Molecular Mechanisms of Bacterial Virulence: Type III Secretion and Pathogenicity Islands

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Recently, two novel but widespread themes have emerged in the field of bacterial virulence: type III secretion systems and pathogenicity islands. Type III secretion systems, which are found in various gram-negative organisms, are specialized for the export of virulence factors delivered directly to host cells. These factors subvert normal host cell functions in ways that seem beneficial to invading bacteria. The genes encoding several type III secretion systems reside on pathogenicity islands, which are inserted DNA segments within the chromosome that confer upon the host bacterium a variety of virulence traits, such as the ability to acquire iron and to adhere to or enter host cells. Many of these segments of DNA appear to have been acquired in a single step from a foreign source. The ability to obtain complex virulence traits in one genetic event, rather than by undergoing natural selection for many generations, provides a mechanism for sudden radical changes in bacterial-host interactions. Type III secretion systems and pathogenicity islands must have played critical roles in the evolution of known pathogens and are likely to lead to the emergence of novel infectious diseases in the future.

Discovery of Two Traits Used by a Broad Range of Bacterial Pathogens

In the past decade, there has been an explosion of new information about bacterial pathogens as researchers have begun to examine the molecular and genetic bases of microbial pathogenicity. Because microbes invade many niches in humans and cause a wide variety of syndromes, it initially appeared that each disease might be created by a distinct molecular mechanism. However, the spectrum of methods is not as broad as first imagined; rather, bacteria exploit a number of common molecular tools to achieve a range of goals (1). Among these tools are pathogenicity islands, which enable bacteria to gain complex virulence traits in one step, and type III secretion systems, which provide a means for bacteria to target virulence factors directly at host cells. These factors then tamper with host cell functions to the pathogens' benefit.

Early in the search for virulence genes, researchers discovered that many of these genes resided on plasmids or phages; however, it was also clear that these genes did not produce all of the physiologic changes induced in host cells by various pathogens (2). Thus, researchers searched the chromosome. Surprisingly, as when found on plasmids, virulence genes often clustered in functionally related groups. Furthermore, these groups often appeared to have been acquired from another organism, as features of their DNA sequence differed from the bulk of the genome. These observations gave rise to the concept of pathogenicity islands—discrete segments of DNA that encode virulence traits and often appear to have a foreign origin (3,4).

Researchers found that a particular set of virulence genes appeared several times on both plasmids and pathogenicity islands (5-8). These genes were discovered in both plant and animal pathogens and were homologous

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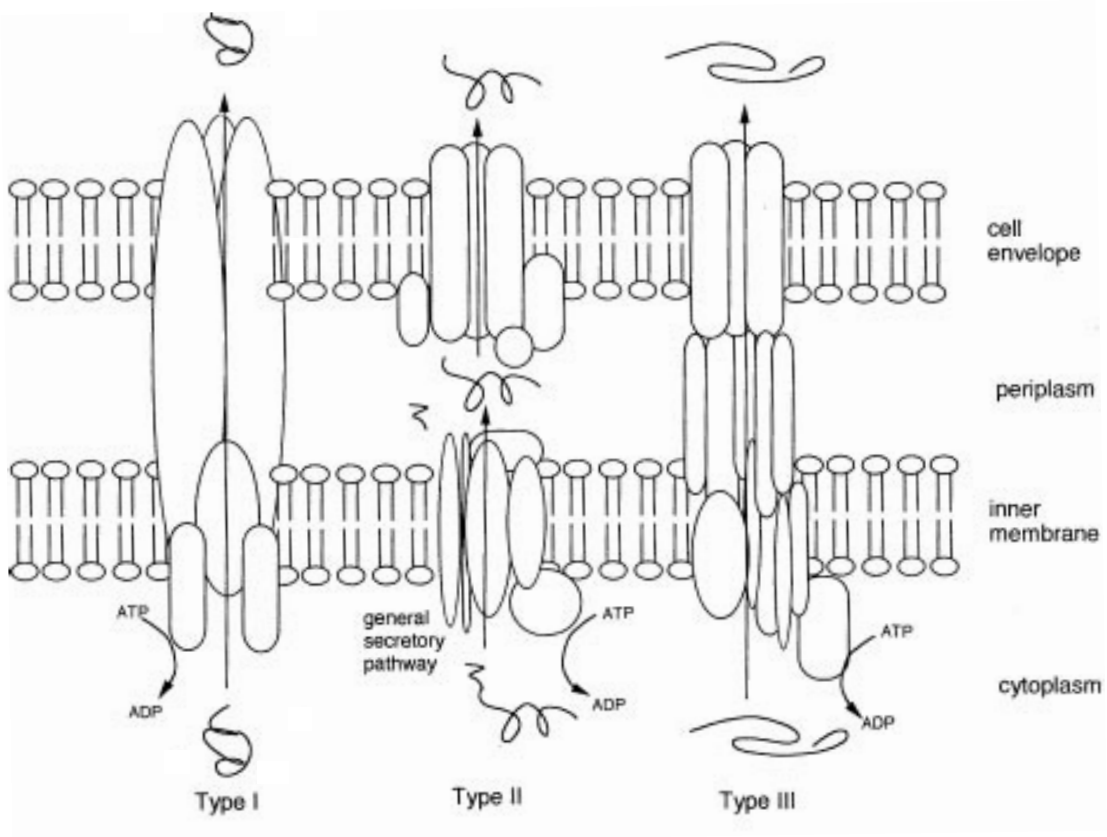

