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Exploitation of Mammalian Host Cell Functions by Bacterial Pathogens

B. Brett Finlay and Pascale Cossart

Interest in bacterial pathogenesis has recently increased because of antibiotic resistance, the emergence of new pathogens and the resurgence of old ones, and the lack of effective therapeutics. The molecular and cellular mechanisms of microbial pathogenesis are currently being defined, with precise knowledge of both the common strategies used by multiple pathogenic bacteria and the unique tactics evolved by individual species to help establish infection. What is emerging is a new appreciation of how bacterial pathogens interact with host cells. Many host cell functions, including signal transduction pathways, cytoskeletal rearrangements, and vacuolar trafficking, are exploited, and these are the focus of this review. A bonus of this work is that bacterial virulence factors are providing new tools to study various aspects of mammalian cell functions, in addition to mechanisms of bacterial disease. Together these developments may lead to new therapeutic strategies.

Despite the extensive use of antibiotics and vaccination programs, infectious diseases, particularly microbial diseases, continue to be a leading cause of morbidity and mortality worldwide. Recent outbreaks and epidemiologic studies predict that their incidence will increase while the world's population continues to grow. The emergence of previously undescribed pathogens has been a feature of the end of this century. Increased global travel has contributed to the dissemination of pathogens previously confined to specific regions. In addition, it is now clear that bacterial pathogens cause diseases previously thought not to be infectious, such as the gastro-duodenal ulcers caused by *Helicobacter pylori*. And old diseases, such as tuberculosis, have returned with a vengeance, particularly in immunocompromised patients, accompanied by the emergence of antibiotic-resistant strains. No new class of antibiotic has been discovered in the past three decades, and derivatives of current antibiotics soon encounter resistance. New anti-infective agents are thus desperately needed to counter diseases previously treated by conventional antibiotics. Development of these reagents, however, requires a better understanding of how bacteria can cause disease.

Knowledge in the field of microbial pathogenesis—the study of the molecular basis of microbial diseases—has increased dramatically in recent years (Table 1) with contributions from several different directions. Research on pathogens such as *Salmonella*, *Shigella*, *Yersinia*, and *Listeria* species that are relatively easy to genetically manipulate has led the way, but new techniques have been developed that allow most bacterial pathogens to be studied at the molecular and cellular levels. Many pathogens share common mechanisms of interaction with the host, but each species has also evolved a repertoire of unique approaches to exploit host processes (*1*). The study of the molecular interactions between bacterial factors and cellular components or signaling pathways in vitro has been called cellular microbiology (*2*). Recent advances in identifying and detecting virulence factors in vivo rather than in culture have also helped open up the field of microbial pathogenesis, with the use of approaches such as sensitive imaging systems to follow light production or green fluorescent protein expression (*3*). More importantly, research with genetic techniques (*4*) to identify genes induced when the bacteria are inside an animal but not in culture, or to identify genes are essential for virulence in an animal, indicates that additional relevant virulence factors will be identified in the near future. Another source of knowledge has come from progress in cell biology. This progress includes new information on cell physiology; the development of in vitro systems; the ongoing development of fluorescence, confocal, video, and electron microscopy; and the development of new techniques such as the ability to generate and express transdominant negative forms of various cytoskeleton proteins or signaling molecules and the ability to change the intracellular composition by microinjection. In turn, bacteria have provided cell biologists with valuable tools to dissect cellular processes, such as cytoskeleton rearrangements and signaling pathways.

This article highlights some of the recent findings concerning the cellular and molecular interactions that occur between bacterial pathogens and their host cells. It is organized according to the successive interactions that occur at different stages during the infectious process, including microbial adherence to host cells, pathogen uptake into mammalian cells, bacterial survival and replication inside mammalian cells, and cell intoxication and death caused by bacterial products.

Adhesion to Mammalian Cells

Bacterial adherence to host cells or surfaces is often an essential first stage in disease because it localizes pathogens to appropri-

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ate target tissues. Adhesion to host cells may result in internalization, either by phagocytosis or by bacterial-induced endocytosis (known as invasion). A variety of molecules and macromolecular structures, collectively known as adhesins, mediate adherence to cell surfaces or cell molecules and these can be broadly divided into fimbrial adhesins (fimbriae or pili), which are filamentous structures on the surface of bacteria, and afimbrial adhesins, which include most other adherence molecules.

The assembly of a pilus is a complex process involving many gene products that guide the structural subunits from their site of synthesis to the bacterial cell surface, where they are assembled into an organelle (5). Pilus biogenesis is a relatively conserved mechanism for many types of fimbriae; that is, the assembly machinery is homologous and often interchangeable for many diverse fimbriae from different pathogens that bind to very different host cell substrates. The bacterial molecule that binds the host component is usually at the tip of these structures, and by varying this molecule, pathogens can vary their host substrate (5).

Afimbrial adhesins are diverse and collectively include all nonpilus adhesins (*1*). Examples include the adhesins AfaD and AfaE from *Escherichia coli*, responsible for attachment of *E. coli* to the urinary tract or the intestinal cells, and the filamentous hemagglutinin (FHA) from *Bordetella pertussis*, responsible for attachment to the lung epithelial and phagocytic cells. Afimbrial adhesins also include the opacity proteins (Opas) from *Neisseria*, which comprise a family of similar proteins responsible for cell-type specificity, and the repeat proteins of Gram-positive bacteria such as the M protein of *Streptococci* or the fibronectin-binding proteins of *Streptococci* and *Staphylococci*. Afimbrial adhesins enable these pathogens to adhere to extracellular matrix components as a first step to tissue colonization.

