to significant levels of apoptosis. The nature of the bacterial ligand and the host cell receptor associated with membrane ruffling is still unknown. It appears, once again, that the common threads of virulence strategy seen with *Salmonella* and *Shigella* are unexpectedly similar, since the induction of apoptosis in macrophages has been well documented for the shigellae (370). The degree of apoptosis seen in invaded host cells varies with the stage of macrophage activation. Indeed, the more activated the macrophage, the higher the degree of apoptosis induced by the entry of both *Salmonella* and *Shigella*. Entry, per se, is the trigger for apoptosis. *S. typhimurium* mutants that enter normally but are incapable of intracellular replication still induce apoptosis. Virtually any gene that interrupts *Salmonella* entry into cells (for example, *hilA*, *orgA*, and *sipD*) nullifies the apoptotic response to *Salmonella* entry.

Several bacterial factors enhance *S. typhimurium* survival within macrophages after entry, including the PhoP/PhoQ system (a two-component regulatory system), which activates at least five bacterial products (*pag*) and represses others (*prg*) (218). Genetic fusions have been used to identify several genes that are induced exclusively within macrophages and are also under PhoP regulation (10, 18). One of the phenotypes that PhoP/PhoQ regulates is the capacity to survive bactericidal cationic peptides, which is involved in killing intracellular bacteria. The ability to resist antimicrobial peptides involves ATP-binding cassette peptide transporters that presumably pump toxic peptides out of the bacteria (242). The *phoP* locus also appears to inhibit antigen processing and the presentation of bacterial antigens expressed by intracellular bacteria (359), which may enhance virulence. Finally, the response of *Salmonella* to an intracellular acidic environment is associated with the prompt synthesis of several bacterial gene products, including PagA. Inactivation of *pagA* leads to a loss of intracellular persistence and a loss of virulence for laboratory animals.

Other *Salmonella*-specific factors appear to be involved in intracellular survival. For example, *recA* and *recBC* mutants in *S. typhimurium* are sensitive to the oxidative burst of macrophages, which indicates that the ability to repair DNA damage is essential for survival within macrophages and for virulence (37).

Salmonella species have the capacity to multiply within vacuoles in nonphagocytic cells after an initial lag of approximately 4 h (192). The lag period that precedes initiation of bacterial replication in nonphagocytic cells indicates that specific bacterial genes may be required for replication in this unique niche. (Nonvirulent *E. coli* never replicates within vacuoles inside epithelial cells.) *S. typhimurium* mutants that are defective for replication within epithelial cells have been identified. A possible function for these bacterial genes has recently been reported (120). At 4 to 6 h after invasion, intracellular *Salmonella* organisms induce the formation of stable filamentous structures (within epithelial cells) that contain lysosomal glycoproteins, which are connected to the vacuoles that contain bacteria. The kinetics of formation of these lysosomal glycoprotein-rich filamentous structures parallel the rate of intracellular replication, including the initial lag period. Filament formation requires viable intracellular bacteria, since addition of antibiotics blocks the formation of these novel structures. These unique structures are never observed in uninfected cells or in those infected with *Yersinia* species, although all *Salmonella* species tested trigger their formation. The role of these structures in survival and replication in phagocytic cells has not been determined. How the bacteria direct the formation of these novel host processes remains a mystery. One potential function for these structures is that they provide access to nutrients for the intracellular bacteria, possibly by intersecting with endocytic or exocytic vesicular transport pathways.

We also do not know how *Salmonella* grows within macrophages. Mutant strains that are unable to grow within epithelial cells are still taken up by macrophages (105). The differences between these two cell types may be due to the internalization routes used, since epithelial cells lack classic phagocytic pathways. It recently has been proposed that two populations of *S. typhimurium* exist within macrophages: one is static, and the other grows rapidly (2). The existence of these two pools perhaps may explain the conflicting data regarding lysosome fusion and intracellular growth within phagocytic cells. It also may reflect the presence of alternative entry pathways into phagocytes; e.g., conventional phagocytosis leads to inhibition or bacterial destruction; invasion leads to apoptosis; or entry leads to persistence and intracellular replication. Presumably, more than a single pathway could operate simultaneously in the same cell.

Mycobacterium uses a different strategy to modify its intracellular environment within phagocytes, although the principles and eventual outcome are similar. Although *M. avium* fuses with early endosomes to a limited extent, it does not fuse with lysosomes; instead, it remains in nonmatured phagosomes or early endosomes (67, 282). *M. tuberculosis* also resides in a compartment lacking several lysosomal markers indicative of an early endosome (50, 51). However, vacuoles surrounding *M.*

avium and *M. tuberculosis* contain lysosomal glycoprotein markers (like *Salmonella*), but the vacuolar membrane lacks the host vacuolar proton-pumping ATPase that is needed for vesicle acidification (51, 328). As a result, the vacuolar environment that surrounds the *Mycobacterium* organisms is not acidified, especially once the bacteria multiply (51, 67, 328). Lack of acidification also has significant effects on lysosomal enzymes that might find their way into bacterium-containing vacuoles, since nearly all of the degradative lysosomal enzymes require a low pH for activation and for optimal activity. As with *Salmonella*, known lysosomal markers such as cathepsin D, as well as mannose-6-phosphate receptor, are excluded from mycobacterial vacuoles. Since persistence and replication of pathogenic mycobacteria in macrophages is a hallmark of virulence, understanding the intracellular biology of this microorganism may hold the key to the development of new anti-infective therapy and to the development of a new generation of vaccines.

Legionella pneumophila, the causative agent of a bacterial pneumonitis called Legionnaire's disease, provides an additional example of an intracellular pathogen that is well adapted to living within vacuoles inside phagocytic cells. It is likely that it learned these tricks by surviving within unicellular phagocytic amoebae found in its natural aqueous habitat. The uptake of some *L. pneumophila* strains occurs via an unusual process termed coiling phagocytosis. During invasion, whirls of pseudopods are seen coiled around the entering bacterium (155). Since less virulent legionellae do not trigger this uptake mechanism, the role of coiling phagocytosis in eventual intracellular survival is suspected but has not been established unequivocally. Regardless, *L. pneumophila* resides within membrane-bound vacuoles within both amoebae and alveolar macrophages. As with the vacuoles in which *Mycobacterium* species reside, the internal pH is not acidified (156). Additionally, like *S. typhimurium*, specific host proteins are excluded from the vacuolar membrane, although the proteins excluded differ between the two pathogens (52, 116). In contrast to both *M. avium* and *S. typhimurium*, only small amounts of lysosomal glycoproteins are found in *L. pneumophila*-containing vacuoles, but, as with *Salmonella*, these vacuoles do not fuse with fluid-phase markers that collect in lysosomes (154), although organisms that are killed by formalin treatment are targeted to lysosomes. Collectively, these results indicate that *L. pneumophila* actively avoids the traditional phagocytic route and directs itself to a protected intracellular niche.

Like *S. typhimurium*, the vacuole that surrounds *L. pneumophila* changes over time. After internalization, host mitochondria are found in close association with the bacterium-containing vacuoles (154), although there is no indication that the bacteria directly parasitize mitochondria for their energy. At later stages of infection, the mitochondria disappear from the vicinity of the vacuoles; the vacuoles now are found associated closely with the rough endoplasmic reticulum (RER), which includes host ribosomes (154, 329). Again, whether host ribosomes play any role for intracellular *Legionella* species remains to be determined. Similar RER colocalization also has been observed with other intracellular organisms, including *Brucella abortus* and the parasite *Toxoplasma gondii*. *L. pneumophila* initiates intracellular replication approximately at the time of RER fusion. *Legionella* mutants grow slowly in macrophages that also are slow to associate with the RER (329). It has been proposed that *Legionella* may exploit the host autophagy machinery, which is used to degrade portions of host cytoplasm and organelles, to form these specialized bacterial replication vacuoles. Conditions that increase autophagy also increase intracellular growth.

Legionella determinants responsible for their unique intracellular routing have been identified recently. There is a cluster of at least four genes (the *icm* locus) that mediates bacterial targeting to prevent phagosome-lysosome fusion. Mutation in these genes lead to a loss of selective routing and their capacity for intracellular replication (34, 202, 330). Another locus, *dotA*, which maps adjacent to and in the opposite direction to the *icm* locus, also is essential for these processes (22). Mutations in *dotA* result in bacterial targeting to a vacuole rich in the lysosomal glycoprotein LAMP-1. The *dotA* gene product appears to function at an early stage of intracellular routing and is not needed once the unique intracellular site has been established. Recent studies suggest that laboratory-grown *L. pneumophila* cells are much less invasive than bacteria that have been passaged through amoebae or macrophages. Amoeba-grown bacilli are more likely to be taken up by coiling phagocytosis and to persist. It is likely that the reservoir for Legionnaire's disease is intracellular bacteria within amoebae found in potable water. These bacteria, therefore, are poised to invade host cells, such as other amoebae or human alveolar macrophages. Infection with some environmental mycobacteria like *M. avium* also is thought to occur through an aqueous reservoir; it is intriguing that *M. avium* grows within amoebae and that amoeba-grown mycobacteria are more invasive for macrophages than are laboratory-grown cultures of the same strain.

Chlamydiae are obligate intracellular bacteria that remain within a nonacidic vacuole throughout their intracellular life cycle and avoid fusion with lysosomes (227). *Chlamydia* differentiates into two forms—one that actively grows inside host cells (the reticulate body), and one that is the infectious (but not dividing) extracellular elementary body. *Chlamydia* utilizes a different intracellular strategy than the other organisms discussed so far, since very few, if any, host proteins are found in the chlamydial inclusion vacuole membrane. The vacuole remains distinct from the normal endocytic and exocytic pathways of the host cell. However, recent evidence suggests that *Chlamydia trachomatis* lives within an inclusion that intercepts anterograde vesicular traffic from the Golgi to the plasma membrane (136). A similar proposal has been made for vesicles that intersect with the vacuole that surrounds *S. typhimurium* inside epithelial cells (115). Sphingomyelin from the Golgi is incorporated into the *Chlamydia* inclusion and into the cell wall of intracellular bacteria. There is growing evidence that these organisms can produce and export bacterial proteins that are incorporated into the host vacuolar membrane, possibly modifying it for bacterial purposes (271). The role of these proteins remains undefined, although they may be involved in directing vacuolar trafficking that would affect intracellular survival or nutrient acquisition necessary for intracellular growth.

Thus, the recurrent theme used by several intracellular pathogens is pathogen-mediated subversion of host cell intracellular trafficking to remain within a privileged niche. However, the mechanisms used to achieve this microbial "nirvana" have not been elucidated. As discussed above, the host receptor used during invasion significantly affects the eventual routing of a membrane-bound pathogen, as well as the response of the host cell. Mammalian cells also have a series of specialized proteins dedicated to budding, fusing, docking, and routing endogenous vesicles appropriately within a cell (270). It is possible that pathogens are able to secrete proteins into or across the vacuolar membrane that affect the host vesicular transport machinery. For example, a large group of small GTP-binding proteins (rabs) that belong to the *ras* superfamily are required for specific vesicle fusion events, which could be a target of a toxin produced by an intracellular pathogen. For

TABLE 3. Organisms that use actin-based motility for intracellular and intercellular spread

Bacterium	Vacuolar escape	Bacterial component	Cytoskeletal linkage	Cell-cell spread
Listeria monocytogenes	Listeriolysin O	ActA	VASP, profilin, actin, α-actinin, fimbrin, filamin, ezrin	PlcB?
Shigella flexneri	IpaB	IcsA (VirG)	VASP?, actin, α-actinin, fimbrin	IcsB, cadherins
Rickettsia rickettsii	?	?	Actin	?

example, it appears that the vacuolating cytotoxin of *H. pylori* causes large amounts of rab7 accumulation in the large vacuoles triggered by this pathogen, although it is not known if the toxin directly or indirectly affects this GTP binding protein (240). Alternatively, nonhemolytic mutants of *Listeria monocytogenes* up-regulate rab5 recruitment to the bacterium-containing phagosome membrane (12). Additionally, other host proteins involved in vesicle budding and fusion include the ADP-ribosylation factor, soluble *N*-ethylmaleimide-sensitive factor attachment proteins (SNAPs), and SNAP membrane receptors, which also are prime targets for intracellular bacteria to exploit. (Tetanus and botulinum toxins proteolytically cleave such molecules, albeit in neurotransmitter cells.) The recent explosion of information about the basic mechanics of vesicle budding and fusion should provide several tools to determine if intracellular bacteria do, indeed, exploit these processes to reroute themselves inside a host cell.