Figure 1: Schematic diagram of type I, type II, and type III secretion systems. All systems use the energy of ATP hydrolysis to drive secretion. Type I and type III secrete proteins across both the inner membrane and the cell envelope (outer membrane) in one step; secreted proteins do not make an intermediate stop in the periplasm, as they do in type II secretion. Type I and type III systems are also similar in that they do not remove any part of the secreted protein. In contrast, the N-terminus of proteins secreted by the general secretory pathway is removed upon transfer to the periplasm; the N-terminal signal sequence can be seen in the periplasm, and the extracellular protein is clearly different from the intracellular protein by virtue of its absence. Type I systems are composed of far fewer components than type III systems; this is indicated by the number of distinct proteins (indicated by shape and size) in the figure. Type II and type III systems share a similar cell envelope component, as indicated by sequence homology; this is reflected in the shape of a cell envelope component in the figure.

to genes encoded on a virulence plasmid of pathogenic *Yersinia spp.* (Table 1) (9-12). The *Yersinia* proteins are the components of a novel secretion system (13), called type III (14). This machinery propels effector molecules toward host cells where they alter host physiology (15,16). The homology suggested that many divergent bacterial pathogens had acquired a similar system from a common source. Pathogens use the type III system to secrete different effector molecules that influence host cells in a variety of ways (16-19).

Secretion Systems in Bacteria

Secreted or surface-exposed bacterial proteins have long been known to play central roles in bacterial-host interactions. In gram-negative bacteria, these proteins must pass through two membranes: the inner membrane, which surrounds the cytoplasm, and the outer envelope, which encloses the periplasm and acts as a barrier to the environment (Figure 1). The general secretory pathway transports proteins to the periplasm. Before the *Yersinia* secretion system was identified, two other specialized secretion systems, type I and type II, were

known to transport molecules to the cell surface (14,20,21). Proteins secreted by the Type I system cross directly from the cytoplasm to the cell surface, bypassing the general secretory pathway completely (Figure 1). Type II-secreted proteins use the general secretory pathway to reach the periplasm and then traverse the outer membrane through distinct channel proteins. Both type I and type II systems secrete proteins involved in various functions, including pathogenesis. For example, α-hemolysin of *E. coli* uses a type I system and bundle-forming pili of enteropathogenic *E. coli* (*EPEC*) and enterotoxigenic *E. coli* (*ETEC*) use type II systems for export.

In the past 5 years, the highly conserved, multicomponent type III secretion system has been found in many gram-negative bacteria that cause disease in animals and plants (8). This secretion system is responsible for transporting effector molecules directly from the cytoplasm to the cell surface, where they interact with mammalian cells and modify host cell proteins (13). This one-step secretion process is reminiscent of the mechanism used by type I systems (Figure 1). The genes that encode many components of type III systems are homologous to those that encode flagellar export machinery in both gram-negative and gram-positive bacteria (Table 1) (22-24). Indeed, these two systems share many structural and functional features. The differences reside at the outer membrane. Flagellar components pass through an outer ring structure that is part of the flagellum itself (23), whereas pathogenic effector molecules traverse the outer membrane through a channel protein that is homologous to those used in type II secretion systems (20,25). While our discussion of type III secretion systems will focus primarily on those used by human pathogens, many characteristics are common among systems found in plant pathogens and bacteria that produce flagella.