A wide range of mammalian cell surface compounds, including proteins, glycolipids, and carbohydrates, can serve as receptors for bacterial adhesins. For example, P pili bind to the α-D-galactopyranosyl-(1-4)-β-D-galactopyranoside moiety present in a series of glycolipids found on cells of the upper urinary tract (5). Other *E. coli* fimbriae called type I pili, which share homologous assembly components with P pili, bind to mannose residues on cell surfaces. *Helicobacter pylori* binds to the Lewis^b blood group antigen, which is expressed on cells in the stomach epithelium (6). *Neisseria* binds to cell surface–associated heparin sulfate proteoglycans and to a CD66 adhesion molecule on epithelial cells and neutrophils (*7*). Thus, bacterial adhesins are capable of binding to a large variety of host cell surface molecules.

The host cell is often an active participant in adhesion, not simply functioning as an inert surface. Indeed, some bacterial pathogens rely on a host response to infection to trigger expression of a target receptor that the bacteria then bind to, at least in vitro. For example, *Streptococcus pneumoniae* adherence to, and invasion of, human umbilical vein endothelial cells is markedly increased after stimulation of the endothelial cells by thrombin or tumor necrosis factor α, two factors produced in response to infection (8). The pneumococcus has a cell wall component, phosphorylcholine, that binds to the platelet-activating factor receptor on activated endothelial cells, leading to enhanced bacterial adherence and invasion.

Many bacterial pathogens activate host cell signal transduction pathways, and although it has long been recognized that these signaling events are involved in mediating invasion, it has only recently become apparent that signal transduction plays a crucial role in bacterial adherence, activating host receptors that the pathogen then adheres to. For example, FHA of *B. pertussis* (the causative agent of whooping cough) binds to a monocyte integrin complex through an Arg-Gly-Asp sequence. This interaction up-regulates the binding activity of another integrin, the complement receptor 3 (CR3), which recognizes a separate FHA domain. Thus the bacterial pathogen enhances its own attachment by co-opting a host cell signaling pathway (9). More dramatic still is the sequence of events involved in the adhesion of enteropathogenic *E. coli* (EPEC), a pediatric diarrheagenic pathogen. EPEC adheres to intestinal epithelial cell surfaces by destroying host microvilli and rearranging the actin cytoskeleton to form a pedestal on the host cell surface, on which the bacterium then resides (Fig. 1). To achieve this, EPEC secretes at least two proteins, EspA and EspB, that activate host cells by inducing calcium flux, inositol phosphate production, tyrosine phosphorylation of a 90-kD membrane protein, and ultimately, cytoskeleton rearrangements. EPEC must activate these host signal transduction pathways to attach to the host cell, indicating that signal transduction, and possibly receptor modification, precedes intimate adherence in cultured epithelial cells (*10*).

The secretion by EPEC of proteins that activate epithelial cells is mediated by a specialized secretion system called the type III secretion system, versions of which are being identified in an ever increasing number of human, animal, and plant pathogens (*11*). Type III secretion systems, which comprise at least 20 gene products, are essential for the virulence of these pathogens; the genes encode both secreted effectors and the machinery necessary for secretion

Table 1. Selected examples of bacterial pathogens and their location with respect to cells.

Bacterial pathogen	Main disease induced	Interactions with host cells
	Extracellular pathogens	
Staphylococci	Skin and tissue infections	Adherence to extracellular matrix
Streptococci	Otitis media, pharyngitis, scarlet fever, meningitis,	Adherence to extracellular matrix
Bordetella pertussis	Whooping cough	Adherence to cells
Neisseria gonorrhoeae	Gonorrhoeae	Adherence to cells
Neisseria meningitidis	Meningitis	Adherence to cells
Helicobacter pylori	Ulcers, gastritis	Adherence to cells
Escherichia coli	Diarrheas, meningitis, urinary tract infections	Adherence to cells
Yersinia species	Plague, mesenteric lymphadenitis, diarrhea	Adherence to cells and matrix
Vibrio cholerae	Cholera	Adherence to cells
	Intracellular pathogens	
Macrophages		
Legionella pneumophila	Legionnaires' disease	Within a vacuole
Mycobacterium tuberculosis	Tuberculosis	Within a vacuole
Mycobacterium leprae	Leprosy	Within a vacuole
Macrophages and epithelial cells		
Salmonella species	Typhoid fever, gastroenteritis	Within a vacuole
Shigella species	Dysentery, gastroenteritis	Intracytoplasmic
Listeria monocytogenes	Listeriosis, meningitis	Intracytoplasmic
Chlamydia species	Trachoma, sexually transmitted diseases, pneumonia	Within a vacuole

and translocation into target cells. Although little is known about the mechanisms of secretion, it has been found that chaperones are needed for secretion and that enhanced secretion of the virulence factors often occurs after contact with host cell surfaces; thus, these systems have also been called "contact-mediated" secretion systems (*12*). Although the secreted virulence factors differ among pathogens (and thus mediate different diseases), the secretion machinery is often interchangeable.

Invasion of Nonphagocytic Cells

Although phagocytic cells are adept at internalizing pathogens, nonphagocytic cells do not usually engulf large particles. However, some bacterial pathogens can induce their own uptake into these cells (invasion), allowing the pathogen to enter a protected niche and, in some cases, enabling the pathogen to pass through cellular barriers such as the intestinal epithelium or the blood-brain barrier. Phagocytosis and bacterial invasion appear mechanistically similar: Both are initiated by ligand-receptor interactions that activate host signaling, with the actin cytoskeleton providing the necessary force to internalize the particle into a membrane-bound vacuole. However, invasive bacteria seem to have evolved two major types of induced uptake: a "zipper" type mechanism involving direct contact between bacterial ligands and cellular receptors that sequentially encircle the organism (used by *Yersinia* and *Listeria*), and a "trigger" mechanism in which bacteria send signals to the cell to induce dramatic membrane ruffling and cytoskeletal rearrangements that result in macropinocytosis and virtually passive entry of bacteria (Figs. 1 and 2). This strategy is used by *Salmonella* and *Shigella*, two species that have been much studied lately.

