Discrimination Between Intracellular Uptake and Surface Adhesion of Bacterial Pathogens

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Most bacterial pathogens initiate infectious diseases by adhering to host cells. Bacterial adherence to nonphagocytic cells usually leads to extracellular colonization; however, many invasive microorganisms enter host cells after binding to the host cell surface. It is unclear why bacterial adherence can result in these two different fates for the microorganism. Analyses of model systems, such as the uptake of enteropathogenic *Yersinia* into cultured cells, indicate that the particular mammalian cell receptors bound and the nature of the binding event dictate whether the bacterium remains extracellular or enters host cells.

HUMAN DISEASES CAUSED BY BACTERIAL PATHOGENS RESULT from an interplay between the invading microorganism and the host. After the bacterium encounters the host, the microbe must colonize a particular tissue. The microorganism may replicate at this site or move deeper into host tissues before significant replication occurs. Damage to the host may occur at any point in this process, either because of products synthesized by the bacterium or the harmful effects of normal host defense mechanisms. The first step in an infectious disease—surface colonization or entry into tissues that are portals for the infection—requires specialized factors encoded by the microorganism that allow binding to host cells. Such colonization factors are not sufficient for the microorganism to cause disease but ultimately help determine the severity of disease and the organ systems that are affected.

Two potential fates await the microorganism after it binds to a host cell. The bacterium usually binds to the external surface of the cell and colonizes, particularly if the encounter occurs in the epithelial layers that line the oral, intestinal, or urogenital tract. Such extracellular adhesion may result from direct binding of the microorganism to receptors located on the cell surface or from binding to host-encoded, secreted polysaccharides and proteins that bind to host receptors (*1*). The alternative route for the microorganism is to be internalized by the target cell after adhesion. Little is known about this pathway, but it appears that the microorganism must bind directly to a host cell receptor (*2–4*). It is not clear why binding to animal cells causes some microorganisms to engage in extracellular adherence and others to enter the cell. In this article I discuss factors that potentially determine the pathway taken by the bacterium after encountering the mammalian cell. Factors that control the fate of the bound bacterium include (i) the type of host cell involved, (ii) the type of host cell receptor bound by the bacterial ligand, and (iii) the nature of the physical interaction between the bacterial ligand and the animal cell receptor.

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Cellular Invasion by Enteropathogenic Bacteria

A variety of pathogenic bacterial species are internalized by epithelial cells, fibroblasts, and endothelial cells, even though these vertebrate cells are not normally considered phagocytic. Some microbial species, such as *Chlamydia trachomatis,* a common agent of sexually transmitted diseases, cannot grow outside of host cells. Others, including the agents of enteric diseases *Shigella flexneri* and *Salmonella typhimurium*, enter host cells but can also grow well in standard bacteriological media. All intracellular organisms must overcome certain problems to enter host cells, so researchers have studied a few microorganisms that are easy to grow in the laboratory in the hope that general principles will be uncovered.

One can study microbial entry into nonphagocytic cells by analyzing the uptake of live bacteria into cultured mammalian cell lines. Bacteria located within mammalian cells (invasive) are not killed by a variety of aminoglycoside antibiotics, whereas those that simply adhere to the host cell surface (noninvasive) are rapidly killed; this behavior facilitates quantitation (*5*). The phenotypic difference between enteroinvasive microorganisms and *Escherichia coli* K12 (or related noninvasive bacteria) in this assay has allowed the isolation of molecular clones (from at least five different bacterial species) that encode proteins responsible for cellular penetration (*5–10*).

Molecular clones have been isolated that encode factors responsible for entry of the enteroinvasive *Yersinia* into cultured mammalian cells. These factors are expressed in *E. coli*, allowing most of the detailed examination of cellular entry to be performed in this easily manipulated genetic background (*11, 12*). Researchers of such clones have identified at least three different gene products responsible for the uptake of enteropathogenic *Yersinia* into mammalian cells (*6, 12*). Each of these gene products promotes entry in the absence of the other two, implying that there are three different routes into mammalian cells.