Our knowledge of the basic factors used by internalized bacteria for growth inside a vacuole remains woefully inadequate. Some attempts have been made to characterize the intracellular environment within a vacuole, but many fundamental questions remain. The source of nutrients (including a carbon source) that are utilized during intracellular growth remains a mystery. It is possible that the internalized bacteria alter the host vacuole in such a way that nutrients are delivered to the vacuole lumen. Alternatively, intracellular microbes could introduce pores in the vacuole membrane that would allow nutrients to diffuse into the lumen. Obligate intracellular organisms, such as *Chlamydia* species, which require host energy to live, presumably use such mechanisms. Alteration of the vacuole by an intracellular organism is not unprecedented, since the intracellular parasite *T. gondii* makes pores in its host vacuolar membrane that allow the diffusion of molecules of up to approximately 1,500 Da into the parasitophorous vacuole (301). Additionally, this parasite secretes a soluble protein that associates and inserts into the vacuolar membrane but does not lyse the vacuole!

The mechanisms used by vesicle-bound bacteria to escape from their host cell also remain a mystery. *Salmonella* can induce apoptosis in phagocytes, which can lead to release of the microorganism. *Mycobacterium* and *Legionella* replicate intracellularly and kill their host cell, presumably freeing the bacteria to infect adjacent cells. However, what one observes in cell culture may or may not reflect the reality of an infectious process. In contrast, although pathogens reside in the cytoplasm and are well adapted to spread from cell to cell (see below), there have been no reports of similar actions by membrane-bound bacteria to spread to an adjacent host cell. It is feasible that infected host cells are engulfed entirely by other phagocytes, which provides a fresh host cell reservoir for the intracellular organisms.

Pathogens That Escape the Phagocytic Vacuole

Several bacterial pathogens that enter host cells have the ability to break out of the phagocytic vesicle to reside and replicate in the host cell cytoplasm. *L. monocytogenes* and *Shigella* species are the best-studied examples, although some *Rickettsia* species follow a similar pattern of host cell cytoplasmic parasitism (335). These bacteria use a common strategy of enzymatically degrading the vesicle that surrounds internalized organisms, followed by intracellular movement that is driven by host cell cytoskeletal components (Table 3). Given that escape into the cytoplasm avoids antibacterial mechanisms and provides the pathogen with accessible nutrients, it might seem surprising that so few intracellular pathogens use such mechanisms, but as we describe below, it is a highly sophisticated bacterium-host cell interaction.

L. monocytogenes is capable of invading both phagocytic and nonphagocytic cells and elicits an immune response that is classic for an intracellular pathogen. Invasion into nonphagocytic cells is mediated by the *inl* locus, which encodes internalin (InlA) and a closely related product, InlB. As also discussed above, internalin binds to E-cadherins to mediate invasion (212). Once internalized, *Listeria* escapes from the phagocytic vacuole. Release from this vacuole is dependent on a hemolysin, listeriolysin O, which is a sulfhydryl-activated toxin that is activated at low pH (39). Two types of phospholipase C also may contribute to this process (315). The significant advantage to the pathogen in escaping the vacuole is exemplified by expressing listeriolysin in the nonpathogenic species *Bacillus subtilus*. These harmless bacilli, which are killed rapidly inside phagocytic cells, are capable of surviving and replicating in the cytoplasm if they synthesize listeriolysin (26)! Although all vacuoles that contain internalized *Listeria* cells are acidified, only a small fraction (14%) of bacteria escape from vacuoles. Others that remain within the vacuole are killed. The presence of two distinct populations of intracellular *Listeria* (one survives, and the other does not) is reminiscent of that discussed above for *S. typhimurium*.

Once free in the cytoplasm, actin begins to condense around *L. monocytogenes*, which is then organized into a polar tail or "comet" of polymerized actin that propels the bacteria through the cytoplasm and probably into adjacent host cells. A single *Listeria* surface protein, ActA, mediates this actin accumulation (72, 181). Proof that ActA causes linkage with actin filaments came from experiments in which *actA* was transfected and expressed in eukaryotic cells (255). ActA was localized on the mitochondrial surface, and the organelles were coated with polymerized actin, although actin tails were not seen. When anchored in the plasma membrane of mammalian cells, ActA also caused the formation of plasma membrane extensions (103). Additional support comes from expressing ActA in *L. innocua*, which also mediates actin-based motility (182). Finally, an ActA fusion protein coupled to cellulose beads can nucleate actin condensation and, when placed on the surface of *Streptococcus pneumoniae*, can mediate unidirectional movement (316). Strains that contain mutations in *actA* do not move intracellularly and are avirulent in a murine model, which demonstrates the importance of this event.

Actin accumulation occurs at one pole on the bacterial surface, presumably due to polar surface expression of ActA. The amino-terminal end of ActA is critical for actin-based motility, while the internal proline-rich repeats appear to affect the rate of intracellular movement (188). The actin polymerizes in a

disorganized manner (instead of in bundles), and profilin (an actin-binding protein) is found at the base of these actin tails (at the interface between the bacterium and the tail). Other host cytoskeletal components, including α-actinin, filamin, fimbrin, and ezrin, are found associated with the actin filaments, but the molecule that bridges the bacteria to the cytoskeleton remains unidentified. A candidate host protein, vasodilator-stimulated phosphoprotein (VASP), that binds directly to ActA recently has been identified (43). VASP also is found in other specialized actin-mediated host structures, including focal contacts and protruding lamellae. It has been proposed that by binding directly to ActA and profilin, VASP bridges the linkage of ActA on the bacterial surface to actin filaments, with profilin mediating the resulting actin nucleation and polymerization (258). Intracellular bacteria appear to be propelled through the cytoplasm by the force generated by continuous actin assembly at one pole of the bacteria, often reaching speeds of 1 μm/s.

The mechanism by which *L. monocytogenes* spreads from cell to cell remains to be defined. It is thought that the actin-directed movement pushes the organism into neighboring cells without the bacteria leaving the cytoplasm. Once inside a neighboring cell, the bacteria must lyse two host membranes to reach the cytoplasm. There is some evidence that a second phospholipase may mediate this escape. Such a mechanism of cell-to-cell transmission would explain the host immune responses to *Listeria*, which mimic those to an obligate intracellular parasite.

Shigella flexneri also is capable of lysing its vacuole after internalization and provides an excellent example of convergent evolution when its intracellular behavior is compared to that of *L. monocytogenes*. As discussed above, *S. flexneri* invades eukaryotic cells via a complex mechanism. Once internalized, the *ipaB* product lyses the phagocytic vacuolar membrane that surrounds the bacteria, releasing the organisms into the cytoplasm (149). As seen with *L. monocytogenes*, actin accumulates around the escaping *S. flexneri* cells. Soon thereafter, the bacteria move through the cytoplasm, propelled by the condensation of actin at one bacterial pole (23). The bacterial locus *icsA* (*virG*) mediates this intracellular spreading. Like ActA of *L. monocytogenes*, IcsA is found on the bacterial surface. However, it also is a secreted protein and, unlike ActA, is found within the actin tails. IcsA alone is sufficient to direct intracellular bacterial actin-based movement, since IcsA also can confer this ability when cloned and expressed in *E. coli* without other *Shigella* gene products (128, 182). Host α-actinin and fimbrin (plastin) are needed for intracellular spread, and fimbrin appears to bundle the actin filaments near the spreading bacteria. The spatial distribution and turnover rates of actin filaments in IcsA-mediated filaments are very similar to those produced by ActA, which confirms that these two organisms utilize similar actin-based mechanisms to move through host cells (128, 182).

Although the mechanisms used by *L. monocytogenes* and *S. flexneri* appear similar morphologically, mechanistically they are very different. ActA and IcsA are not homologous proteins and share no structural similarities. Additionally, IcsA is secreted and incorporated into the actin tail, while ActA remains bound to the bacterial surface. Thus, two different pathogens find different mechanisms to achieve the same strategic goal—lysis of the phagocytic cell and intracellular spread by using host actin.

S. flexneri has an additional mechanism to move inside host cells. These bacteria can orient parallel to preexisting actin filaments and move unidirectionally along these filaments. This organelle-like movement of the bacteria is distinct from movement mediated by the IcsA-mediated mechanisms used to spread from cell to cell (345).

Another *S. flexneri* gene, *icsB*, affects intracellular spread (6). Strains that contain mutations in *icsB* invade, escape the vacuole, and form protrusions by which bacteria normally spread and enter adjacent host cells. However, the mutants remain trapped in the protrusions surrounded by two host cell membranes and mutant cell-to-cell spread is impaired, which indicates that IcsB may be analogous in function to the phospholipases of *L. monocytogenes* and may mediate the lysis of these membranes after projection into neighboring cells.

The mechanism by which *S. flexneri* spreads from cell to cell recently has been found to be dependent upon host cadherins, which are cell surface molecules that are components of intermediate junctions that link adjacent cells (289). *S. flexneri* did not spread intracellularly in cultured cells that lacked cadherins. Whether *S. flexneri* mediates signaling between two cells via intermediate junctions or stimulates endocytic uptake of cell protrusions that contain intracellular bacteria remains to be answered. However, this work emphasizes the intimate relationships that intracellular pathogens develop with host cells and the extent to which these pathogens exploit normal host cell biology.

IpaB, a multifunctional secreted protein that is necessary for invasion and lysis of the vacuolar membrane, also mediates the apoptosis of host macrophages (369, 370). Purified IpaB induces apoptosis when microinjected into the cytoplasm of macrophages (47). We now know that IpaB binds to interleukin-1β (IL-1β)-converting enzyme, a host protein that promotes apoptosis in macrophages. Apoptosis is a common pathogenic strategy. As noted above, *Salmonella* species trigger apoptosis in macrophages, even though they do not escape the phagocytic vacuole. Recent experiments indicate that *Yersinia* infection of macrophages also leads to apoptosis (224a).

Several *Rickettsia* species, including *R. rickettsii* and *R. conorii*, also cause actin polymerization and "tails" around intracellular bacteria (to different extents). Actin polymerization also mediates the cell-to-cell spread of *R. rickettsii* (144, 334). Whether this thematic mechanism is similar to those used by *S. flexneri* or *L. monocytogenes* remains to be determined. A recent observation indicates that actin-based, intracellular mobility also may be used by a virus for intracellular spread (64). The fully enveloped form of vaccinia virus induces the formation of actin tails that are very similar to those caused by the above-mentioned bacterial pathogens, which indicates a common mechanism used by these diverse intracellular pathogens.

BACTERIAL ENTRY INTO "REAL" HOSTS: IN VIVO INVASION

Strains of *Salmonella* that are noninvasive for cultured cells, as well as defined genetic mutants of *S. typhimurium* (110), are less virulent in an animal model (124). However, the actual sites of entry into the host for many pathogens are not well defined. Recent work indicates that several enteric pathogens breach the mucosal barrier in the small intestine through a specialized epithelial cell, the M cell (Table 4) (308). M cells are found in Peyer's patches and are thought to internalize luminal material to underlying antigen-presenting cells. The M cells are thought to provide an early-warning system. Functionally, M cells exhibit a reduced mucus layer, shortened microvilli, and active endocytic and pinocytotic uptake mechanisms when compared to columnar epithelial cells. These terminally differentiated epithelial cells contain relatively few lysosomes and internalize luminal particles without degradation for presentation to underlying macrophages. Although M

TABLE 4. Interaction of various enteric bacterial pathogens with M cells

Bacterium	Type of interaction with M cell
E. coli RDEC-1	Microvillus effacement, pedestal formation, no invasion
Vibrio cholerae	Phagocyte-like uptake into vacuole
Campylobacter jejuni	Active invasion into vacuole
Yersinia enterocolitica and *Y. pseudotuberculosis*	Active invasion into vacuole
Shigella flexneri	Invasion into vacuole, then release into cytoplasm and intercellular spread, M-cell destruction
Salmonella typhi and *S. typhimurium*	Ruffling, microvillus degeneration, uptake into vacuole, followed by M-cell destruction

cells evolved as part of a host protective strategy to prevent incursion of foreign bodies, their functional properties seem to provide an entry target—an Achilles' heel of the host bowel—to many pathogens; thus, many pathogens gain access to underlying cells and tissues through the M cell by capitalizing on its unique properties.

S. typhimurium preferentially invades M cells in murine ligated intestinal loops (48, 171) and triggers membrane ruffling similar to that seen in cultured cells. M cells invaded by *S. typhimurium* die. Cell death enhances additional bacterial penetration of subepithelial tissue; apical and basolateral invasion of adjacent columnar epithelial cells is seen at later times. A mutant of *S. typhimurium* that does not invade tissue culture cells also does not invade or disrupt M cells (171). Similar morphological disruptions of M cells have been observed for *S. typhi* (183). *S. typhi* often causes intestinal perforations during the course of disease, and it is possible that lesions caused by invasion of Peyer's patches are the source of these perforations. Although *S. typhi* enters the murine M cell, it fails to cause a productive infection in mice due to its host specificity (humans). Hence, crossing the M cell barrier is only the first step in the pathogenesis of *Salmonella* infection.