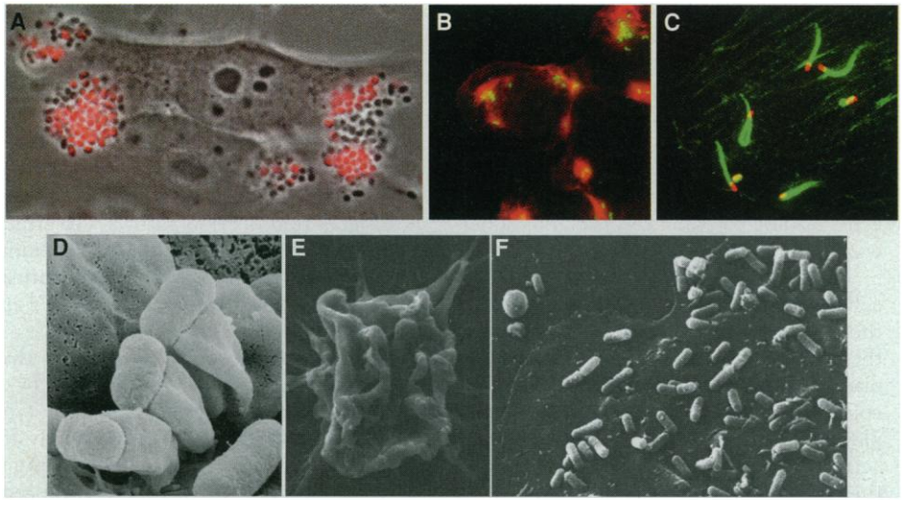

Fig. 1. Bacterial interactions with cultured mammalian cells. (**A**) Phosphotyrosine immunofluorescence staining (red) was overlaid on a phase contrast micrograph of HeLa cells infected with enteropathogenic *E. coli* (EPEC). (**B**) An immunofluorescence micrograph of actin (red) in Madin Darby canine kidney (MDCK) epithelial cells infected with *Salmonella typhimurium* (green) showing areas of actin condensation and ruffling around invading bacteria. (**C**) *Listeria monocytogenes* (red) in infected Vero cells exhibit characteristic polymerized actin tails (green) that propel bacteria inside the cell. (**D**) Scanning electron micrograph (SEM) of EPEC attaching and effacing pedestals on the surface of HeLa cells. (**E**) Ruffles surrounding *Shigella flexneri* as it invades a HeLa epithelial cell. Reprinted from *Trends in Microbiology* (*14*) with permission. (**F**) SEM of *L. monocytogenes* invading cultured Caco-2 cells. Reprinted from *Cell* (*25*) with permission. (A) to (C), bar, 2 μm; (D) to (F), bar, 1 μm.

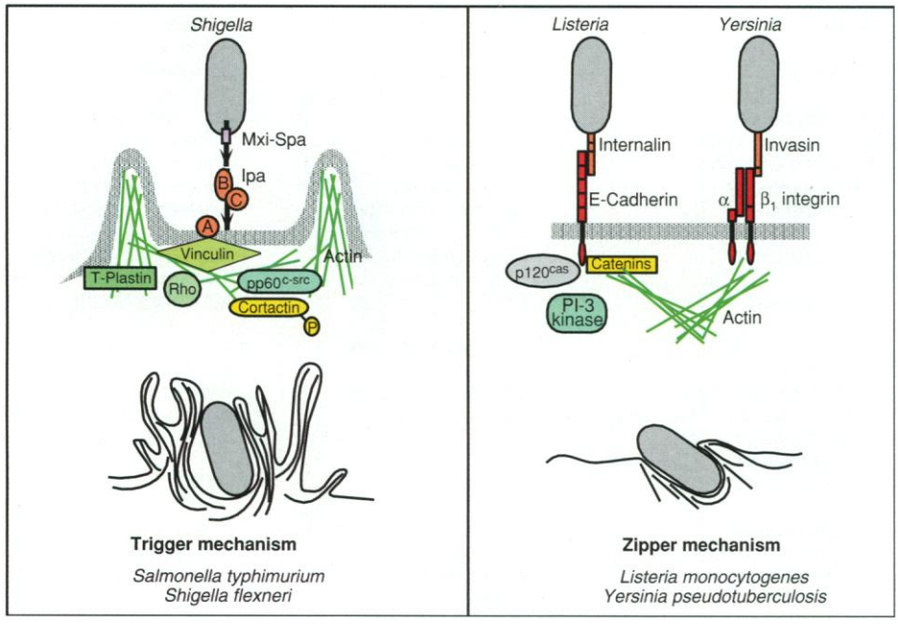

Fig. 2. Mechanisms of bacterial invasion.

The bacterial components that mediate signaling and invasion of *Salmonella* and *Shigella* into cultured cells are surprisingly similar, although the two mechanisms also show marked differences. Both species use type III secretion systems (Figs. 2 and 3). In *Salmonella*, the genes encoding the secretion system and effector proteins are known as the *inv-spa* complex and are located at centisome 63 on the chromosome, forming a "pathogenicity island," a cluster of virulence genes inserted at one site in the genome (*13*). In *Shigella*, the *mxi-spa* secretion system and genes encoding its secreted proteins (IpaA, -B, -C, and -D) are found on a large virulence plasmid (*14*). Secretion of these mediators of bacterial invasion activate host signaling pathways, resulting in bacterial uptake. Unlike adherent EPEC, which induce focused reorganization of the actin cytoskeleton under the bacteria, both *Shigella* and *Salmonella* stimulate major rearrangements of cellular actin that result in large membrane projections similar to "membrane ruffles" induced by some growth factors or oncogenes. The process culminates in bacterial uptake through the formation of a membrane-bound vacuole, which in the case of *Shigella* is subsequently lysed (Fig. 1) (*15*).

What are the cellular signaling events associated with *S. typhimurium* entry? Increased concentrations of intracellular Ca^{2+} and increased inositol phosphate production have clearly been demonstrated. It seems likely that the bacterium stimulates host phospholipase C, which induces inositol trisphosphate production, which in turn

mobilizes Ca^{2+} from intracellular stores. This idea correlates with the role of calcium and phosphoinositides in affecting many actin-binding proteins, several of which (α-actinin, talin, ezrin) are recruited at the site of entry. Tyrosine kinase inhibitors do not block entry of *Salmonella*; additional cell signals are involved, although defining the contribution of each signal to invasion has been difficult (*16*).