The most efficient pathway for entry is promoted by the product of the *inv* locus. Expression of this single gene is sufficient to render a noninvasive *E. coli* strain capable of entering cultured cells. The gene is expressed in all virulent enteropathogenic *Yersinia* species that have been examined (*13*), and its product, invasin, has been analyzed from both *Y. pseudotuberculosis* and *Y. enterocolitica*, which encode highly related *inv* genes (*14*). The *Y. pseudotuberculosis* invasin protein is a 103-kD product found in the outer membrane of both the parental organism and the engineered *E. coli* strains that express it. Invasin binds directly to mammalian cell receptors, and this binding is required for bacterial entry (*15*).

The second characterized pathway for *Yersinia* entry is promoted by the 17-kD product of the *ail* gene (*6, 16*). Bacteria that harbor the *ail* gene from *Y. enterocolitica* bind to a wide variety of cultured cells, but in contrast to the behavior of invasin, only in a few cell types (for example, Chinese hamster ovary cells) does binding result in efficient entry by the microorganism. Although the basis for this

cell line specificity is unknown, efficient entry is dependent on specific culture conditions.

Yersinia pseudotuberculosis strains that contain null mutations in *inv* are able to use a third pathway to enter mammalian cells, even under conditions in which *ail* is not expressed (*12*). All pathogenic *Yersinia* harbor a large plasmid that is required for virulence in mice (*17*). Entry via this third pathway is dependent on the presence of the *Yersinia* virulence-associated plasmid; *inv* mutants that lack this plasmid are defective for entry into a number of cell lines (*12*).

We do not know why there should be multiple pathways for entry, but their expression is tightly regulated by temperature in a fashion that suggests that the pathways work in a sequential order during infection of animal hosts. Expression of invasin is highest when bacteria are grown at ambient temperature or lower, which are conditions that the microorganism encounters before infecting an animal (*18*). In contrast, there is little expression of the Ail protein before the bacteria begin to grow within the host. These observations indicate that invasin is utilized early in the infection process, whereas Ail is important for cellular interactions at later stages.

The use of multiple pathways for entry may be a general tactic used by invasive bacteria. For example, two loci present in a wide variety of *Salmonella* species encode factors that promote entry into cultured mammalian cells (*9, 10*). These loci probably act independently of one another, because at least one of them is sufficient to promote entry in the absence of the other (*9*).

Extracellular Localization of Pathogenic Bacteria by Adhesion to Cell Surface Polysaccharides

Not all bacterial–host cell interactions result in cellular entry. Most bacteria that are pathogenic for mammalian hosts adhere to the surface of host cells (particularly to epithelial layers) (*19*) and remain extracellularly localized (*20*). Surface adhesion allows microorganisms to colonize on epithelial layers at the initiation of the disease process and facilitates gaining a foothold in a variety of tissues as the infection process continues (*21*). Mutants defective for the synthesis of microbially encoded adherence factors are either unable to colonize in appropriate tissues, unable to compete with resident microbial flora, or attenuated for virulence relative to isogenic wild-type strains (*22*).

Much of what is known about the adhesion of bacteria to epithelial surfaces is a result of the analysis of pili (fimbriae), which are found coating a variety of Gram-negative bacterial species (*23*). The best characterized of these are the *E. coli* pyelonephritis-associated pili (Pap). These pili are encoded by a large gene cluster and are primarily found in strains of *E. coli* associated with urinary tract infections (*24*). The surface appendages encoded by these strains consist of a repeated polymer called pilin and at least three other gene products that are minor constituents of the structure. The PapG protein, one of the minor components located at the tip of the pilus, is responsible for binding to receptors on human cells (*25*). Such a structure, in which adherence to animal cells is mediated by a minor protein component located at the tip of the pilus, appears to be common for pili encoded by *E. coli* and its relatives (*26*). However, in both *Pseudomonas aeruginosa* and some porcine-pathogenic *E. coli* strains, the major pilus subunit can be an adhesin (*27*), so two major strategies may exist for forming adhesive pili.

Studies of mammalian cell-encoded receptors for Pap pili indicate that the pilus has a lectinlike activity (*28*). Adhesion of Pap-piliated *E. coli* to uroepithelial cells is inhibited by the presence of saccharides that contain galactose (α1–4) Gal residues (*29*), and only human erythrocytes containing this sugar moiety, which is present in the globoseries of glycolipids, can be agglutinated by such bacteria (*29*). The mode of presentation of the Gal (α1–4) Gal on a particular glycolipid determines whether the PapG adhesin will bind, and therefore confers species and tissue specificity. For instance, the PapG adhesin is able to bind to human but not canine uroepithelial cell lines, even though both lines express glycolipids with the Gal (α1–4) Gal structure (*30*). The neighboring sugar residues on the glycolipid determine whether this digalactoside is recognized by the PapG adhesin, and these residues differ for canine and human cells (*30*).