Many other organisms have been reported preferentially to infect or to associate with M cells in Peyer's patches (Table 4) (308). M cell uptake is not as active a process as that seen for *Salmonella* species in all cases. For example, RDEC-1, the rabbit enteropathogen that resembles EPEC by forming attaching/effacing lesions, remains adherent to M cells throughout its infection cycle (160). Other enteric pathogens that are taken up by M cells, including noninvasive *Vibrio cholerae*, *Campylobacter jejuni*, and *Yersinia* species, do not appear to harm the M cell. In general, the mechanism of bacterial entry into M cells closely parallels the mechanism observed with cultured epithelial cells in the laboratory, including pseudopod extension and internalization of bacteria into membrane-bound vacuoles. It remains to be determined whether all of these organisms target a specific M cell receptor by using specific bacterial adhesins or, instead, interact predominantly with M cells because of their specialized role in sampling luminal contents and their underlying biological properties (reduced mucus, shortened microvilli, and increased pinocytotic activity). In *S. typhimurium*, the *lpf* fimbrial operon is involved in targeting the pathogen to murine Peyer's patches and may mediate specific adherence to M cells (17). These fimbriae are needed for virulence if mice are inoculated orally but not if the mice are inoculated intraperitoneally with *S. typhimurium* cells, which indicates a role for these M-cell adhesins in pathogenesis.

The equivalent of M cells is found in other organized lymphatic regions adjacent to the mucosal surfaces throughout the body, including the large bowel, nasopharynx, and bronchus. Whether pathogens like *Bordetella pertussis*, the meningococcus, and virulent streptococci exploit these analogous structures to colonize and breach mucosal barriers of the respiratory tract remains to be seen. A recent report indicates that in swine, *S. typhimurium* initially may enter the tonsils (and lungs) prior to systemic dissemination of the bacteria and their appearance in the gut (85). Given the large area of exposed mucosal surface in mammalian hosts, it seems likely that more than a few pathogens will exploit mucosal lymphoid tissue to breach the mucosal barrier and to gain entry into the host.

The role of *Yersinia* invasin in pathogenesis provides an informative example of the complexity of defining the role of a virulence determinant in pathogenesis. An early report unexpectedly demonstrated that *inv* mutants of *Y. pseudotuberculosis* are as virulent as the parental strain in a mouse model when they are administered orally or intraperitoneally (278). Subsequent examination of *Y. enterocolitica* revealed that although the 50% lethal doses of *inv* and parental strains were similar, the initial course of infection was significantly altered (249). The number of *inv* mutants in the Peyer's patches was significantly reduced. Inv mutants penetrate the liver and spleen slowly compared to the parental strain. Death is a stringent and not always revealing virulence end point.

The ability of *S. flexneri* and other organisms (including *Yersinia* species) to invade only the basolateral (bottom) surface but not the apical surface of epithelial cells poses a dilemma for these organisms (and the scientists who study them). If these microbes have access only to the apical surface of the intestinal surface after oral ingestion, how do they breach the luminal epithelial cell barrier? Crossing the epithelial barrier through M cells surely provides immediate access to the basolateral surface of adjacent cells. Moreover, although *S. flexneri* cannot invade the apical surface of epithelial cells, it can provoke a powerful chemotactic response and induce the transmigration of basolaterally located PMNs through the tight junctions between epithelial cells (251). Once these PMNs begin to migrate, the bacteria can invade the neutrophils that are located at the intercellular junctions between epithelial cells, thereby penetrating the epithelial monolayer and gaining access to the basolateral surfaces of the polarized epithelial cells. Such a mechanism that appears to occur in vivo remains and is consistent with the pathophysiology of bacillary dysentery (252).

Addition of *S. typhimurium* to the apical surface of polarized epithelial cells also causes recruitment of PMNs from the basolateral surface across an in vitro polarized epithelial barrier (204). PMN transepithelial migration requires active protein synthesis by both bacteria and epithelial cells and induces IL-8 production, although IL-8 and *N*-methionyl-1-leucyl-1-phenylalanine are not necessary for PMN migration. Like shigellae, apical recruitment of PMNs after *Salmonella* adherence in vivo may provide an additional pathway for invading *Salmonella* cells to penetrate across host epithelial barriers.

Once bacteria have penetrated host epithelial barriers, their journey has often just begun. They must target appropriate cells or specific organs that are most satisfactory for their multiplication. It is hardly surprising that this targeting appears to be driven by specific bacterial adhesins and invasins. *L. monocytogenes* accumulates predominantly in the liver of mice following penetration through the gastrointestinal tract. The *inlAB* locus of *L. monocytogenes* promotes entry into hepato-

cytes in the liver but not into the resident Kupffer cells (108). The bacteria multiply in these hepatocytes, while avoiding the innate immune defense mechanisms of the host. The *inlAB*-mediated invasion is required for full virulence. *L. monocytogenes* also penetrates the central nervous system in mice, which causes severe meningoencephalitis. A recent study demonstrated that central nervous system penetration was highly dependent on the level and duration of bacteremia (21), which again emphasizes the relationship between dose and successful tissue colonization and disease production.

INTERACTION OF PATHOGENS WITH THE HOST IMMUNE SYSTEM

We (the hosts) and the microbes that surround us are the products of a long-standing interrelationship. When a bacterial pathogen comes into contact with a host, a struggle between the pathogen and the local, innate host defense systems ensues. The resolution of this encounter is critical and determines whether the outcome leads to infection or overt disease. Localized host defense systems are only the first line of defense and are in close communication with the humoral host immune system. Thus, microorganisms that survive the initial interaction with local host defenses usually activate other imposing immune functions that attempt to control the infection. Because of this selective pressure, successful bacterial pathogens developed strategies to avoid or circumvent host defense mechanisms. Several pathogens can directly alter components of the host immune response (such as certain immune cells or cytokine production) and modulate this response. Alternatively, pathogens can attempt to elude the surveillance mechanism of the host immune system. Pathogens that reside on mucosal surfaces and in the superficial cell layers, like the gram-positive pyogenic cocci, bind host proteins on their surface to disguise their identity or alter their surface proteins to confound detection by the immune system. Many pathogens also possess mechanisms to outwit other host defense strategies, including mechanisms to defeat resident phagocytes, serum killing, and Fe^{2+} sequestration.

Mammalian immune systems are finely honed to detect unique prokaryotic cell components like LPS and mucopeptides that serve as early-warning systems to initiate the immune cascade. Bacterial pathogens have developed several strategies to circumvent an inevitable host immune response. Opportunistic pathogens "seize the moment" when the host defenses are weakened. Other organisms affect key effector cells and directly alter the host immune response cells (203). Thus, *Staphylococcus aureus* produces toxins called superantigens that are associated with toxic shock syndrome and food poisoning. These toxins can stimulate certain T cells that bear specific Vβ sequences, while other T-cell types remain unaffected (95). In addition to several other candidate superantigens (94), the *Yersinia pseudotuberculosis* superantigen (224) and the *Mycoplasma arthritidis* superantigen (54) affect other subsets of T cells. Why these pathogens stimulate a particular subset of T cells and the role of this stimulation in infection remain a mystery. We still do not know whether these microbial factors actually influence the virulence of the microorganism. It does not seem likely that these factors were designed to cause toxic shock syndrome or food poisoning. Rather, we believe that superantigens were developed by the microorganism to overcome local host defense systems. So far, it appears that if superantigens do enhance bacterial virulence, they do so to a small extent (279).

Adherence of a pathogen to host cell surfaces, including epithelial cells, evokes modulators of the immune response (141), which serve to signal and recruit cellular reinforcements to the site of infection (80). Thus, when uropathogenic *E. coli* bind to host cells, several cytokines, including IL-1, IL-6, and IL-8, are produced. Stimulation of these cytokines is dependent upon binding via the P fimbriae. Since IL-8 is a potent chemoattractant and activator for neutrophils, this mucosal cytokine response may initiate the localized inflammation seen in urinary tract infections.

Bacterial entry into epithelial cells commonly elicits cytokine production. *Salmonella* species, *L. monocytogenes*, and *H. pylori* (63) stimulate IL-8 secretion in intestinal and cultured epithelial cells (79). Sometimes pathogens suppress host cytokine production. For example, *Y. enterocolitica* YopB suppresses the production of tumor necrosis factor alpha, a cytokine that is central to host immune and inflammatory responses in macrophages (24).

As noted above, the stimulation of chemoattractant cytokines at the mucosal surface actually may potentiate establishment of infections by some invasive organisms. By using in vitro systems, it has been shown that both *S. typhimurium* and *S. flexneri* cause transepithelial migration of PMNs from the basolateral surface of polarized monolayers to the apical surface when these bacteria are added apically (204, 251). Although the *Salmonella*-induced PMN recruitment does not use the classical chemotaxic peptide, *N*-formyl-methionine-leucine-phenylalanine, to trigger chemotaxis, PMN transmigration does require bacterial adherence and protein synthesis by both bacteria and host cells, although the exact mechanism has not been defined. The recruitment of these phagocytic cells to the mucosa may enhance bacterial penetration across epithelial barriers. There is a good correlation of the ability of various *Salmonella* species to cause transepithelial signaling to neutrophils and their capacity to cause disease (enteritis) (205).

S. flexneri does not invade the apical surface of epithelial cells. Thus, it is important for it to gain access to the basolateral membrane (228). Similarly, PMN transmigration, which disrupts tight junctions and exposes the basolateral aspect of cells, is required for successful *S. flexneri* penetration of the polarized monolayers (228, 251) similar to that which also occurs in colonic crypts during shigellosis. *S. flexneri* also induces apoptosis in macrophages via an IpaB-mediated mechanism that leads to the release of IL-1 and an inflammatory response (288, 369).

Stealth Technology

Pathogens that remain adherent on mucosal surfaces throughout their infection cycle have developed strategies to avoid the ever-present immune system surveillance. Perhaps the simplest way is to bind host proteins on their surfaces, which disguises the bacterial surface antigens. Many pathogens are capable of binding numerous host proteins; however, defining the role of such actions in virulence has not been easy. As discussed in a previous section, many pathogens bind basement membrane components, such as fibronectin and collagen. Pathogens bind several other host proteins. Mucosal pathogens, such as *Haemophilus influenzae* and *Neisseria meningitidis*, have specific receptors for the iron-binding proteins transferrin and lactoferrin (60). Although it is thought that these receptors provide a mechanism for the bacteria to acquire iron, they also may play a role in masking the bacterial surface. *S. aureus* binds immunoglobulins via protein A and protein G, which may confer protection from other antibodies and complement. The *B. pertussis* adhesin FHA can bind several host molecules, including heparin and host glycoproteins and glycolipids. Alternatively, the pathogen may produce a surface

molecule that mimics a normal host component. For example, *H. pylori* expresses a complex surface carbohydrate, Lewis X, which may contribute to the down-regulation of T-cell responses to this self-antigen seen in *H. pylori*-infected individuals (307).

Pathogens also produce factors that inhibit the binding of complement and antibodies on their surface. Such actions prevent activation of the immune response and also prevent opsonization that facilitates phagocytosis. Most clinically important mucosal pathogens, including *H. influenzae*, *Streptococcus pneumoniae*, *E. coli* K1, and *N. meningitidis*, make carbohydrate capsules that surround the organisms. Although the carbohydrate composition and biosynthetic pathways vary among organisms, it is well established that such structures protect the organism from complement lysis, antibody deposition, and phagocytosis. Bacterial S-layers and some types of LPS also can inhibit antibody binding and phagocytosis, presumably by similar mechanisms. It is known that long side chains of *S. typhimurium* LPS inhibit complement lysis by causing complement to deposit distal to the bacterial surface, which forms inactive complexes that do not insert into the bacterial membrane (170). Varying the amount of sialylation in the variable portion of *N. gonorrhoeae* LPS affects antibody deposition and complement activation, in addition to affecting invasion (344). In gram-positive organisms that lack LPS, bacterial proteins that extend from their surface may cause improper complement deposition distal to the bacterial surface; for example, the M protein of *Streptococcus equi* interferes with complement deposition on the bacterial surface (33).