In the case of *Shigella*, host proteins that become tyrosine phosphorylated upon entry include cortactin, pp125FAK, and paxillin (*17*). Cortactin is an actin-associated protein (Fig. 2) and a substrate for the nonreceptor tyrosine kinase Src. Src is activated during *Shigella* invasion, colocalizing with the site of entry. Transient overexpression of Src in transfected cells induces membrane ruffles and mediates entry of noninvasive *Shigella* mutants, strongly suggesting a role for this kinase in bacterial entry. T-plastin, which is an actin-bundling protein, appears to play a central role in mediating bacterial uptake, possibly by bundling newly formed actin filaments in the membrane extensions (*15*). Another actin-binding protein, vinculin, colocalizes to the site of entry and can be coimmmunoprecipitated with IpaA. However, bacteria that do not express IpaA still recruit vinculin but are impaired in recruitment of α-actinin, a vinculin-binding protein. These results suggest that IpaA affects vinculin activity after the recruitment step (*17*).

In mammalian cells, major rearrangements of the actin cytoskeleton upon receptor stimulation or other stimulation lead to either membrane ruffling, filopodia formation, or actin stress fiber formation. These rearrangements are controlled by specific small guanosine triphosphate (GTP)–binding proteins belonging to the Ras superfamily, namely, Rac, Rho, and CDC42. It was thus anticipated that invasive bacteria would depend on these molecules to mediate their uptake, and indeed *Salmonella* requires CDC42 but not Rac or Rho for invasion, whereas *Shigella* needs Rho but not Rac or CDC42 (*18*).

The entry processes of *Salmonella* and *Shigella* into nonpolarized epithelial cells in vitro appear, morphologically, to be very similar. However, one major difference is that *Salmonella* interacts with the apical epithelial surface, whereas *Shigella* enters only by the basolateral face, although both penetrate the intestinal epithelium. The integrin $\alpha_5\beta_1$, which is found only on the basolateral surface of epithelial cells, is a receptor for *Shigella* invasion in CHO cells (*17*), but no cell surface receptor has been identified so far for *Salmonella*. How does *Shigella* get to the basolateral surface? In vivo studies reveal that one major site of entry is the M cells of the Peyer's patches. M cells are specialized epithelial cells capable of internalizing inert particles at their apical surface and delivering them to underlying macrophages. By targeting this cell type, which is used by the immune system to sample antigens from the intestine, *Shigella* can cross the epithelium to invade the basolateral surface of enterocytes. Alternatively, it may transmigrate between epithelial cells once the cell junctions have been opened up by the migration of neutrophils in response to the presence of *Shigella* on the apical face of colonic cells (*19*). Thus, both the M cells and the paracellular pathway allow *Shigella* to reach and infect the basolateral surface of epithelial cells in intestinal crypts. *Salmonella* also enters through the M cells in vivo, at least in the mouse. However, it has cytotoxic effects that result in M cell destruction and invasion of adjacent enterocytes at both the apical and basolateral face.

Perhaps the best studied invasion system is that of *Yersinia* species. *Yersinia enterocolitica* and *Y. pseudotuberculosis* have an outer membrane protein, invasin, that mediates attachment and entry into epithelial cells. By binding tightly to a subset of β_1 integrins on cell surfaces, invasin mediates bacterial uptake through a zipperlike mechanism, zippering the host cell membrane around the bacterium as it enters (Fig. 2) (*20*). Host signal transduction mechanisms are used in invasin-mediated entry, because, for example, tyrosine kinase inhibitors inhibit entry (*16*). Host actin is also needed for this process, but the local cytoskeletal rearrangements near the site of entry are not as dramatic as for *Shigella* and *Salmonella*. The fact that the intracytoplasmic domain of the β_1 subunit of integrin interacts with the cytoskeleton by binding to actin-binding proteins such as talin and α-actinin and the fact that actin is required for entry led to the speculation that direct association of integrins with the cytoskeleton is required during internalization. However, mutations that reduced the interaction of integrin with the cytoskeleton increased bacterial uptake, whereas mutations that disrupted a NPIY sequence (N, Asn; P, Pro; I, Ile; Y, Tyr) [related to the consensus NPXY motif (X represents any amino acid) implicated in localization of receptors mediating endocytosis to clathrin-coated pits] resulted in integrins that were deficient in bacterial uptake (*21*). In fact, large lattices of clathrin and AP2 adaptor complexes are formed beneath bound bacteria in the early stages of internalization, suggesting that integrin-mediated internalization may share common features with clathrin-mediated endocytosis. *Yersinia* species have at least two other molecules, Ail and YadA, that can mediate invasion into cultured epithelial cells; however, much less is known about their mode of action.

In addition to invasion systems to enter epithelial cells, *Yersinia* species have evolved sophisticated mechanisms to avoid uptake by phagocytic cells. The anti-phagocytosis strategy relies on the expression of Yop proteins, the archetype of type III secretion systems (*12*). Upon contact of bacteria with the host cell surface, several Yops are injected into phagocytic cells and impair phagocytosis (*22*). YopE paralyzes the cellular actin cytoskeleton; its target is unknown, but YopE shares homology to exoenzyme S (ExoS) of *Pseudomonas aeruginosa*. ExoS is also secreted by a type III secretion system, and it elicits the same cytotoxicity as YopE when present in a recombinant *Y. pseudotuberculosis*, suggesting that the two proteins have the same target. ExoS modifies small G proteins involved in the regulation of the actin network. YopH also contributes to the blockade of phagocytosis. YopH is a broad-spectrum tyrosine phosphatase that dephosphorylates several host proteins, interrupting