Type III Systems Secrete Effector Proteins Upon Contacting Host Cells

In contrast to the secretion process in type I and type II systems, type III secretion is triggered when a pathogen comes in close contact with host cells (18,19,26,27), and hence, has been called contact-dependent secretion (28). Temperature, growth phase, and salt conditions are environmental cues known to induce synthesis of the secretion apparatus and effector molecules in various pathogens (29-31). When the pathogen comes into close contact with tissue culture cells, effector molecules move to the external surface of the bacterium, sometimes forming appendages suggestive of flagellae (18). In some cases, the bacterium binds to the host cells and these molecules are delivered into the host cell (32). The effector molecules cause changes in host cell function, which facilitate the pathogen's ability to survive and replicate (15-17,33).

How Pathogens Use Type III Secretion Systems in the Host

The best studied bacterial pathogens that use type III secretion are *Yersinia pestis*, which causes plague, and a number of enteropathogens. Although these various enteropathogens (*Yersinia spp.*, *Salmonella spp.*, *Shigella spp.*, and EPEC) cause diarrhea and, in some cases, systemic disease, they produce distinct syndromes because their secreted proteins target different host cells and molecules (Table 1) (34).

Yersinia spp. use their effector molecules to destroy key functions of immune cells and render them innocuous (35). When these bacteria bind to tissue culture cells, approximately 10 different effector molecules are secreted (13) and at least three are injected into cells (27,36-38). Two of these injected molecules, YopE and YopH, modify macrophage proteins and destroy the cells' abilities to engulf and kill bacteria (16,39). During the course of disease, immune cells are presumably neutralized by these effector molecules, which enables *Yersinia spp.* to flourish in the reticuloendothelial environment.

While effector molecules in *Yersinia* destroy normal cellular functions, those from *Shigella spp.* and from one of the *Salmonella spp.* type III secretion systems, encoded by genes located in SPI I (Table 2), stimulate

Table 1. Function and location of components in Type III systems[a]

Yersinia (p)[b]	*S. typhimurium* SPI I(c)	*S. typhimurium* SPI II(c)	*S. flexneri* (p)	*EPEC* (c)	Flagella[c] (c)	Plant[d] (c)	Possible Function[e]	Possible Cellular Location[f]	Reference
YscN	SpaL	orf1	MxiB	SepB	FliI	HrpE	ATPase	cytoplasm, associated with inner membrane (I.M.)	8, 43, 63
LcrD	InvA	orf9	MxiA	SepA	FlhA	HrpO	structural/ regulatory	I.M.	28, 43, 63
YscR	SpaP	orf5	Spa24		FliP	HrpT	structural	I.M.	8, 43
YscS	SpaQ	orf6	Spa9		FliQ	HrpU	"	"	28, 43
YscT	SpaR	orf7	Spa40		FliR	HrpC	"	"	8, 43
YscU	SpaS	orf8	Spa40		FhlB	HrpN	"	"	8, 43, 63
YscC	InvG	orf11	MxiD	SepC		HrpA	structural/ channel forming	outer membrane (O.M.)	28, 43, 63
YscJ	PrgK	orf10	MxiJ	SepD	FliF	HrpI	lipoprotein/ structural	O.M.	8, 28, 43, 63
VirG							"	"	52
YscD							essential, but unknown	I.M.	48
YscE							"	unknown	51
YscF	PrgI		MxiH				needed for YopB and D secretion	"	28, 51
YscG							essential, but unknown	equally distributed between membrane and soluble fractions	48
YscI							"	unknown	51
YscK							"	"	51
YscL						HrpF	"	"	8
YscO	SpaM	orf2	Spa13				unknown	"	12, 28, 43
YscP	SpaN	orf3	Spa32				"	"	12, 28, 43
YscQ	SpaO	orf4	Spa33		FliN	HrpQ	SpaO needed for secretion of Sips (see below)	Spa O: secreted	8, 28, 43
YopB	SipB		IpaB				homology to pore forming toxins/ delivery of effector molecules to host cells	secreted/targeted to host cell	26, 28, 55

[a]Listed are names of proteins in type III secretion systems
[b]p-proteins are encoded by genes on a plasmid; c-proteins are encoded by genes on the chromosome.
[c]Protein names are those from the *S. Typhimurium* flagella; for list of flagellar protein names from other bacteria, see ref. 23.
[d]Protein names are those from the plant pathogen *Pseudomonas solanacearum*; for a list of components of type III secretion systems in other plant pathogens, see ref. 8.
[e]These are the possible functions for these factors in *Yersinia*, unless noted.
[f]These are the possible locations for these factors in *Yersinia*, unless noted.