Other bacterial surface proteins serve to protect microorganisms from complement-mediated lysis and therefore provide serum resistance. The *ail* locus of *Yersinia* species is one such example. Although this molecule mediates low levels of invasion, it also enhances resistance to serum (27). A plasmid-encoded locus in *Salmonella* species, *rck*, also confers serum resistance by blocking formation of a fully polymerized membrane attack complex by complement (143). Ail and Rck share homologous sequences. Other abundant outer membrane proteins also confer serum resistance (145, 355) by altering the bacterial outer cell membrane, which blocks the ability of complement to insert pores into the bacterial membrane.

Enzymatic Degradation of Immune Components: a Frontal Approach

An alternate mechanism to prevent the binding of complement and antibodies on bacterial surfaces is to produce enzymes that degrade host molecules. As discussed in a previous section, bacteria like *P. aeruginosa* and *S. aureus* produce several proteases that degrade host proteins. However, pathogens such as *N. meningitidis*, *H. influenzae*, *Clostridium* species, *Streptococcus sanguis* (a dental pathogen), *Streptococcus pneumoniae*, and *Proteus mirabilis*, secrete IgA protease, which has a much narrower target spectrum and is often host specific (180).

Pathogens also produce enzymes that degrade complement. For example, *S. pneumoniae* is capable of degrading and inactivating C3 (14). Group A and B streptococci also have a C5a convertase that blocks the chemoattractant capabilities of this molecule (49). The parasite *Entamoeba histolytica* also possesses a cysteine proteinase that cleaves C3a (267), which indicates that a broad spectrum of pathogens use such strategies.

Antigenic Variation

Strain variation effectively can nullify the immune response and is a common microbial theme used to avoid the host immune response. Such variation ensures that subsequent infections are not recognized fully or efficiently by the host immune memory (35). As a consequence, certain pathogens have been selected to vary their surface components, including pili, flagella, LPS, capsules, S-layers, secreted enzymes, and several outer membrane proteins. This is not to say that most surface components vary in most pathogens. Instead, extensive variation usually is seen only in a small number of highly expressed (or exposed) immunodominant surface proteins in pathogens that reside on mucosal surfaces. Examples of antigenic variation within intracellular bacterial pathogens are comparatively less common, presumably because the host immune response is different and is activated by antigens found anywhere in a bacterium, not just on its surface.

The pathogenic *Neisseria* species (*N. gonorrhoeae* and *N. meningitidis*) provide a paradigm for microorganisms that constantly change their surface by a novel genetic mechanism. One of the best-studied examples of such antigenic variation is the gonococcus pilus (214, 303, 367). Although there are potentially a large number of genes that encode distinct pilus types in a given gonococcal strain, only one at a time is expressed. However, as the organism grows, whether in culture or in the urethra, the pilus type switches continually. Only one transcriptionally active pilin expression locus (*pilE*) is expressed per cell, but there are many (>50) other different truncated pilin genes (*pilS* loci) that are not transcribed and are located throughout the chromosome. Frequently, genetic rearrangement (via RecA) occurs, which removes the expressed gene from *pilE* and places a copy of one of the silent genes into the expression locus, generating a different pilin product. *Neisseria* species also can express any number of a variety of opacity (Opa) proteins on their surface (25, 168, 324). Expression of each *opa* gene is independent of expression of the other *opa* genes and is governed by an on-off genetic switch. Each *opa* gene has a series of repeats of the sequence CTCTT within the region that encodes a hydrophobic signal sequence. The number of CTCTT sequences determines the translational frame of the gene and, ultimately, whether a complete Opa protein is expressed. Recombination between CTCTT sequences varies the number of CTCTT repeats. PilC, another gonococcal protein that can vary, is a molecule involved in pilus assembly (173). There are two variant copies of *pilC* on the chromosome, which contain a poly(G) tract in the region that encodes a signal sequence. Like *opa*, variation in the number of Gs in this tract affects the reading frame and expression of each PilC, which results in phase variation. Finally, as discussed above, *N. gonorrhoeae* LPS varies its level of sialylation, which affects its levels of serum resistance, opsonophagocytosis, and invasion (139, 344).

Borrelia species, including the causative agent of relapsing fever, *Borrelia hermsii*, provide another example of antigenic variation (283). These organisms harbor linear plasmids that encode multiple (27) silent copies of the variable major proteins. Like *Neisseria* pili, translocation of these sequences through DNA rearrangements into an expression site on another linear plasmid results in expression of different variable major proteins. Constant alteration of the antigenic profile of these organisms (and the corresponding immune responses) presumably results in the characteristic relapsing fever, much like that seen in the trypanosome-mediated sleeping sickness. In *B. burgdorferi*, which causes Lyme disease, the size and amount of surface-associated proteins (OspA, OspB, and OspC) vary among strains, although there is no evidence of antigenic variation. This strain variation appears to be caused by genetic recombination events and impacts reinfection by

different strains since each strain appears immunologically different.

Strain-to-strain variation occurs in many bacterial surface components. Some examples include *Pseudomonas* pili, *Salmonella* LPS O side chains (>60 types), *S. pneumoniae* capsule (>80 types), *H. influenzae* IgA proteases (>30 variants), the streptococcal M protein (>80 serotypes), and the major outer membrane protein of *Chlamydia trachomatis*. Most of the variations are caused by small nucleotide substitutions, insertions, and deletions in the genes that encode these virulence factors that result in antigenic drift. Interspecies gene transfers facilitate such variations, and it is noteworthy that several pathogens that exhibit antigen activation, such as *Neisseria* species and *H. influenzae*, are transformed readily by extracellular DNA. In this way, potentially useful genetic variations are shared effectively among members of an entire microbial population. In general, the clinical diseases caused by most antigenically variant strains of the same species are not different. However, epidemiological investigations (and experience) teach us that while some diseases caused by the same microbial species recur, some degree of resistance accumulates with time as a result of multiple insults to the immune system. Nevertheless, the organisms that exploit their capacity to vary have a powerful advantage in the interplay between the host and the invading microbe in "immunized" hosts.

An obvious way for intracellular bacterial pathogens that reside within host cells to avoid the host immune response is to block antigen processing and presentation. For example, the *phoP* locus of *S. typhimurium* is part of a two-component regulator that controls the synthesis of several proteins needed for *Salmonella* to survive within macrophages. *phoP*-regulated genes also affect antigen processing and presentation of bacterial antigens, which inhibits the induction of specific immunity (359). On the other hand, *Salmonella* strains with mutations in the *phoP* locus persist within the infected host and permit the development of a strong, specific anti-*Salmonella* immune response; not unexpectedly, these mutants are excellent candidates for live vaccine strains. Other intracellular pathogens, such as *Legionella pneumophila*, inhibit the amount of major histocompatibility complex class I and II antigens that are contained in the vacuolar membrane, which affects the ability of macrophages to present bacterial antigens (234). (In contrast, both *M. tuberculosis* and *S. typhimurium* contain major histocompatibility complex molecules in the vacuolar membrane that surrounds them.) Some viruses inhibit the peptide transporters (TAP) that deliver peptides to the lumen of the endoplasmic reticulum and subsequently to the cell surface for antigen presentation (150). Most probably, some intracellular bacterial pathogens utilize similar mechanisms to inhibit specific antigen presentation, although no examples have been reported. This is especially likely for pathogens that cause persistent infections in their host.

CONSERVED MACHINERY FOR SECRETION OF VIRULENCE FACTORS

Nearly all bacterial virulence factors are located on the bacterial surface or are secreted. In the past few years, there has been an explosion of information identifying bacterial factors that are needed as accessories to transport virulence factors to the cell surface and into the surrounding environment. Many of these systems are highly homologous, and unrelated virulence factors often share related basic transport mechanisms. Perhaps this commonality indicates that there are only a limited number of ways to move a protein out of the bacterium, so that virulence factors use one of a few such general mechanisms. The common thread seen in the secretion of pathogenicity factors has led to their classification into families according to the transport pathways used. It also has led to optimism that new classes of novel, broad-spectrum anti-infective compounds that might inhibit such systems may be developed.

Organelle assembly on the bacterial surface requires several factors in addition to the subunits that compose the organelle, such as the Pap/type I pilus system (discussed above). The Pap/type I pilus system uses its own molecular chaperones and ushers that move the pilus components through the cell and into their correct locations within the pilus. These mechanisms are conserved among many different pilus types throughout the gram-negative bacteria.

Type I Secretion Systems

Three general secretion pathways have been found in gram-negative bacteria that export bacterial virulence factors (286). There is, in addition, a group of bacterial proteins that mediate their own transport. The type I system is a *sec*-independent pathway whose prototype member is the *E. coli* hemolysin (discussed above) (356). Other molecules, including adenylate cyclase and FHA from *B. pertussis*, alkaline protease from *P. aeruginosa*, leukotoxin by *Pasteurella haemolytica*, and proteases of the plant pathogen *Erwinia chrysanthemi*, are exported via this pathway. Molecules transported by type I systems lack classic signal sequences and require three or four accessory molecules that form a transmembrane channel through which the secreted protein moves. The genes that encode these accessory molecules usually are found clustered with those that encode the secreted molecule. The accessory molecules are homologous and often interchangeable. One of the accessory proteins contains an ATP-binding domain that probably energizes the system. The carboxy-terminal region of the secreted molecule encodes the information necessary for secretion.

Type II Secretion Systems

Several virulence factors use a more general secretion system that is found commonly in gram-negative organisms (262). The type II system exports many different types of molecules, including some virulence factors. Mutations in this system are pleiotropic and generally affect several exported products. Members of this family include the *N*-methyl-Phe (type 4) pili of *P. aeruginosa* and other related pili discussed above, the pullulanase system of *Klebsiella*, DNA uptake by *H. influenzae*, DNA conjugative transfer in *B. subtilis*, M13 phage formation in *E. coli*, S-layer formation, and secretion of virulence factors in several plant pathogens (151). This general system uses a traditional *sec*-dependent pathway to move the exported molecules across the inner membrane to the periplasm. Although not well characterized, additional accessory molecules assist in moving exported proteins across the outer membrane. A formidable array of accessory molecules (approximately 14) are required for this process and usually are clustered in an operon. The type II secretion system is thought to be the major export pathway in most gram-negative organisms, although *E. coli* K-12 does not have such a system (286)! Like the type I secretion system, one component of this system has a consensus ATP-binding site (Walker box), which indicates that energy for export may come from this high-energy nucleotide. These systems also encode a peptidase that cleaves off a small N-terminal sequence that appears to be involved in the export process.

TABLE 5. Examples of type III virulence factor secretion systems

Bacterium	Molecules secreted	Role in virulence
Yersinia species	Yops	Inhibition of phagocytosis by affecting host signaling (YopH and YpkA) and cytoskeleton (YopE), thrombin binding (YopM), injection of Yops into host cell
Shigella species	IpaA to IpaD	Mediation of invasion in nonphagocytic cells
Salmonella species	SipA to SipD, InvJ, SpaO (InvK)	Mediation of invasion in nonphagocytic cells, invasome organelle formation
Salmonella species	? (encoded within pathogenicity island SPI2)	Survival in host tissue, critical for virulence
Attaching/effacing organisms (EPEC, EHEC, RDEC-1, *Citrobacter rodentii*)	EspA, EspB, GAPDH-like protein (EPEC), ≥1 other	Mediation of signal transduction in host cells; required for pedestal formation and intimate contact
Plant pathogens (*Pseudomonas solanacearum*, *Xanthomonas campestris*, *Pseudomonas syringae*, and *Erwinia* species)	Harpins	Plant tissue damage and disease
P. aeruginosa	Exoenzyme S, others (?)	Dissemination to bloodstream, inhibition of T-lymphocyte host defense?

Type III Secretion Systems

More recently, a third major export system, which plays an active role in the specific secretion of virulence factors in both human and animal pathogens, has been identified (343). The type III export system is responsible for secretion of Yops in *Yersinia* species (215, 354), *Salmonella* and *Shigella* invasion and virulence factors (8, 9, 111, 131, 305), EPEC signal transduction molecules (167), and virulence factors (hairpins) in several plant pathogens including *Pseudomonas solanacearum* and *Erwinia* species (Table 5) (343). Additionally, homologs are found in genes involved in flagellar assembly, which indicates that this pathway may be involved in surface organelle biosynthesis (41). Secretion of *P. aeruginosa* exoenzyme S (and possibly other *Pseudomonas* secreted proteins) also appears to use such a secretion system (364). Each secreted protein appears to have a corresponding cytosolic chaperone that recognizes a portion (usually the amino-terminal region) of the secreted protein that is critical for mediating secretion (352, 354, 363). Conservation of these systems is such that if the appropriate cloned chaperones and secreted proteins are placed in other organisms that contain a different type III system, heterologous secretion occurs (131, 277). The interchangeability of components of these systems was demonstrated with *Shigella*, *Salmonella*, and *Yersinia* and their corresponding secreted proteins (131, 277). Little is known about the molecular mechanisms of the type III export pathway. Approximately 20 linked genes are necessary to achieve functional secretion. One component of this *sec*-independent system shares homology with the catalytic subunits of F_0F_1 ATPase, contains an ATP-binding site (the Walker box), and may be the energizer of the system (362). There is also some relation between the type II and type III export systems. At least one component of the two systems is homologous, but most of the other components are distinct. It appears that both types of export systems are used to assemble organelles on bacterial surfaces. In addition, the secreted effector molecules of type III secretion affect host cell signaling. At least for *Yersinia* species, several of the Yops, including a tyrosine phosphatase (YopH), an actin cytotoxin (YopE), and a Ser/Thr kinase (YpkA), are transported directly into the host cell following host cell contact (see above). It is probable that the secreted products of other type III secretion systems, including the invasins of *Salmonella* and *Shigella* species and the signaling molecules secreted by EPEC-like organisms (EspA and EspB), also are injected into the host cell following cell contact. Clearly, the type III secretion system is "contact mediated" and acts to deliver bacterial virulence factors into host cells.