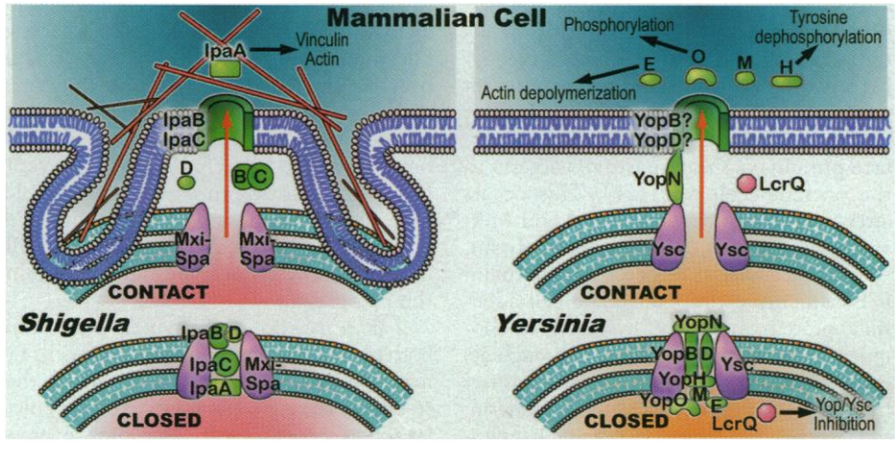

Fig. 3. Comparison of the type III secretion systems and secreted products of *Shigella* and *Yersinia*.

early phosphotyrosine signaling associated with phagocytosis. It also inhibits the Fc receptor mediated oxidative burst (Fig. 3). Another Yop, YopO (also called YpkA) probably also interferes with some signal transduction pathway of the eukaryotic cell, although its substrates have not been identified. YopO/YpkA has homology to mammalian serine and threonine kinases and like YopH is targeted to the inner surface of the plasma membrane of the eukaryotic cell.

In vivo, upon reaching the intestine, *Yersinia* are taken up by M cells, and invasin plays a critical role in this process. After translocation across the intestinal barrier, in order to avoid uptake into the phagocytic cells that underlie M cells, the bacteria may interfere with phagocytosis by injecting Yops. In this way, bacteria remain extracellular, allowing their survival and multiplication as extracellular microcolonies in lymphoid tissues. Phagocytosis generally results in death of the bacteria.

Thus, there are multiple uses for bacterial products secreted by a contact-mediated type III secretion systems, ranging from enhanced adherence (EPEC) and invasion (*Shigella* and *Salmonella*) to cell damage and blockage of phagocytosis (*Yersinia*). Similar systems have not been identified in Gram-positive bacteria.

Among Gram-positive bacteria, only the invasive properties of *Listeria monocytogenes* have been studied in detail (*23*). This food-borne pathogen enters a variety of mammalian cells and tissues during disease and in cell culture (Figs. 1 and 2). A surface protein, internalin, mediates entry into cultured intestinal epithelial cells, and it confers invasiveness to the noninvasive species *Listeria innocua*, suggesting that, like *Yersinia*'s invasin, it is sufficient to promote entry. Internalin contains multiple copies of a 22–amino acid leucine-rich tandem repeat (LRR), a feature of several eukaryotic proteins that are generally involved in protein-protein interactions. The COOH-terminal region of internalin contains an LPXTG motif preceding a hydrophobic membrane-spanning region. The LPXTG motif (L, Leu; T, Thr; G, Gly) permits covalent linkage of surface proteins of Gram-positive bacteria to the bacterial cell wall (*24*). This occurs after cleavage of the T-G bond and linkage of the T residue to the peptidoglycan.

The mammalian receptor for internalin is E-cadherin, a transmembrane cell adhesion protein normally involved in homophilic cell-cell interactions through its extracellular domain (*25*). Internalin is not the only heterophilic ligand for E-cadherin; the integrin $\alpha_E\beta_7$ of intraepithelial lymphocytes also binds to it (*26*). The intracytoplasmic region of E-cadherin, which through a complex with catenins is linked to the cytoskeleton, is critical for homophilic interactions and cell-cell adhesion. Interestingly, it is not required for the interaction between $\alpha_E\beta_7$ and E-cadherin (*26*); whether it is required for internalin-mediated entry is unknown.

Internalin is not required for the entry of *L. monocytogenes* into a number of other cultured cell lines, suggesting that this bacterium has evolved additional strategies for invasion (*23, 27*). Indeed, InlB, another surface protein belonging to the internalin multigene family and displaying similar LRRs, mediates entry in cultured hepatocytes, HeLa cells, and CHO cells. The InlB receptor has not been identified.

The morphological events associated with entry of *L. monocytogenes* are very different from the macropinocytosis triggered by *Shigella* and *Salmonella* and are more reminiscent of the zipper mechanism mediated by *Yersinia* invasin-integrin interactions (Fig. 2) (*25*). Cytoskeletal rearrangements are critical for *Listeria* internalization. Among the signaling events that occur between initial contact and the actin cytoskeletal rearrangements is the activation of the lipid kinase p85/p110 (*28*). This activation requires the InlB protein, tyrosine phosphorylation in the host cell, and association of p85 with at least one tyrosine-phosphorylated protein. How this phosphatidlyinositol 3-kinase (PI 3-kinase) mediates uptake is unknown. One attractive possibility is that PI-3 kinase lipid products directly act on actin, by uncapping barbed ends of actin filaments as previously shown in platelets (*29*).

Intracellular Life of Bacterial Pathogens

The theme of exploitation of host functions continues when bacterial pathogens become intracellular parasites. Nearly all invasive bacteria enter a membrane-bound vacuole as part of their invasion process, but their subsequent fates vary. Certain bacteria thrive within vacuoles that fuse with lysosomes, although little is known about their survival mechanisms. Others have developed mechanisms to prevent fusion of the pathogen-containing vacuole with lysosomes, thereby maintaining a protected niche inside the host cell. Still others lyse the vacuole and survive within the cytoplasm.