Other bacterially encoded proteins that mediate surface adherence have sugar-binding activity (for example, the adhesin encoded by *Neisseria gonorrhoeae*) (*31*). Bacteria-encoding lectinlike adhesins, after promoting attachment to normally nonphagocytic cells, remain localized on the extracellular surface and are not taken efficiently into an intracellular locale. No explanation has yet been tendered for this contrast to the efficient penetration of enteropathogenic *Yersinia* into mammalian cells.

Table 1. Selected examples of the binding of microorganisms to extracellular matrix components and identified receptors. This list is not exhaustive and includes only examples in which receptors have been identified. For a more comprehensive list see (*54*).

Organism	Extracellular matrix protein	Receptor	Reference
S. aureus	Fibronectin	210-kD binding protein	(*33*)
S. aureus	Collagen	135-kD binding protein	(*55*)
S. pyogenes	Fibronectin	Lipoteichoic acid	(*56*)
E. coli	Fibronectin	Curli	(*40*)

Adhesion to Extracellular Matrix Proteins

Pili-mediated adhesion is the best studied strategy for surface colonization by pathogenic microorganisms, but it may not be the most commonly used. Gram-positive bacteria and eukaryotic microorganisms that cause infectious diseases do not have pili. Thus, some other modes of adhesion must be used by these organisms. A variety of pathogenic organisms, ranging from *Staphylococcus aureus* and *E. coli* to *Treponema pallidum*, bind to extracellular host proteins that, in turn, attach to host cells (*1, 32*). The proteins most often bound by these microorganisms are extracellular matrix (ECM) components and include fibronectin, laminin, collagen, and vitronectin (*33*). The binding of these host proteins to the microorganism is probably due to the presence of specific receptors on the bacterial cell surface, because binding is often saturable and of high specificity and avidity (*1, 33*). Some examples of bacteria able to bind ECM components are detailed in Table 1.

The prevalence of bacterial pathogens that bind to ECM proteins suggests that this interaction functions in colonization of host tissues. Researchers have studied this adhesion process by analyzing the binding of microorganisms to solid surfaces that are coated with extracellular matrix proteins. Results of these studies indicate that ECM protein binding could facilitate tissue colonization in two potential ways: (i) direct attachment of the microorganism to the extracellular matrix and to plasma clots found in wounds (*34*), or (ii) adhesion of the microorganism (with receptors that bind extracellular matrix components) to mammalian cells (*35*). Binding to ECM proteins could thus provide an indirect mechanism for bacterial adhesion to host cells.

The binding of fibronectin to *S. aureus* has been the most studied

Table 2. Members of the integrin family that bind invasin. The table is not meant to be exhaustive; most integrins can be found on more than one cell type so only one or a few typical cell types are given for each receptor. For complete review see (*54*).

Integrin	Typical cells	Characterized ligands
$\alpha_3\beta_1$	Epithelial cells	Fibronectin, laminin, collagen
$\alpha_4\beta_1$	Lymphocytes, monocytes	Fibronectin, VCAM-1
$\alpha_5\beta_1$	Endothelial cells, fibroblasts	Fibronectin
$\alpha_6\beta_1$	Platelets	Laminin

of bacterial-ECM interactions (*33*). Fibronectin is a large dimeric protein that is a major component of the extracellular matrix and is often found in plasma clots (*36*). The NH_2-terminal 29-kD region of fibronectin binds to several types of bacteria, including *S. aureus*, whereas other regions of this protein are capable of binding to mammalian cells (*37*). Fibronectin binds with high affinity to a protein on the *S. aureus* surface (*38*). This fibronectin-binding protein is a 100-kD primary translation product that contains a region of 38 amino acids that is repeated three times intact and an additional time in part (*39*). The 38–amino acid repeats are responsible for binding to fibronectin. Hybrid proteins that contain these repeats bind to fibronectin, and synthetic peptides covering the 38–amino acid region inhibit binding of fibronectin to *S. aureus* (*34, 39*). This structure may be unique to Gram-positive bacteria, even though microbial binding to fibronectin is prevalent. For instance, a fibronectin receptor encoded by *E. coli* has recently been analyzed at the molecular level (*40*). This protein, called curlin, shows no sequence similarity to the *S. aureus* protein.