Type IV Secretion Systems: Autotransporters

Gonococcal IgA protease (180) is representative of a family of secreted proteins that mediate their own transport out of bacteria. Other members of this family include the IgA proteases of *H. influenzae* (259); a serine protease from *Serratia marcescens* (223); the vacuolating cytotoxin of *H. pylori* (62); Tsh, a hemagglutinin expressed by an avian *E. coli* pathogen (260); SepA, a secreted protein from *Shigella flexneri* (20); EspC, a secreted 110-kDa molecule of EPEC (320); a recently described molecule from *H. influenzae* that mediates in vitro adherence and invasion into cultured cells (325); and a family of outer membrane proteins in *B. pertussis*, including pertactin and BrkA, a serum resistance protein (86). Like the type II secretion system, molecules that belong to the IgA protease family use a *sec*-dependent pathway and cleavage of a classic signal sequence to translocate across the inner membrane. However, these molecules can direct their own passage across the outer membrane (autotransporters), apparently by forming a pore through which they pass. Autoproteolytic cleavage at three closely spaced sites at the carboxy-terminal end of the surface-bound protein releases the IgA protease and another protein (α-protein) into the medium. α-Protein contains a nuclear targeting sequence and has homology to eukaryotic transcriptional factors (257). Fusions with the IgA protease gene result in secretion of these heterologous molecules out of the bacteria.

As more and more sequences of virulence factors are determined, it is becoming abundantly clear that most can be divided into families, based either on their function or on their mechanism of export to the bacterial surface. For example, invasin from *Yersinia* species shares homology with intimin from EPEC and other organisms that cause attaching/effacing lesions (169). Both proteins are surface localized, although their functions differ. The homology between these two molecules does not occur at the end that recognizes the host receptor (C-terminal region); instead, it occurs in a region that appears to mediate export to the bacterial surface (N-terminal region). Similarly, the homology that occurs between the complement resistance proteins Ail and Rck, PagC (*phoP* activated gene), OmpX, and Lom (a lambda outer membrane protein)

may be due to common export mechanisms and insertion into the outer membrane (142, 208).

Of course, many common components involved in general protein synthesis and maturation are also necessary for the expression of virulence factors. For example, there is a family of related proteins with homology to *E. coli* DsbA that are involved in the formation of disulfide bonds within the periplasm. These molecules are necessary for maturation of cholera toxin and heat-labile enterotoxin, pili in *V. cholerae* and EPEC, some *H. influenzae* outer membrane proteins, and even secretion of a *Shigella*-invasion protein that uses the type III secretion system (351). Other molecules that assist in maturation of proteins include members of the heat shock protein family, which are broadly distributed in microorganisms. Nonetheless, they are necessary for expression of virulence factors due to their ability to correctly fold these molecules.

Gram-Positive Protein Secretion

Export of proteins to the surface and beyond is less complex in gram-positive organisms. A signal sequence is usually sufficient to ensure secretion. Most surface or secreted proteins of the streptococci, staphylococci, and *Listeria monocytogenes* contain analogous structural features that are found in diverse proteins that mediate host cell contact or cell recognition. In addition to a classic signal sequence, these proteins usually have a region of several tandem repeats followed by a carboxy-terminal region that contains a proline/glycine-rich segment and a conserved hexapeptide (LPXTGE), followed by a hydrophobic stretch of approximately 20 amino acids and a positively charged tail (77). Such sequences are thought to anchor these proteins in the membrane with the hydrophobic sequences that span the cell membrane. The charged tail forms an anchor on the cytoplasmic face of the membrane. A cell wall-spanning sequence usually is found upstream of the LPXTGE sequence; however, some of these proteins also are secreted into the supernatant. The carboxy termini of the secreted form of these proteins terminates in the LPXTGE sequence (239). This is a common motif of virulence factors found in gram-positive organisms (i.e., the fibronectin binding molecule protein F, the adhesin protein M, and the G protein from *S. pyogenes*; the P1 adhesin from *S. mutans*; the fibronectin binding proteins FnBPA and FnBPA and A protein from *S. aureus*; and internalin from *L. monocytogenes*). Although the sequence hepapeptide is found in nearly all surface proteins, there are exceptions, including ActA from *Listeria monocytogenes*.

Because of the conservation of several basic mechanisms that are critical for virulence factor expression, there is increasing interest in defining inhibitors that could block such systems. Identification of such factors might provide new antibacterial agents that specifically target machinery that is necessary for virulence factor expression. However, whether such agents could arrest an established infection (as opposed to blocking bacterial growth or killing the organisms, which is characteristic of most antibiotics) remains an untested hypothesis.

IRON ACQUISITION INSIDE THE HOST

All bacteria require iron for their growth, and this requirement is no less stringent for pathogenic organisms that must proliferate inside the mammalian host. However, the iron in mammalian hosts is tightly sequestered by high-affinity, iron-binding proteins such as transferrin and lactoferrin, making it difficult for most bacteria to acquire iron inside a host. Pathogens that live on mucosal surfaces, on deeper tissue, and inside host cells have acquired specialized mechanisms to obtain iron inside the host (248, 254). For example, *Neisseria* species, *H. influenzae*, *Moraxella catarrhalis*, and *Actinobacillis pleuropneumoniae* can directly bind host iron-binding proteins, such as transferrin and lactoferrin, that use a conserved family of bacterial receptors (60, 296, 297), which allows them to obtain iron. Thus far, the host specificity of the iron-binding protein appears to be specific. For example, *Neisseria* species and *H. influenzae* can utilize only human transferrin and lactoferrin. It has been proposed that this requirement may contribute to their strict host specificity (for primates). *N. meningitidis* also has a hemoglobin receptor that is part of the bacterial transferrin and lactoferrin receptor family that provides this organism with an additional capacity to capture iron (326). Mutations in this receptor adversely affect virulence, which emphasizes the importance of this iron acquisition mechanism. A dental pathogen, *Porphyromonas gingivalis*, has the capacity to transport an intact hemin moiety into the bacteria (123).

Alternatively, bacteria acquire host iron by secreting molecules (siderophores) that can bind iron with an even higher affinity than host iron-binding proteins, thereby "stealing" it from the host (230). The iron-bound siderophores can be taken up by bacteria via a specialized transport system and subsequently utilized. Although siderophores are found in several pathogenic bacteria, they are not restricted to pathogens. They are found as an iron-scavenging mechanism throughout the prokaryotic kingdom. The contribution of siderophores to virulence varies among pathogens.

Since iron is found in such low concentrations within the host, many bacterial pathogens use low iron concentrations as a cue to activate certain virulence factors (195). Such virulence factors are diverse and include toxins, adhesins, invasins, and several other outer membrane regulatory proteins that are regulated by Fur (discussed below).

How bacterial pathogens acquire iron from within vacuoles inside host cells has not been well studied. For example, free iron does not reach the intracellular bacteria when added at high concentrations to cultured cells that contain intracellular *S. typhimurium* (118). It is possible that intracellular bacteria secrete siderophore-like molecules that can penetrate the host vacuole membrane, sequester iron in the cytoplasm, and then return iron to the vacuole. Perhaps the vacuoles that contain the intracellular bacteria fuse with exocytic or endocytic vesicles that contain host iron; the bacteria can then salvage this iron. Finally, bacteria, like the protozoan *Toxoplasma*, may insert pores into the vacuole membrane that allow host molecules such as iron-binding components to enter the vacuole (301). However, none of these proposed mechanisms have been documented to occur inside infected host cells. It is likely that intracellular pathogens have evolved different or additional iron acquisition strategies than those used by pathogens that live extracellularly on mucosal surfaces or inside tissue.

REGULATION OF VIRULENCE FACTORS

Nearly all bacterial virulence factors are tightly regulated, with their expression linked to various environmental signals. Some parameters that affect virulence factor regulation include temperature, ion concentrations, osmolarity, iron levels, pH, carbon source availability, growth phase, and oxygen levels (132). Pathogens use one or more of these environmental factors to sense which microenvironment they currently occupy within a host or even within a specialized compartment inside a single host cell. Some pathogenic bacteria are schizophrenic. The expression of virulence factors is not ordinarily needed outside of the host; however, other factors equally essential for microbial survival are required outside of the preferred host.

The microorganism presumably senses both environments and acts accordingly.

Once the pathogen encounters a host and senses certain biochemical and physical parameters, such as an increase in temperature or a change in pH, a cascade of virulence factors are induced. At each step in the infectious cycle in response to a kaleidoscope of host responses, different genes are turned on even as others are turned off. For example, invasion genes usually are turned on early in the infection but are repressed once bacteria are inside host cells. A single regulatory element often controls the expression of many unrelated genes (global regulators), including effectors of virulence, as well as associated genes that are optimized to provide survival within a particular environmental niche. For example, in *L. monocytogenes*, PrfA regulates nearly all of the identified virulence factors (44).

The regulation of virulence factors, as expected, has common motifs used for a variety of normal bacterial functions. As a result, virulence factor regulators can be grouped into a relatively small number of families based on their conserved sequences and mechanisms. The study of virulence factor regulation has benefited greatly from knowledge of basic microbial regulatory mechanisms (and vice versa). In the not too distant future, inhibitors of these conserved bacterial regulatory systems also might be used as novel anti-infective agents.

We have begun to appreciate that bacterial virulence factors do not operate in a biological vacuum. Rather, they are often expressed coordinately and are interdependent to achieve their biological effects on the host. Hence, upon receiving the appropriate cues, virulence genes are activated and proteins are synthesized and transported to precise cellular locations, where they are assembled into complexes that may be activated upon contact with the host or in response to a unique "cellular signature." Thus, virulence factors may be controlled simultaneously by several regulators that measure different parameters, and several regulatory systems can regulate a single virulence factor. Thus, virulence expression is a sum of the signals that are sent by various cell regulators and sensing systems. The challenge in the future will be to define which of these mechanisms work in vivo and to determine their activities on virulence factors in a given location within the host.

Two-Component Regulatory Systems

"Two-component" regulatory systems are found in gram-negative bacteria that regulate many bacterial functions, including those involved in pathogenicity (217). These systems usually consist of (i) the sensor protein, which spans the cell membrane, where the extracellular domain "senses" a given external signal and the intracellular domain is a histidine kinase, and (ii) the regulator protein, which can function as a transcriptional activator and/or repressor. Upon sensing a certain stimulus, the sensor undergoes autophosphorylation and then transfers the phosphate residue to the amino-terminal domain of the regulator protein that changes the ability of this protein to bind to specific DNA sequences, which initiates the transcription of genes that contain a conserved upstream regulator-binding sequence.

Many examples of bacterial virulence factors that are regulated by two-component systems (132) include global regulators of virulence factors in *Bordetella pertussis* (BvgA/BvgS) (340) and *V. cholerae* (ToxR/ToxS) (71), *Salmonella* survival inside macrophages (PhoP/PhoQ) (122, 218), and outer membrane porin regulation in *Salmonella* and *E. coli* (EnvZ/OmpR) (98). The environmental factors that regulate two-component systems that affect virulence are varied and often not known. For example, the PhoP/PhoQ system of *S. typhimurium*, which regulates a number of virulence factors (see above), is affected by starvation for phosphate and carbon, low pH, and the macrophage intracellular environment and appears to be directly regulated by Mg^{2+} (121). PhoP/PhoQ also represses several genes in *S. typhimurium*, and their products also are needed for virulence. However, it has been estimated that only 25% of the PhoP-regulated genes are involved directly in virulence, and, of course, nonpathogenic microbes (such as *E. coli*) also can contain the PhoP/PhoQ operon. Note that when PhoP is modified genetically to be constitutively turned on, it renders the host microbe avirulent (219). Collectively, this work suggests that PhoP/PhoQ regulated genes are turned off in the environment and in the early stages of infection. Once the bacterium enters phagocytic cells, PhoP/PhoQ activates the expression of some products that are involved in survival within macrophages while repressing the expression of other genes whose products are no longer needed, including some involved in the initial invasion of host cells.