Life in the vacuole. Salmonella enter into both phagocytic and nonphagocytic cells by macropinocytosis. They often reside in the resulting large membrane-bound vacuoles (spacious phagosomes), and they express several gene products that enhance intracellular survival by neutralizing lysosomal killing mechanisms that are mediated, for example, by cationic peptides. Within epithelial cells, the *S. typhimurium* vacuole appears to be uncoupled from the main endocytic route, and after a lag period, the bacterium replicates within it (*30*). Associated with these vacuoles are filamentous host structures that contain lysosomal glycoproteins; the role of these structures is uncertain, but *Salmonella* have at least one virulence factor that is necessary for triggering their formation.

A general theme among pathogens that remain within membrane-bound vacuoles is their ability to avoid fusion with lysosomes, although the mechanisms used to achieve this differ. For example, vacuoles containing *Mycobacteria* remain within the early endosomal compartment, thereby avoiding the process of development into lysosomes. The vacuolar adenosine triphosphatase, which is responsible for acidifying vesicles, is not incorporated into the membranes of intracellular M. *avium*–containing vacuoles (*31*) so that the vacuole is not acidified, a prerequisite for activation of several lysosomal degradative enzymes. Most lysosomal markers, including those that are delivered by a mannose-6-phosphate receptor, do not reach *Mycobacterium*-containing vacuoles. *Chlamydia trachomatis*, an obligate intracellular pathogen, resides within a vacuole that remains completely uncoupled from the main endocytic route. The chlamydial inclusion contains no specific vesicle markers, but acquires and incorporates sphingomyelin into the vacuole, probably by intercepting an anterograde vesicle export pathway (*32*). Growth of the Chlamydia in the vacuole requires adenosine triphosphate that is pumped in from the host cell by an unknown mechanism. *Legionella pneumophila*, the causative agent of Legionnaires' disease, also inhabits a unique intracellular niche within a membrane-bound vacuole. It enters phagocytes by an unusual phagocytic mechanism called "coiling phagocytosis," during which a phagocyte pseudopod coils around the bacterium as the organism is internalized (*33*). After internalization, host mitochondria accumulate around the bacterial vacuole, and these are later replaced by ribosomes lining the unacidified vacuole. The ribosomes are associated with host endoplasmic reticulum. It appears that the *Legionella* vacuole fuses with the rough endoplasmic reticulum, probably by exploiting autophagy machinery to establish an intracellular niche favorable for its replication. Several bacterial genes including *icm* and *dotA* have been shown to be critical for intracellular survival and growth of *Legionella* (*34*).

Other possible mechanisms by which

pathogens influence the maturation of bacteria-containing vacuoles are only beginning to be studied. For example, vesicular trafficking is normally mediated by a family of small GTP-binding proteins called Rabs. One possibility is that bacteria-containing vacuoles interact with Rabs or their effectors, thereby altering their trafficking. Another potential mechanism is the engagement of a surface receptor that does not target the vacuole to become a lysosome. The development of new techniques, such as the isolation of vacuoles containing intracellular pathogens and the use of confocal microscopy to label vacuolar membranes, coupled with the identification of bacterial genes that mediate these processes, will yield information about the mechanisms.

Escape from the vacuole and cell-to-cell spread. Not all intracellular bacteria remain within a vacuole. *Shigella*, *Listeria*, and *Rickettsia* rapidly gain access to the cytosol, where they replicate. In the case of *Shigella*, the bacterial factor used to breach the vacuolar membrane is IpaB, one of the secreted proteins used to invade cells, but the mechanism of lysis is unknown (*35*). *Listeria* use a pore-forming toxin, listeriolysin O. This potent membrane-damaging toxin, when expressed in *Bacillus subtilis*, is sufficient to allow this soil organism to reach the cytosolic compartment (*36*).

When free in the cytoplasm, these three species have evolved a phenotypically similar mechanism to propel themselves through the cytosol (Fig. 1) (*2*, *37*). A continuous actin polymerization process takes place at one pole of the bacterium and provides the driving force for movement. Actin assembly is visible as a tail of polymerized (F) actin, which remains stationary within the cytosol ("the actin tail") while bacteria move ahead (*2*, *37*). In this tail, filaments are short and randomly oriented, with the barbed (fast polymerizing) end oriented toward the bacterium, indicating that actin polymerization was initiated at the bacterial surface. Bacterial actin-based motility has no connection with bacterial chemotaxis; rather, it is highly reminiscent of other cellular actin-based motility events, such as the migration of neutrophils toward a site of infection or metastasis of cancer cells. In these cases, actin polymerization takes place at the leading edge of the moving cell. However, the molecular basis for these cellular events remains elusive, explaining why bacterial motility, when discovered, received a great deal of attention because it provides simplified and genetically manipulatable systems to study a complex phenomenon.

Actin-based motility is mediated by a single bacterial protein: ActA in the case of *Listeria* and IcsA (also called VirG) for *Shigella* (*38*). ActA is a 610–amino acid surface protein characterized by a central region made of proline-rich repeats. IcsA is an 120-kD outer membrane protein that also has a region of repeats, albeit glycine-rich. One interesting feature of these two proteins is their polar distribution on the bacterial surface. Establishment of ActA polarity is clearly linked to bacterial replication (*39*). For *Shigella*, the protease SopA contributes to polarity (*40*). Polarized distribution determines the site of actin assembly and direction of movement, demonstrating that bacteria can target proteins to particular locations to execute specific programs [see (*41*) in this issue].

How ActA and IcsA mediate actin assembly is a challenging problem that has been more extensively studied for ActA and has been tackled using cell-free systems (such as *Xenopus* egg extracts or platelet extracts) that support actin-based bacterial motility. Genetic analysis has revealed that the NH_2-terminal portion of ActA is necessary and sufficient for movement and the central proline-rich region increases the efficiency of the process, whereas the COOH-terminus plays no role (*42*). Bacteria expressing ActA do not nucleate actin efficiently, suggesting that ActA recruits an actin-binding protein or must be modified inside the host to interact with actin, or possibly both. Purified ActA binds VASP, a cellular protein that is associated with microfilaments and that can bind profilin, a small actin-binding protein that plays a critical role in the control of cellular actin assembly (*42*). Thus VASP could bring in the vicinity of bacteria, polymerization competent profilin/actin complexes. However, in the infected cell, VASP is recruited by the proline-rich region of ActA and can thus be considered as not absolutely essential. Also involved in the process are Arp2 and Arp3, two actin-related mammalian proteins (*43*) that may interact with actin and create the link between ActA and actin.