Staphylococcus aureus bound to fibronectin may face a different fate than that of piliated bacteria when encountering the surface of the host cell. Instead of binding to carbohydrates, the microorganism can directly associate with specific mammalian cell surface proteins that may facilitate uptake. The prevailing evidence, however, is that fibronectin binding yields the same results as binding by piliated microorganisms because the bacteria continue to adhere to the mammalian cell surface without efficient internalization (*41, 42*).

Relation Between Cellular Adhesion and Uptake

Why a binding protein such as invasin promotes uptake into mammalian cells while pili or ECM components mediate extracellular adhesion is unknown. Clearly, the nature of the host cell encountered can be important, as for the *Yersinia ail* gene product (*6*). However, it cannot be the only factor that causes one route to be favored over the other because a single cell line can internalize invasin-encoding *E. coli* yet leave piliated microorganisms on the extracellular surface. Three more likely explanations for these different fates are the following: (i) the bacterial-encoded protein that binds to the cell may make an important contribution to the entry process, actually triggering the bound bacterium to enter; (ii) the mammalian cell receptor may have characteristics that route the microorganism intracellularly; or (iii) the physical nature of binding to this receptor may determine the fate of the microorganism.

To investigate the first model, researchers determined if entry was the result of invasin having two domains with distinct functions. In this model, one domain binds to a receptor, whereas the other domain triggers uptake by causing a conformational change that stimulates the process. Inconsistent with this hypothesis is the fact that a distinct COOH-terminal 192–amino acid region of the 986–amino acid invasin molecule binds to animal cells (*43*), and this small region is sufficient to promote entry (*44*). This inability to separate the regions of invasin involved in binding mammalian cells from those involved in the cellular penetration of the microorganism suggests that the protein has a relatively passive function in the entry process; that is, the protein serves only to bind to the appropriate mammalian cell receptor. From this result, it follows that the receptor performs the active function in internalization.

Receptors for invasin have been identified as members of the β_1 chain integrin family of cell adhesion molecules (*4*) (Table 2). The integrin receptor family is a large collection of $\alpha\beta$ heterodimeric cell surface proteins that are found on most mammalian cells and are involved in ECM binding and in cell-cell adhesion (*37, 45*). Well-characterized integrins include receptors for fibronectin, fibrinogen, and complement component C3b (Table 2). In most models for integrin function it is proposed that members of this family function in communicating intracellular events with those outside the cell.

The finding that integrins are involved in invasin-mediated uptake was provocative for a number of reasons. First, integrins interact in some fashion with the mammalian cell cytoskeleton and with extracellular ligands, allowing surface adhesion processes to be coupled to cytoskeletal rearrangement (*45*). Integrins colocalize with a variety of actin-associated proteins when mammalian cells are allowed to adhere to ECM components immobilized on solid surfaces (*46*). This close coupling is likely to function in invasin-mediated entry because uptake of the microorganism is dependent on the presence of an intact cytoskeleton (*47*). Second, the identification of integrins as invasin receptors allows a conceptual connection to other phagocytic processes. Uptake of microorganisms by normally phagocytic cells can be initiated by binding to the complement receptor CR3 ($\alpha_{mac}\beta_2$), an integrin that has sequence similarity to invasin receptors (*48*). Payne and Horwitz have shown that this receptor participates in the internalization, by macrophages, of the intracellular bacterium *Legionella pneumophila* (*3*). This result implies that the mechanism of bacterial entry into nonphagocytic cells, such as epithelial cells, is probably related to phagocytic uptake by macrophages and neutrophils and that many of the same factors may be involved in both processes.