Another well-studied two-component regulator is BvgA/BvgS in *Bordetella* species. BvgA/BvgS regulates its own expression, as well as the expression of other genes, including FHA, fimbriae, and several toxins. BvgS senses different stimuli, including temperature, magnesium sulfate, and nicotinic acid, although the factors that activate BvgA/BvgS in vivo have not been characterized. It has been shown that if the BvgA/S regulatory system is "rewired" by reversing regulatory conditions, virulence is lost (5). This and the PhoP/PhoQ story described above indicate that regulation of virulence factors and of other factors that are repressed when virulence genes are expressed is critical and finely tuned and that altered expression of these genes affects virulence.

AraC Transcriptional Activator Family

In addition to the two-component regulatory family, several other families of regulatory factors recently have been shown to influence virulence expression. A rapidly growing family of regulators is the AraC transcriptional regulatory protein family, initially named after the *E. coli araC* product that controls the arabinose operon. Proteins in this family contain helix-turn-helix motifs, which bind to specific DNA sequences upstream of genes that are actively transcribed upon regulator activation. Many diverse virulence factors are regulated by AraC-like molecules; several different stimuli can activate such systems. One of the better-characterized systems is VirF (LcrF), found in *Yersinia* species (152, 313). VirF functions as a global regulator of Yops. Yops are membrane-localized or secreted proteins that function as virulence factors. VirF binds to a sequence in several Yop promoters (353). VirF also is regulated by temperature, and Yops are expressed only at 37°C (without Ca^{2+}). It has been proposed that higher temperatures are needed to modify the DNA structure of these promoters to enhance transcriptional activation (187).

Another important AraC-like virulence regulator is found in *Shigella flexneri*; coincidentally, it is called VirF (284). VirF activates *virB* transcription; VirB, in turn, activates transcription of several invasion genes. *Shigella* VirF, like its *Yersinia* counterpart, also is strictly temperature regulated and is active only at 37°C. AraC-like proteins also regulate several other virulence factors. For example, in *V. cholerae*, ToxR (a member of the two-component regulatory family) activates transcription of *toxT* (148). ToxT (also called TcpN) is an AraC-like molecule and regulates expression of the toxin-coregulated pili in *V. cholerae*. (Other virulence factors are regulated by the ToxR/ToxS system directly, independent of ToxT.) AraC-like

virulence regulators include AggR, which regulates aggregative adherence fimbria I in enteroaggregative *E. coli* (229); ExsA, which regulates exoenzyme S in *P. aeruginosa* (102); PerA, which regulates EPEC virulence factors (129); and RNS in enterotoxigenic *E. coli* (40). Several AraC-like homologs also transcriptionally activate virulence factors found in plant pathogens. As more virulence systems are characterized, it is likely that several additional AraC-like transcriptional regulators will be added to this family.

LysR Transcriptional Regulators

A family of virulence factor transcriptional activators are homologous to LysR described initially in *E. coli*. Members of this family also contain a helix-turn-helix domain that mediates DNA binding and subsequent transcriptional activation of downstream genes. One member of the LysR family is SpvR in *Salmonella* species (38), which is a plasmid-encoded molecule that regulates several other *Salmonella* plasmid-encoded virulence factors (SpvA to SpvD) that mediate long-term survival in mice (133, 135). SpvR is positively controlled by itself and negatively controlled by SpvA and SpvB. SpvR normally is repressed when *Salmonella* is grown in the laboratory, although it is induced under carbon starvation conditions. Additionally, the *spv* genes are transcriptionally activated when *Salmonella* bacteria enter into epithelial or macrophage cells; and this activation is induced by SpvR (87, 269). Another member of the LysR family that regulates virulence factors is IrgB, which is repressed by iron (127). However, low-iron conditions in a host permit *irgB* transcription; its product then activates transcription of *irgA*, a *Vibrio* virulence factor. LysR also regulates virulence in plant pathogens, including *Pseudomonas solanacearum* and *Agrobacterium tumefaciens*, which indicates the common use of this transcriptional activation system.

Fur and Other Regulatory Systems

Fur is a DNA-binding protein that binds to specific sequences only if iron is present to repress transcription of the downstream genes (195). Without iron, Fur does not bind and gene transcription is activated. Fur regulates several factors within most bacteria. However, determining the role of Fur in virulence has been difficult. For example, the *S. typhimurium fur* mutant is still virulent in an animal model (117). However, since Fur is a repressor (rather than an activator), this regulation may not be needed within a host for virulence.

Clearly, the regulation of virulence factors is not dependent upon specialized mechanisms but, instead, uses mechanisms that are common in most bacteria that adapt to virulence factor regulation. For example, cyclic AMP and catabolite repression, a common regulatory mechanism, also regulates certain virulence factors, and *cya crp S. typhimurium* mutants can be used as attenuated live vaccine strains.

DNA Topology: a Global Regulator of Virulence Factors

Prokaryotic DNA usually is maintained in a negatively supercoiled form (underwound) by topoisomerases and histone-like DNA binding proteins. The level of supercoiling can be affected by several environmental parameters, which, in turn, affects the transcriptional activity of several promoters, including those upstream of virulence factors. Thus, environmental changes can play a regulatory role in virulence gene transcription by affecting DNA supercoiling (75).

As noted above, the invasion genes of *S. flexneri* (and the closely related enteroinvasive *E. coli*) are positively regulated by the AraC homolog, VirF, which activates the transcription of *virB* and the invasion genes. *virB* is transcribed at 37°C but not at 30°C. Another chromosomally encoded regulatory protein, VirR, negatively regulates the invasion system. VirR is a histone-like DNA binding protein, also called H-NS, that represses *virB* at lower temperatures, which indicates that the *virB* promoter is controlled by temperature through changes in DNA topology induced by VirR (337, 338).

An analogous regulatory system is found in the invasive *Yersinia* strains. The *yop* genes are transcriptionally activated at 37°C by the AraC homolog VirF. It is thought that a thermally regulated, topological change activates VirF-mediated transcription. Additionally, a histone-like protein, YmoA, negatively regulates *virF* transcription, presumably by affecting the DNA topology of this promoter (58, 216). Because of their global nature, changes in DNA supercoiling affect the expression of many other virulence factors. Some examples include *S. typhimurium* invasion, *Neissera* opacity protein expression, type 1 and Pap pilus expression, and elementary body formation in *Chlamydia trachomatis* development. In these cases, there is involvement of a DNA topological effector molecule that is either an enzyme that affects supercoiling (gyrase or topoisomerase) or a histone-like protein that alters promoter structure, thereby affecting transcription. Thus, by varying DNA supercoiling in response to the environment, the transcription of several virulence genes can be controlled.

Alternate Sigma Factors

Several bacterial pathogens use alternate sigma factors to regulate the transcription of virulence genes. One such example is RpoS (or KatF), which is a sigma factor in *E. coli* and *Salmonella* that is induced and regulates several genes operative during the stationary phase and during nutrient starvation (84). A pathogen also could encounter these environmental conditions within a host. It has recently been shown that this sigma factor affects the transcription of SpvR (a LysR homolog) in *Salmonella* species, which, in turn, activates transcription of plasmid-encoded genes (*spv*) that are needed for long-term survival in the host (134).

Other alternative sigma factors involved in virulence gene expression are a heat shock sigma factor, RpoH (HtpR or sigma 32), that regulates ToxT expression in *V. cholerae* (243); RpoN (sigma 54) and AlgU (sigma H), which mediate "mucoidy" in *P. aeruginosa* (300); and sigma F, which affects flagellar expression in *Bordetella bronchiseptica* and is homologous to FliA in *E. coli* and *S. typhimurium.*

Population Density Detection Plays a Newly Defined Role in Virulence

Bacterial pathogens need to reach a critical density within their host if they are to be transmitted effectively or to establish themselves within their host. An intriguing mechanism used by some pathogens to "measure" their population density, has recently been described. Accordingly, these bacteria can sense the need to express virulence determinants once bacteria reach a certain concentration (245, 285, 331, 361). This phenomenon was originally identified in the regulation of bioluminescence in marine *Vibrio* species. In this system, a small molecule, acyl-homoserine lactone, is synthesized and secreted by the bacteria. Once this chemical, called an autoinducer, reaches a sufficient concentration, it induces a transcriptional regulator, LuxR, which activates the genes necessary for bioluminescence and further autoinducer biosynthesis (LuxI). Thus, by sensing the density of the secreted autoinducer, bacteria can sense if

there is a "quorum" of their population sufficiently present to initiate the appropriate biochemical action.

Similar quorum-sensing systems have been identified in several microbial pathogens. In some plant pathogens (such as *Erwinia carotovora*), such systems are extensively used to regulate the biosynthesis of antibiotics and exoenzymes that damage plant tissues. A similar mechanism signals Ti plasmid conjugation in *Agrobacterium tumefaciens*, although the nature and length of the lipophylic-acyl chain vary among systems (159). More recently, a similar system has been identified in *P. aeruginosa*. LasR is a regulator in this opportunistic pathogen that activates transcription of the gene which encodes elastase (*lasB*) and several other virulence factors (245). LasR is a homolog of LuxR from *Vibrio* species. Additionally, the structure of the *Pseudomonas* autoinducer has been established as another homoserine lactone. When this autoinducer reaches a critical concentration, it activates LasR-mediated transcription of these virulence genes. This clever regulatory mechanism ensures that the bacteria reach a certain density before initiating gene activity that is required for growth and replication in the host. It is likely that this is a more common mechanism than we realize. For example, it has recently been demonstrated that *Y. enterocolitica* synthesizes two *N*-acylhomoserine lactone signal molecules, although the function of these molecules has not been characterized (336).

Interconnections between Regulatory Systems

The regulation of pathogenicity is complex. Several regulatory mechanisms affect simultaneously virulence gene expression. Presumably, the virulence level is determined by an average of all of these signals. Additionally, regulatory factors usually regulate themselves, which gives a hierarchy to regulation and exquisite control over virulence factor expression. Two examples of the complexity of these systems are provided above. Regulation of the *spv* virulence operon is controlled by SpvR (a LysR homolog), which is regulated by RpoS (an alternate sigma factor that is induced during starvation). In *E. coli* (and presumably *Salmonella* species), RpoS (or sigma S) is regulated by a homoserine-lactone-dependent signaling pathway. The *spv* operon also is controlled by cyclic AMP levels via a *crp*/*cya*-mediated mechanism. Alternatively, *V. cholerae* pili are regulated by ToxT (an AraC homolog). ToxT is regulated by ToxR (a two-component system) and an alternate sigma factor, sigma 32. These two examples provide only a cursory indication of the complexity of the interactions between regulatory mechanisms that affect virulence gene expression.

Methods To Study In Vivo Regulation

Investigators have begun to analyze virulence gene expression and regulation in the presence of host cells or even inside host animals. Although this is technically more difficult, it is thought that additional relevant virulence factors will be identified, since the environmental conditions inside a host are quite different than those found in a laboratory. For example, two-dimensional gel systems have been used to characterize gene expression of *S. typhimurium* inside host cells (1). Alternatively, fusions to *lacZ* and Pap pili have been used to demonstrate that SpvR activates *spv* transcription in *Salmonella* inside epithelial cells and macrophages (87, 269). *lacZ* and luciferase fusions also are used to monitor the regulation of known genes inside host cells and animals (for *lacZ*) and to identify genes that are induced inside host cells (10, 57, 118, 179, 198). Fusion to antibiotic-resistant genes also can enrich for promoters that are induced inside host cells (199). Quite recently, the *Aequorea victoria* green fluorescent protein has become a promising tool to study gene expression in bacterial pathogens (70, 184, 342). The advantage of this marker is that it fluoresces constitutively, without addition of substrate or use of an enzymatic assay and without fixation. This marker is especially well suited for examining gene expression inside cells and even in tissues. Fluorescent-cell sorting can be used to fractionate infected cells containing bacteria expressing green fluorescent protein.

In addition to using gene fusions as reporter probes to measure in vivo gene expression, other workers have begun to make subtractive RNA banks from organisms grown intracellularly after subtraction with RNA from organisms grown extracellularly. This technique has been used with *M. avium* and *L. monocytogenes* to identify genes that are induced inside host cells (256, 341).

A clever method has recently been developed to select for promoters that are activated inside animals. This method, called in vivo expression technology, uses a strategy that ensures that bacteria that survive within a mouse contain a promoter that is induced in vivo (198). Several different promoters have been identified by this technique. Interestingly, several housekeeping promoters also were identified, again emphasizing the relationship between virulence and bacterial growth.