Initial models for ActA-mediated F-actin assembly were largely based on the idea that this protein nucleated, or recruited a nucleator of, actin monomers. Although this is still an attractive possibility, at present there is little evidence for it. Another possibility is that ActA controls movement by directly or indirectly generating or regulating the availability of free barbed ends of actin filaments. This is supported by the observation that bursts of actin polymerization inside cells in response to stimuli can result from the transient appearance of free barbed ends, resulting either from the uncapping or severing of actin filaments. The recent finding of an ActA deletion mutant moving discontinuously suggests that ActA may play a role in protecting free barbed ends from capping proteins. This mutation also suggests that there is a threshold in the number of free barbed ends, which, when not attained, leads to stalling of the bacteria (*42*).

The recruitment of actin by bacteria that replicate inside the cytosol is a good example of the use of cellular components by bacterial pathogens (*1*, *2*). Recalling that the actin cytoskeleton is also appropriated during entry and even adhesion, it is clear that pathogens have evolved a wide range of approaches to manipulate this highly dynamic cytoskeletal network, indicating a lengthy coevolution between cells and pathogens.

Generally, when intracellular bacteria have actively replicated inside the host cell, the cell dies, often by lysis. This releases the bacteria, which then either invade other cells or are engulfed by phagocytic cells. For *Shigella* and *Listeria*, an important consequence of actin-based motility is direct spreading to neighboring cells. On reaching the plasma membrane, these bacteria induce the formation of protrusions that invaginate into the neighboring cell, resulting in the formation of a two-membrane vacuole containing the bacterium. After vacuole lysis, the bacterium starts a new cycle of infection. Engulfment of the bacterium-containing protrusion by the neighboring epithelial cell requires the presence of the cell adhesion molecule E-cadherin, at least in the case of *Shigella* (*44*). This direct cell-to-cell spread allows dissemination within tissues while the bacteria remain sheltered from bactericidal cells or host components such as circulating antibodies or complement.

Host Damage by Bacterial Pathogens

Killing the host may involve several factors. In a few cases, including tetanus, diphtheria, botulism, and cholera, the clinical symptoms of disease are caused by a single secreted toxin. Usually, however, toxins are only one of several contributors to disease. The bacterial protein toxins can be divided into three groups according to their site of action (*45*). (i) Toxins acting at the plasma membrane, where they interfere with transmembrane signaling pathways. This group includes the *E. coli* heat-stable enterotoxin ST, which acts directly on the transmembrane guanylate cyclase of intestinal cells. (ii) Toxins that alter membrane permeability, such as pore-forming toxins of the streptolysin O/listeriolysin O family, the toxin alpha from *Staphylococci*, and the RTX toxins such as *E. coli* hemolysin. (iii) Toxins that

act inside cells, where they enzymatically modify a specific cytosolic target. These toxins can be subdivided into six categories according to their enzymatic activity (Table 2). Toxins that modify host substrates are among the most potent because of their catalytic nature, in addition to their being confined to the intracellular environment. These toxins are often involved in changes to the host cell cytoskeleton or signaling pathways. In some cases, because of their specificity, they have become valuable tools for cell biologists; for example, the C2 toxin of *Clostridium botulinum* adenosine diphosphate (ADP)–ribosylates actin monomers, preventing actin filament elongation and resulting in complete depolymerization of cellular actin (*46*). The C3 toxin from *C. botulinum* inactivates the small guanosine triphosphatase Rho, causing disorganization of the cytoskeleton (*46*), whereas the *E. coli* toxin CNF constitutively activates it (*47*).

Another type of host cell damage resulting from bacterial infection is the activation of the cellular program of apoptosis or programmed cell death. The first bacterium shown to induce apoptosis was *Shigella flexneri*, which specifically kills cultured macrophages but not epithelial cells (*48*). The *Shigella* protein IpaB is sufficient to induce apoptosis, one of its several functions (along with participating in invasion and escape from the vacuole) (*48*). IpaB binds to interleukin-1β (IL-1β)–converting enzyme (ICE), a cysteine protease that can initiate apoptosis when expressed in cells (*48*); ICE is known to be activated during *Shigella* infection, and its inhibition abolishes *Shigella*-mediated apoptosis and IL-1β release. (As an aside, the role of secreted IL-1β may be to induce inflammation and neutrophil migration in epithelial tissues, a process that disrupts epithelial cell junctions and favors the translocation of bacteria through the intestinal barrier from the apical face of epithelial cells.) In vivo, *S. flexneri* induces extensive apoptosis of macrophages, B cells, and T cells, which are located under M cells (*48*). Thus, induction of apoptosis may be an important step in the pathogenesis of *Shigella* as it breaches the gastrointestinal barrier.

Salmonella typhimurium also induces apoptosis in macrophages. Mutants that are unable to induce host cell membrane ruffling or to express the type III protein secretion system that is used to invade nonphagocytic cells fail to induce apoptosis (*49*). However, cytotoxicity does not require bacterial invasion because apoptosis can be detected after treatment of macrophages with cytochalasin D, which prevents internalization.

Apoptosis is also one of the early events in listeriosis, occurring in infected hepatocytes in vivo as well as in cultured hepatocytes (*50*). But in this case it may be working to the advantage of the host. It is associated with the release of neutrophil chemoattractants, and during the early stages of infection, neutrophils clear the apoptotic cellular debris and kill the *Listeria* in apoptotic cells. Studies in vitro have demonstrated that apoptosis also takes place in cultured dendritic cells (antigen-presenting cells present in the lamina propria of the mouse intestine) and is mediated by listeriolysin O (*50*).