The unique relation between integrins and the cytoskeleton suggests that the second model given above is correct, that binding of a microorganism to an integrin is sufficient to promote entry. Researchers have addressed this model numerous times with seemingly contradictory results, usually by analyzing the interaction of fibronectin-coated organisms with either phagocytic or normally nonphagocytic cells (*41, 49*). In general, microorganisms coated with fibronectin (which recognizes multiple integrin receptors) are not efficiently phagocytosed, although the adhesion of phagocytes to surfaces coated by fibronectin enhances the ability of other receptors to internalize microorganisms (*42*). On the other hand, baby hamster kidney (BHK) cells, lifted into suspension, are able to internalize latex beads coated by fibronectin (*49*). Recent data indicate that this result can only be reproduced if there is excessive expression of the integrin fibronectin receptor or if the cells are lifted from the extracellular matrix (*50*). So it would seem that uptake of particles coated by ECM components can take place only under special circumstances. Therefore, adhesion mediated by invasin interacting with integrins is not identical to the binding of extracellular matrix components to integrins, and particles coated by these two proteins usually face different fates.

It remains to be determined why fibronectin-coated organisms face a fate different from that of invasin-coated microorganisms, even though both ligands bind to members of the same receptor

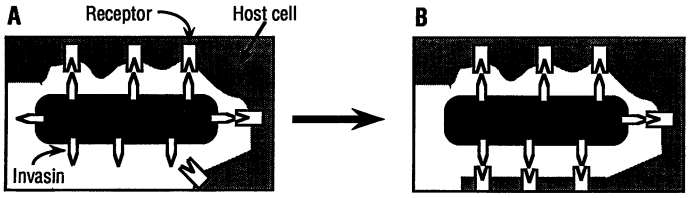

Fig. 1. Invasin-promoted entry by binding of the mammalian cell around the circumference of the bacterium. Bacterium coated by invasin attaches to host cells (**A**), and uptake occurs as the integrin receptor binds the entire surface of the bacterium (**B**) (*51*).

family. The simple explanation that invasin and fibronectin bind to different sites on the integrin receptor seems unlikely (*50*). Monoclonal antibodies of integrins that block fibronectin binding also block invasin binding. Furthermore, the tripeptide sequence Arg-Gly-Asp (RGD), which is a well-characterized competitive inhibitor of receptor binding to fibronectin (*37*), also inhibits binding to invasin. Finally, fibronectin itself is a competitive inhibitor of invasin binding, which means that invasin and fibronectin probably bind to the same site on the receptor. Thus, something else distinguishes invasin from ECM components.

The most readily apparent physical difference between invasin and fibronectin is the fact that cultured mammalian cells bind much more efficiently to immobilized invasin than to fibronectin (*43*). The difference in attachment efficiency is quite large: the dissociation constant (K_d) for the binding of soluble fibronectin to the purified integrin receptor $\alpha_5\beta_1$ (Table 2) is two orders of magnitude greater than that for the binding of soluble invasin to the same receptor (*50*). This disparity between the two ligands suggests that receptor affinity determines whether an integrin-bound bacterium is internalized or remains on the extracellular surface.

Analysis of the steps leading to the internalization of invasin-encoding bacteria may give insight into why ligand-receptor affinity determines whether the microorganism will be routed toward efficient internalization or simple surface adhesion. After a bacterium binds to a mammalian cell, internalization requires that the integrin receptors bind invasin throughout the entire circumference of the microorganism. If only one portion of the bacterial cell surface is allowed to contact the mammalian cell surface, the microorganism will remain extracellularly localized (*44*). This behavior presumably occurs because the mammalian cell surface will wrap around the bacterium only if the receptors are able to make a series of contacts over the entire surface of the microorganism (Fig. 1). This model for uptake is identical to the "zipper mechanism," proposed many years ago by Griffin and Silverstein (*51*), for the uptake of opsonized particles by phagocytes, which holds that the mammalian cell zippers up around the surface of the particle in this fashion (Fig. 1). Some factors may hinder the efficient zippering of the mammalian cell around the surface of the bacterium, and a low affinity of the bacterial-encoded ligand for the integrin receptor is one such problem that would destabilize the zipper and inhibit internalization.

Low-affinity receptor-ligand interaction may result in inefficient uptake because integrin receptors are involved in competing processes, such as maintenance of the host cell shape or adhesion to the extracellular matrix. Anything that stabilizes the zippering process, such as increased affinity of the microorganism for the receptor, will allow more effective competition with these other processes; hence, uptake will be favored over simple adhesion. By this formulation, the failure of fibronectin-coated microorganisms to be efficiently internalized is due to competition with other normal processes involving integrins. Because other processes that involve integrins probably tie up cytoskeletal components, bacteria coated with low-affinity ligands are unable to efficiently recruit these key components for the entry process. Moreover, BHK cells released from tissue culture dishes and placed in suspension are able to internalize fibronectin-coated particles (*49*), presumably because they have been freed from ligand competition for the identical receptors to which fibronectin binds.