More recently, another method has been developed to identify *S. typhimurium* genes that are needed for replication in mice (146). By using sequence-specific tags to identify each transposon insertion, bacterial mutants that are attenuated in virulence (i.e., unable to persist inside a mouse) can be identified. This method identified several known virulence factors, as well as other previously unidentified factors that are related to known virulence factors, and led to the discovery of a second type III secretion pathway in *Salmonella* contained within a second pathogenicity island (305).

EVOLUTION OF BACTERIAL PATHOGENS

It is well established that many pathogenicity factors (and antibiotic-resistance genes) that are plasmid encoded are often found adjacent to each other, often flanked by repetitive sequences or transposable elements. Examples cited above include the plasmid-encoded *Yersinia* Yops and the *Salmonella* and *Shigella* invasion systems. Recent information suggests that pathogenic bacteria evolved from related nonpathogenic organisms by genetically acquiring relatively large blocks of genetic material that encode virulence factors rather than by slow, adaptive evolution of preexisting genes. A disproportionate number of essential virulence determinants, particularly toxins and adherence factors, are found on mobile genetic elements, which can be disseminated to other bacteria by bacterial conjugation, transformation, and transduction. The mechanisms of transfer and the integration and establishment of transferred DNA are extensively reviewed elsewhere (45). In principle, since many virulence genes are encoded on "mobile DNA" genetic elements, they have the ability to spread to other microorganisms or, more probably, to nonpathogenic members of the same or closely related species. The paradigm for this kind of genetic spread is readily seen in the dissemination of R-plasmids and the transposition of antibiotic resistance genes.

Within the past decade, a common thread has been observed in a number of distinct microbial pathogens that suggests a major evolutionary mechanism by which pathogenic bacteria have evolved. In a surprising number of cases, virulence genes are found in large contiguous blocks found as chromosomal inserts or pathogenicity islands (30, 189) (Table 6). These islands are often bound by sequences that suggest that the

TABLE 6. Selected examples of pathogenicity islands

Bacterium	Name	Size (kb)	Point of insertion	Function
Uropathogenic *E. coli* 536	PAI I	70	*selC* (82 min)	Hemolysin production and P-related fimbrae
	PAI II	190	*leuX* (97 min)	Hemolysin production and P-related fimbrae
Enteropathogenic *E. coli*	LEE	35	*selC* (82 min)	Attachment and effacement, pedestal formation (encodes type III secretion system)
Salmonella typhimurium	SPI1	40	*fhlA*, *mutS* (60 min)	Invasion into nonphagocytic cells (encodes type III secretion system)
	SPI2	40	31 min	Bacterial survival in host (encodes type III secretion system)
Helicobacter pylori		40		Encodes CagA and VacA regulator, needed for full virulence (peptic ulcer, adenocarcinoma)
Yersinia pestis		102		Iron acquisition, hemin utilization, pesticin sensitivity
Clostridium difficile		19.6		Toxins A and B
Listeria monocytogenes		10		Virulence regulation, escape from vacuole, cell-cell spread

DNA segment was acquired by an illegitimate recombinational event that resembles transposition or phage insertion. In line with this conclusion, these DNA blocks also often are inserted into hot spots in the chromosome that are presumably more susceptible to incursion by foreign DNA or that represent a phage attachment site. For example, uropathogenic *E. coli* strains typically possesses one or two pathogenicity islands that encode hemolysin and P-related fimbriae. These large (70- and 170-kb) pathogenicity islands are inserted in two tRNA genes that encode leucine and selenocysteine tRNAs (29, 30). In EPEC and EHEC, nearly all of the known virulence genes (such as *eaeA* and *espB*) are found within a 35-kb insert that also is inserted at the same site within the selenocysteine tRNA gene as the 90-kb uropathogenic *E. coli* insertion (206). Thus, two *E. coli* pathogens that cause remarkably different diseases both contain large segments of DNA that encode different virulence factors inserted into the same site in the *E. coli* chromosome. The sequences within the pathogenicity island are not homologous to those found in a nonpathogenic clone like *E. coli* K-12, but sequences immediately adjacent to the pathogenicity island are found in pathogens and nonpathogens alike.

The chromosomal organizations of *E. coli* and *S. typhimurium* generally are similar. It has been shown recently that many *Salmonella* genes involved in epithelial and macrophage cell entry are found within a 4-kb segment of DNA between 59 and 60 min on the *Salmonella* chromosome that is absent from the *E. coli* chromosome (221). Sequences adjacent to this insertion are found in both *Salmonella* and *E. coli*. Signature-tagged mutagenesis (see above) has led to the identification of a second *Salmonella* pathogenicity island that is involved in survival of the bacteria within the host after entry and is located close to 31 min on the *Salmonella* chromosome (146, 232). One can imagine that these large DNA insertions were acquired during evolution by an ancestral microbe (shared with *E. coli*) that gave the prototypic *Salmonella* the capacity to translocate the mucosal barrier and the genes necessary to survive within the host in particular target organs. Regions of chromosomal DNA that encode several clustered virulence factor genes are common among pathogens ranging from plant pathogens to *H. pylori* and *Y. pestis* (207a).

Although virulence is often multifactorial and possibly acquired en masse, the inheritance or change of a single virulence factor also can drastically alter the type of infectious process or disease that an organism causes. For example, it is thought that EHEC contains most, if not all, of the EPEC virulence factors (including the aforementioned EPEC-specific pathogenicity island inserted into the selenocysteine tRNA gene), yet it causes bloody colitis and sometimes hemolytic-uremic syndrome rather than gastroenteritis in humans. In addition to the EPEC virulence factors, EHEC contains a Shiga-like toxin that is thought to cause the EHEC-specific disease. Recent experimental results support the singular role of Shiga toxin in this disease. RDEC-1 is an *E. coli* strain that is similar to EPEC but causes an EPEC-like disease in rabbits. (It is used as an animal model for EPEC.) Shiga-like toxin I was introduced into RDEC-1 via a bacteriophage (312). When rabbits were infected with this strain, the illness closely resembled EHEC-induced colitis seen in humans rather than the diarrheal disease normally seen with RDEC-1. Shiga-like toxins usually are encoded within bacteriophages. Thus, EHEC probably has arisen by a simple genetic event, with the Shiga-like toxin moved from some enteric organism that normally harbors this toxin (such as *Shigella dysenteriae*) via its bacteriophage into EPEC. This single genetic event would then generate a new pathogen that causes a remarkably different disease and presumably gives the host microbe a selective advantage at some level of survival or transmissibility. In accessing such changes, however, it is instructive to see that EHEC strains are as often as not carried in adult animals asymptomatically and that the disease in humans is as much a reflection of human technology (how we prepare hamburger) as it is the presence of a distinct constellation of virulence factors.

Another recent example of a toxin-encoded phage illustrates the selection pressure that occurs on virulence factors and the interrelationship between various virulence factors (347). Although cholera toxin originally was not thought to be genetically motile, this work demonstrates that, instead, it was encoded within a M13-like bacteriophage and was capable of moving between bacteria. Even more surprising, the cholera toxin phage receptor was a toxin-coregulated pilus (TCP), thus ensuring that the phage could infect only *Vibrio cholerae* organisms that expressed another virulence factor, the pilus. It is instructive that phage was transferred more efficiently in the gastrointestinal tracts of mice than in the laboratory setting.

Bacterial chromosomes are not constant but are continually changing. Such dynamic changes also may modify the evolution

of pathogenicity within different clonal variants of a pathogenic species. As mentioned above, the chromosomes of *E. coli* and *S. typhimurium* are remarkably conserved. However, the chromosome of *S. typhi*, which is a human-specific pathogen, has undergone major genomic rearrangements during its evolution when compared to other nontyphoidal salmonellae; these include inversions, transpositions, and insertions through homologous recombination events (196). It has been proposed that some of these events may affect *S. typhi* virulence, possibly contributing to its human-specific host adaptation. Shuffling of chromosomal genes also could affect the regulation and expression of chromosomal virulence factors.

We should not forget that the selective pressure of infection and disease also has affected the evolution of host genes, presumably by selecting for genetic changes that enhance survival. We cannot do justice to this point here. However, the impact of infectious diseases on the genetic selection of humans is considerably broader in scope than the usual textbook examples that sickle cell anemia provides a selective advantage for resistance to malaria. For example, it has recently been proposed that defects in the CFTR gene product (which, in homozygous individuals, results in cystic fibrosis) provide resistance to cholera toxin. Mice that do not express CFTR do not secrete fluids in response to cholera toxin, while heterozygotes only secrete 50% of the normal amount of fluids in response to cholera toxin, which provides a possible selective advantage of resistance to cholera and therefore selective pressure for these mutations to remain in the population (104). This might explain the prevalence of defective CFTR genes in the Caucasian population. Conversely, individuals with the O blood type are more likely to succumb to cholera infection than others. As the host acquires added ways to resist infection or to limit transmissibility, so must pathogens adapt to these changes.

Many of the genes we recognize as virulence determinants may have ancient roots. We tend to think of infectious diseases in terms of ourselves or as relatively recent events. Yet encapsulation is as effective for preventing phagocytosis by amoebae as it is for human neutrophils. Similarly, *P. aeruginosa* has a formidable array of virulence determinants, but it is an opportunistic pathogen of humans and rarely causes disease in immunocompetent individuals. The recent finding that mutations in some virulence factor genes in *P. aeruginosa* (*toxA*, *plcS*, and *gacA*) affect virulence in both mice and a leaf infiltration model (264) suggests that the molecular basis of *Pseudomonas* pathogenesis may have had its evolutionary origins in plants rather than animals.

Apparent redundancy of function appears to be a common theme that emerges in the experimental evaluation of virulence factors. By having more than one virulence factor that can mediate a particular function, a pathogen is better able to resist host cell defenses. For example, *L. monocytogenes* has two phospholipase genes, *plcA* and *plcB*, and a hemolysin. Either gene product can mediate escape from the intracellular vacuole (201, 315, 346). Deletion of either phospholipase gene does not significantly affect virulence. However, when both genes are deleted, virulence is lost. Similar overlapping functions can be seen in *Yersinia* species, which have four functional adhesins—invasin, Ail, YadA, and the pH6 antigen. All seem to provide attachment directly or indirectly to the host cell. Loss of any one of these may not be devastating to the virulence of the bacterial cell. Indeed, loss of two (invasin and YadA) has been reported to increase virulence. We still are not sure that each of these provides a similar function or whether they act at different points in the infectious process. It is useful to remember that while we use mutations to reveal function in experimental systems, virtually all clinical isolates of *Y. enterocolitica* from nature express all of the adhesins while *Y. pestis* actually has conserved only one functional adhesin, the pH 6.0 antigen.

Bacterial virulence factors can often encode multiple functions. In evolutionary terms, acquisition of a single virulence factor may bestow several functional attributes on an organism. For example, FHA of *B. pertussis* encodes several functions (197). The invasin protein of *Yersinia* acts as an adhesin that is a prerequisite for the transduction of other bacterial virulence factors that affect host cell signaling, yet it also mediates invasion. Similarity, the Ail protein of *Yersinia* is a representative member of a class of membrane proteins that encode both adherence and serum resistance in several bacterial pathogens (see above).

It appears that pathogenic organisms evolve in quantum leaps, usually by acquiring a genetic segment that encodes multiple virulence factors from an unrelated organism, possibly even eukaryotic sequences (as seen with tyrosine phosphatase acquisition [YopH] in the *Yersinia* species). This genetic information is then integrated into the chromosome or a stable plasmid. Appropriate selection for virulence factors ensures that pathogens maintain such sequences. By sharing this genetic information via mobile genetic elements, the organisms ensure that other organisms also can acquire selective advantages. Information that is not needed is presumably lost, since there is no selective advantage to maintaining it. Organisms also can vary this genetic information, altering it for a specific purpose. Conserved gene families, including secretion mechanisms, are good examples of this. Chromosomal shuffling and rearrangements provide an alternate way to alter and shuffle genetic expression of various virulence factors.