The strategy of activating programmed cell death may be widespread. Certain bacterial toxins, including diphtheria toxin, *Pseudomonas* exotoxin A, and cholera toxin, can induce apoptosis in vitro (*51*). Indeed, the induction of apoptosis in cells directly involved in the immune response, as shown in the case of TSST1 from *Staphylococcus aureus*, which induces B cell apoptosis and inhibits immunoglobulin G production (*51*), may be of considerable benefit for the incoming microbe.

Control of Bacterial Virulence Factor Production

Controlled expression of virulence factors is a key point in the adaptation of pathogens to their host or their environment. This control can be accomplished at different levels: In addition to the classical transcriptional regulation, recently discovered types of control allow pathogens to regulate expression of their virulence factors (*1*).

Transcription of virulence genes is controlled by a series of regulators including AraC-like and LysR-like transcriptional activators; small proteins that affect DNA topology such as H-NS; alternate σ factors; and the classical two-component regulatory systems. Recently, this latter system was shown to be induced after pilus-mediated adherence of uropathogenic *E. coli* (*52*).

One sophisticated strategy for a bacterial population to coordinately turn on expression of a virulence factor is "quorum sensing": bacteria measure their population density and only produce virulence factors when they have reached a critical density, presumably that needed to overcome the ensuing host defenses. The signal that activates the generalized expression of the virulence factor is triggered by the accumulation of a diffusible small molecule to a certain threshold. This molecule acts as a cofactor to promote transcription. Such quorum sensing systems have been identified in *P. aeruginosa* (*53*) and several other pathogens.

Two other ways of regulating production of virulence factors are found in type III secretion systems. First, as demonstrated in the Yops systems and in the Ipa system, there is a protein (YopN or IpaD) that acts as a cork to prevent secretion of the Yops or Ipas (Fig. 3). Upon contact with the mammalian cell, this protein is released and the Ipas or Yops proteins are secreted. Second, there is control of LcrQ, a negative regulator of the *Yersinia* Yop system (*54*). Upon contact with

Table 2. Examples of bacterial toxins.

Enzymatic activity (reference)	Toxin	Target*	Effect
ADP ribosyl transferase (*45*)	Diphtheria toxin	EF2	Blockade of protein synthesis, cell death
	P. aeruginosa exotoxin A	EF2	Blockade of protein synthesis, cell death
	Cholera toxin, *E. coli* LT	Gs	Increase in cAMP, alteration of permeability
	Pertussis toxin	Gi, Gt	Increase in cAMP, various effects
	Clostridium botulinum C2	Actin	Actin depolymerization
	C. botulinum C3	Rho (−)	F-actin disorganization
Depurinase (*45*)	Shiga toxin	rRNA 28S	Blockade of protein synthesis, cell death
Adenylate cyclase (*45*)	*B. pertussis* AD, hemolysin	None	Increase in cAMP
Zinc protease (*45*)	Tetanus toxin	VAMP	Blockade of exocytosis
	Botulinum toxins B, D, F, G	VAMP	Blockade of exocytosis
	Botulinum toxins A, E	SNAP25	Blockade of exocytosis
	Botulinum toxins C	Syntaxin, SNAP25	Blockade of exocytosis
UDP-glycosyl-transferase (*56*)	*Clostridium difficile* toxins A, B	Rho(−), Rac(−), CDC42(−)	F-actin disorganization
	Clostridium sordellii LT	Ras(−), Rap(−), Rac(−)	Cell shape changes
Deamidase (*57*)	*E. coli* CNF, *B. pertussis* DNT	Rho(+)	Membrane ruffling, actin polymerization

* (−), inhibition; (t), constitutive activation.

mammalian cells, LcrQ is exported through the Yop–type III secretion system, thereby lowering LcrQ concentrations inside the bacteria and enhancing Yop expression (Fig. 3). This regulatory system allows the bacteria to tightly coordinate virulence factor expression and contact with mammalian cell surfaces.

Conclusions

The progress described in this article, much of it from developments at the interface with other disciplines, is not an end in itself. Rather, it serves to open up a large number of possibilities for the understanding of the biology of bacteria and the diseases they cause. It should not be forgotten that the study of a bacterial pathogen in cultured cells is a reductionist approach and an artificial situation, and that diseases need to be considered in the context of their complex mammalian hosts. There is now an urgent need to apply the new information in animal models, bearing in mind that animal infections may be different from human infections and that single-strain infections often do not necessarily reflect the real situation. The issue of interbacteria competition also needs to be addressed: As a pathogen colonizes a mucosal surface, it is usually in competition with the normal flora, and even with other pathogens. Finally, integration of host genetics, physiology, and immune system, which play critical roles in the outcome of infection, is necessary for a full understanding of pathogenesis.

The identification and understanding of bacterial virulence factors is providing useful information for the development of new vaccines. Specific virulence factors, usually toxins (either wild type or mutated), can be used in component vaccines, and genetic mutation of virulence factors often cripples a pathogen sufficiently for it to be considered for use as a live attenuated vaccine strain, and even to use it to express and deliver heterologous cloned antigens. Knowledge of where the pathogen targets are in the body and the ensuing host immune response to these infections provides additional opportunities for vaccine development; for example, pathogens that target mucosal surfaces are being used to develop mucosal vaccines.

Are we on the way to developing new therapeutics? A better understanding of the whole infectious process should aid the design and targeting of drugs for various infections. Discoveries concerning the molecular events involved in the expression of virulence factors, the targeting of virulence factors to the bacterial surface or their secretion, and the mechanisms regulating their expression, will generate new targets to test for various inhibitors. With the continued progress in cellular microbiology and microbial pathogenesis, and the prospect of completing the genomic sequences of most of the pathogens [see (55) in this issue], information will increase exponentially. The major challenge for the future will be how to exploit this wealth of information to develop new therapeutics.

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