If high-affinity ligand-receptor interaction and the involvement of the cytoskeleton in zipper formation are critical for the uptake of microorganisms, these factors may provide an explanation for why piliated bacteria remain localized on the extracellular surface. The lectin activity of the pilus allows the bacterium to adhere to a variety of glycoconjugates, many of which are linked to membrane lipids. The binding of mammalian cell glycolipids to the bacterium may not recruit the cytoskeletal components necessary to stabilize a zipper, or else the affinity of the lectin may not be sufficient for uptake. On the other hand, some of the glycoconjugates recognized by the pilus lectin can probably be found on cell surface proteins, such as integrins, that can communicate with the cytoskeleton. Perhaps the number of contacts made with these glycoproteins is insufficient, the affinity of the interaction is too low, or the large number of glycolipids binding to the pilus efficiently competes with binding to glycoproteins, which routes the microorganism toward extracellular adherence.

Conclusions and Potential for General Principles

Although most mammalian cells are not normally phagocytic, many internalize microorganisms when they encounter the appropriate bacterial pathogen. The host cell must encode and be able to mobilize factors that are critical for the uptake process. A relatively large surface area of the cell must be reorganized in order to internalize a bacterium. This idea, together with results from inhibitors of microfilament polymerization, suggests that one of these critical factors is the host cell cytoskeleton. Perhaps a major determinant of whether a bacterial pathogen will be internalized or remain extracellularly localized is its ability to induce cytoskeletal-dependent movement of the host cell surface around the microorganism. The *Yersinia* invasin protein appears to communicate with the cytoskeleton by binding to four members of the integrin family. Other bacterial proteins that promote uptake probably bind to cell surface components that allow similar communication to occur. Integrins may not be the only receptors capable of coupling these cell surface adhesion processes to the cytoskeleton (Table 3). This

Table 3. Bacterial-encoded factors identified as promoting uptake into normally nonphagocytic cells. Noted below are loci that encode factors to promote uptake or have been implicated in promoting uptake. All have been identified genetically, either by the analysis of mutations or by direct isolation of molecular clones that confer the ability to enter mammalian cells. Full names of loci are as follows: *inv*, enteropathogenic *Yersinia* gene encoding invasin protein; PDE, *Yersinia* plasmid-dependent entry; *ail*, *Yersinia* adhesion and invasion locus; and EAE: *E. coli* attaching and effacing locus.

Organism	Locus	Reference
Y. pseudotuberculosis	*inv*	(*7*)
	PDE	(*12*)
Y. enterocolitica	*ail*	(*6*)
	inv	(*16*)
S. typhimurium	*invA-invD*	(*10*)
S. typhi	*inv*	(*9*)
Enteropathogenic *E. coli*	EAE	(*53*)
Shigella flexneri	*ipaB-ipaC*	(*57*)

coupling may occur because interaction between the bacterial-encoded ligand and the mammalian cell receptor may induce a conformational change in the receptor that initiates a cascade of intracellular signals leading to uptake. Perhaps the high-affinity interaction between the bacterium and the mammalian cell receptor facilitates this conformation change.

It is important to determine if observations made in the model uptake systems are general. In the case of enteropathogenic *Yersinia*, there is some evidence that both the Ail and the invasin proteins represent specific families of proteins that are found in many bacterial species. For instance, proteins of high sequence similarity to Ail are encoded by enteropathogenic *Yersinia* and many *Salmonella* species (*52*), and some evidence suggests a functional similarity of the proteins encoded by these different species. A protein with high sequence similarity to invasin, called *E. coli* attaching and effacing protein (EAE), also has been identified in enteropathogenic *E. coli* strains, and mutations that affect the function of this protein are defective for bacterial uptake into nonphagocytic cells (*53*) (Table 3). These recent findings indicate that the same pathways for uptake of enteropathogenic *Yersinia* are utilized by a variety of bacterial species. Other strategies are probably used for entry into mammalian cells that are very different from those found in enteropathogenic *Yersinia*, and the identification of these strategies should generate much future excitement.

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