Pathogens are remarkably adept at capitalizing on new environments. We recently have seen several "nonpathogenic" organisms that cause much morbidity and mortality due to such capitalization. Opportunistic pathogens are good examples, as are the increases in the number of cases of nosocomial (hospital-acquired) infections caused by commensal organisms. As technology develops, many new "environments" are created that can affect the incidence of disease. For example, Legionnaires' disease became commonly recognized only after the widespread use of air conditioners and other widespread aerosolization of water in human environments. Contamination of water systems in major institutions also enhanced the spread of this organism and other parasites. Several technological developments in health care altered the spectrum of pathogenic organisms. For example, immunocompromised people now are kept alive for much longer periods, providing a new niche for nosocomial and opportunistic pathogens. Surgical implantation of medical devices also provides a new niche within the body for these organisms to colonize. Worldwide, there has been a rapid increase in the number of nontyphoidal *Salmonella* cases, due to the automation of food processing in developing countries. Related to this, there has been a significant decrease in typhoid cases as sanitation improves and with the dissemination of a better vaccine. The normally innocuous enteric gram-negative organisms have become a major cause of nosocomial pneumonias due to their direct introduction in the lung by intubation. Thus, we can expect to see a changing spectrum of pathogens as we continually alter our environment and other organisms evolve to capitalize on these new niches.

CONCLUSIONS

It is now clear that pathogenic bacteria share many mechanisms that cause infection and disease. In part, this conclusion could be reached because virulence determinants of bacteria

tend to be clustered in discrete regions of the chromosome, as well as on bacterial plasmids. PCR and DNA sequencing technology, together with the widespread availability of an ever-growing genetic sequence data bank, quickly led to the discovery of shared contact-dependent secretion systems, common regulatory mechanisms, and widespread homology among the virulence effector molecules of seemingly diverse microorganisms. This, in turn, led to the appreciation that bacterial pathogenicity genes commonly are found as discrete islands in the chromosomes and extrachromosomal elements of pathogenic species but absent from nonpathogenic members of the same genus or species.

If there is satisfaction with the relatively rapid discovery of these common themes, there also is the caution that we are perilously close to molecular sequencing leading the biologist, rather than the biologist directing the sequencing. The full DNA sequences of a number of pathogens are now available, and it has been confidently predicted that the full sequences of the most common 100 microbial pathogens will be available to scientists before the first decade of the new millennium is over (314). However, nucleotide sequence is not a function, and homology suggests, but does not provide, definitive proof of a particular biochemical activity. In this sense, it is informative to discover that functional analysis of the oldest known virulence factors, the bacterial toxins, provide us with more of a sense of the utility of a common motif in the microbial strategy for survival than does an analysis of their toxic effects or even clinical symptomatology.

There are new genetic methods that are available to identify relevant genes on a functional basis from pools of mutant bacteria; these methods can be of extraordinary value because they can exploit known sequences. Even so, it is sobering to realize that the full sequence of the *E. coli* K-12 chromosome provides a mere handful of the known virulence determinants of pathogenic *Escherichia*. Until recently, we did not know that the vast majority of virulence genes were in a sense "foreign DNA." Presumably, new molecular detection systems will permit us to probe the microbial universe to detect the relatives and possible origins of the common pool of genes found in pathogenic microorganisms. It seems plausible that the original type III secretion system evolved from flagellar assembly systems, while other assembly systems, like those seen in *Bordetella* and *Helicobacter*, were derived from conjugative transfer systems. Attempts to establish any of the known pathogenicity islands as "the" progenitor have been fruitless; each known organism can be ruled out as the source. This leads to the uncomfortable conclusion that each of the known pathogenicity islands and virulence-associated plasmids seems to have been introduced independently into each of these bacteria. Moreover, a single bacterial strain can harbor more than one pathogenicity island (it has been estimated that *Salmonella* has at least five), and the specialized insertion of virulence genes does not need to be large and complex. Thus, pathogenicity seems to have arisen by the inheritance of blocs of DNA despite the presence of multiple barriers to chromosomal gene transfer between species and even among strains of the same species. Bacterial plasmids, bacteriophages, and other mobile genetic elements play a key role in a haploid world as the seminal effectors of metabolic diversity and specialization. It is no surprise, therefore, that they are often essential components of pathogenicity. Extrachromosomal inheritance, genetic transposition, lysogeny, and transduction provide a genetic means for a microorganism to have the best of both a genetically stable and unstable world.

If one accepts that many of the genes associated with pathogenicity reside on discrete blocs of added DNA, it might be supposed that bacterial pathogenicity results in part from the unregulated expression of some of these alien sequences. Nothing seems further from the truth, however. Virulent bacteria express the genes that are appropriate for each host cell environment. As noted above, the expression of virulence factors at the wrong time can have a devastating effect on the invading microbe. The control is complex; expression of pathogenicity is choreographed by a cascade of regulatory factors. For example, *Salmonella* invasion of epithelial cells is coregulated by oxygen, osmolarity, and the levels of Mg^{2+} and pH. Regulation by these environmental cues is mediated indirectly through a genetic locus, *hilA* (a two-component regulator), that, together with the type III secretion apparatus and the effectors of epithelial cell entry, resides on the pathogenicity island SPI1. Additionally, invasion is regulated by the distal second pathogenicity island, SPI2. What is admirable about this system is that the invasion genes are expressed only when all of the environmental and regulatory conditions are optimal. If even a single condition is unfavorable, invasion gene expression is repressed. This is coordinate regulation indeed, and it is not exclusive to the salmonellae but seems to be a common theme used by most primary bacterial pathogens. This finding seems to imply that while the blocs of genes inherited by bacteria during their evolution to pathogenicity were acquired from a foreign source, many of the regulatory links were in place. This is not surprising. All prokaryotes respond to temperature, carbon dioxide, oxygen, iron, pH, stationary phase, specific nutrients, etc., whether this is experienced at the site of a root hair, in the gut of an animal, or in an aquatic environment. One has the distinct impression that response to these factors is not a unique evolutionary motif for the expression of bacterial pathogenicity, as much as it is a common theme for the evolution of bacterial specialization in the general sense. Furthermore, it should be noted that the blocs of genes, even the large ones, are concerned with getting relatively few effector molecules to the surface of the cell, assembling them, and delivering them specifically and precisely. Thus, the essence of the pathogen is in the sequences of the relatively small number of effector molecules. Closely related virulence factors can work within different pathogens because the delivery systems are so similar. Within the specific host species, the kinds of differences that one sees between *Salmonella* and *Shigella*, for example, are the expression of distinct differences in effector function. The life of an adapted pathogen, therefore, can be visualized by this scenario as a series of steps of gene activation in response to a complex set of simultaneous environmental conditions. As a result of direct transcription or posttranslational modification, one particular cue—often contact with the host—results in a directed assembly most probably at a unique location of the bacterial cell envelope of several virulence factors that interact with and modify the host cell. Under optimal conditions, the normal goal of the pathogenic strategy, i.e., sufficient replication to establish or to be successfully transmitted to a new host, is achieved. There is commonality in the "nuts and bolts" of sensing and reacting to the environment and a commonality in the limited number of ways to bring the effectors of pathogenicity to the surface of the cell to be assembled or to be directed to a cellular target. The distinct pathogenic signature, however, is in how the microorganism has crafted the effectors of virulence to its own particular utility.

The parallel discoveries in the cell biology of bacterial infection also lead to the conclusion that pathogenic bacteria use only a handful of motifs to survive within their preferred host. Although a recent discovery, it is noteworthy that the interaction between pathogenic bacteria and host cells leads quite

often to programmed cell death. Whether this is a strategy of the bacteria or a survival tactic of the host cell is still not altogether clear. However, some microbes want and need to survive and replicate within host cells. While there clearly are variations on whether the invading organism tolerates or exploits the pH of the initial vesicular compartment in which it finds itself, the organisms, with few exceptions, modify their environment to become a privileged niche and avoid the defense barriers that eukaryotic cells have developed to inhibit microbial incursion. If we have learned a good deal about actin and the cytoskeleton from the study of *Listeria* and *Shigella*, we are just beginning to learn about signal transduction, intracellular targeting, protein transport, and other host cell factors from the study of small GTP-protein toxins, as well as learning from the perturbations that *Shigella*, *Salmonella*, *Chlamydia*, and *Mycobacterium* cause when they are proliferating intracellularly.

Similarly, we are beginning to explore the cellular immunology of the early stages of bacterial infection. We no longer dwell on immunity to infection while ignoring the initial host-parasite encounter. For example, we now appreciate that for pathogenic enteric species, there is a reiteration of a strategy that involves exploiting a common entry pathway: the M cells of the Peyer's patch. At the same time, we are beginning to learn more about the important, but largely ignored, M cell per se from studying this interaction. We also are beginning to understand that the distinct pathogenicity islands we see in species like *Salmonella* hold clues to study different aspects of the cell biology and cellular immunology of infection. For example, it appears that invasion genes of *S. typhimurium* might not be expressed after the bacteria translocate from the ileum into the Peyer's patches following passage through M cells. However, the genes of the second pathogenicity island are needed for the bacteria to gain access to the spleen when the bacteria are no longer found inside nonphagocytic cells. At the same time, there is evidence that the virulence plasmid of *Salmonella* also carries genes that are important for the bacteria to survive during systemic infection, including in the spleen. Is this simple redundancy or possibly a reflection of both the need to replicate within the reticuloendothelial system and host adaptation? The point is that the availability of defined mutations allows us to investigate both the pathogenesis of infection and the cellular and humoral elements in play during bacterial infection. We need no longer dwell on the disease, but, rather, we need to investigate the biology of the interaction. We believe that the real revolution in the treatment of infectious diseases will come from understanding this biology. The more we learn about the fundamental basis of the host-parasite interaction, the more likely it is that we can devise immune system modifications of the infectious process and find new potential targets to inhibit bacterial growth. It is not too far-fetched to believe that it will be possible also to modulate the expression of virulence by invading bacteria so that they become more susceptible to anti-infective compounds and normal host cell antibacterial factors. At the same time, the more we discover about the basic aspects of bacterial pathogenesis, the better and more precise will be our diagnostic tools, as well as our understanding of the epidemiology of infection. Despite our optimism, one cannot emerge from the discovery of the genetic mechanisms involved in the origin of pathogenicity as a bacterial specialization without understanding that "new pathogens" will continue to evolve. We are not dealing with genetic fossils but, rather, with constantly evolving systems. Indeed, the recent devastating emergence of *V. cholerae* O139 seems the likely product of a recent genetic acquisition of a small pathogenicity island.

What does the future hold for the study of bacterial pathogenicity? As noted, we expect to have the sequence of all of the genes of the most important pathogens, and these will be extensively analyzed through known computer algorithms to identify homologous sequences and known biochemical motifs. The molecular clues of evolution will be sought through the analysis of these sequences. The functional analysis of the genetic sequences cannot be accomplished without understanding when and how they are expressed. The genes were not designed for the comfort of investigators. Increasingly, we will need to devise ways to examine genetic expression at the cellular level within infected animals. Genetic reporter fusions are in use. Through the use of newer reporter molecules like the green fluorescent protein and others under development, we will be able to explore microbe-host cell interactions in real time in living hosts. Medical microbiologists have increasing access to transgenic animals that provide the means to investigate specific immunologic and cellular defects and their role in the pathogenesis of infection from the standpoint of both the host and the parasite. If there has been utility in using molecular Koch's postulates as an approach to the study of bacterial pathogenicity, so the combination of examining precise mutations in bacterial genes, coupled with precise modifications in the genetic complement of the host, will yield even greater rewards. In parallel, we have to begin to take note that we still remain ignorant of many members of our own microbial flora and the role that they play in disease. If we have discovered that a bacterium causes gastric cancer and ulcers in the last decade, we shall probably find that this is not a unique case. Is it not likely that some level of colon cancer has a microbial etiology? The use of transgenic knockout mice suggests to us that while ulcerative colitis is surely an autoimmune disease, the incitant is some component of the bowel flora. Is there not an infectious component of many autoimmune disorders and other perturbations of the immune system? It is unlikely that simply any bacterium will do; the organisms involved will most probably have unique determinants that give a distinctive interaction with its host. There is no doubt about the future. We have just begun to look at the most exciting features of the interactions of microbes with humans.

ACKNOWLEDGMENTS

We thank our many colleagues who have provided us with so many interesting findings and many helpful comments, and we thank members of our laboratories for critical reading of the manuscript. We thank Sara Fisher for her editing assistance. We apologize to those who do not find their favorite research work (generally their own) referenced here. It was simply impossible to find, let alone read and assimilate, all the important literature in this "age of information." On the other hand, it is probably fair to say that much of the important early work in pathogenicity gets overlooked and underappreciated because MEDLINE does not have citations earlier than 1966.

Work in the B.B.F. laboratory is supported by a Howard Hughes International Research Scholar Award, the Canadian Bacterial Diseases Network Center of Excellence, the Medical Research Council of Canada, and the National Sciences and Engineering Research Council of Canada. B.B.F. is an M.C. Scientist. Work in the S.F. laboratory is supported by the National Institutes of Health and unrestricted gifts from Praxis and Microcide Pharmaceuticals, Inc.

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