Table 1. Function and location of components in Type III systems[a] (continued)

Yersinia (p)[b]	*S. Typhimurium* SPI 1(c)	SPI II(c)	*S. Flexneri* (p)	EPEC (c)	Flagella[c] (c)	Plant[d] (c)	Possible Function[e]	Possible Cellular Location[f]	Reference
YopD							delivery of effector molecules to host cells	secreted	55
	SipD		IpaD				"	secreted	26
SycD	SicA		IpgC				chaperone for YopB and D/ IpaB and IpaC in *Shigella*	cytoplasm	56
SycE							chaperone for YopE	"	56
SycH							chaperone for YopH	"	56
YopN	InvE		MxiC				regulatory: cell-contact sensing pathway	O.M./secreted	28
LcrG							"	cytosol/I.M.	59, S. Straley pers. comm.
LcrQ							regulatory	cytoplasm/ secreted	54
VirF	InvF		MxiE			HrpB	regulatory: temperature	cytoplasm	28
YopE							effector molecule: depolymerizes actin anti-phagocytic	host cell	27
YopH							effector molecule: tyrosine phosphatase anti-phagocytic	"	36
	SipC		IpaC				effector molecule: induces entry into epithelial cells	secreted	17, 28
				EspB (EaeB)			effector molecule: induces AE lesions		33, 63

[a]Listed are names of proteins in type III secretion systems
[b]p-proteins are encoded by genes on a plasmid; c-proteins are encoded by genes on the chromosome.
[c]Protein names are those from the *S. Typhimurium* flagella; for list of flagellar protein names from other bacteria, see ref. 23.
[d]Protein names are those from the plant pathogen *Pseudomonas solanacearum*; for a list of components of type III secretion systems in other plant pathogens, see ref. 8.
[e]These are the possible functions for these factors in *Yersinia*, unless noted.
[f]These are the possible locations for these factors in *Yersinia*, unless noted.

Table 2. Characteristics of several pathogenicity islands

Organism	Name	Location	Borders	Stable ?	Foreign origin G+C: % island/ % chromosome	Functions	Size	Ref.
Uropathogenic *E. coli* 536	Pathogenicity island I, Pai I	*selC*[a], 82'	•16 bp direct repeats, derived from *selC* • shared motif with Pai II repeats	no	•direct repeats •absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin I	70 kb	3, 82, 84
	Pai II	*leuX*[a], 97'	•18 bp direct repeats, derived from *leuX* • shared motif with Pai I repeats	no	•direct repeats •absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin II •prf (fimbriae: adherence to host cells) •transcriptional activators of chromosomal genes	190 kb	3,4, 67, 82, 84
Uropathogenic *E. coli* J96	Pai I	near *pheV*[a], 64'			•absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin I •psp (fimbriae: adherence to host cells) •IS element sequences •R plasmid sequences •P4 phage sequences	>170 kb	96
	Pai II	*pheR*[a], 94'	135 bp imperfect direct repeats	no	•direct repeats •absent from normal fecal and laboratory strains of *E. coli*	•α-hemolysin II •prs (fimbriae: adherence to host cells) •cytotoxic necrotizing factor type 1 •IS element sequences •P4 phage sequences •OmpR homolog	106 kb	4, 96, 97
Entero-pathogenic *E. coli* (EPEC)	Locus of enterocyte effacement, LEE	*selC*[a], 82'	no repeats or IS elements found	yes[b]	•G+C: 39%/51% •not present in closely related, non-AE-producing bacteria	•mediates formation of AE lesions •type III secretion system	35 kb	63, 83
Salmonella typhimurium	*Salmonella* pathogenicity island 1, SPI 1	between *fhl* and *mutS*, 63'	no repeats or IS elements found in *S. typhimurium*; IS3 on one border in certain *Salmonella* serotypes	yes[b,c]	•G+C: 42%/52% •absent from *E. coli*	•invasion into cultured epithelial cells •type III secretion system	40 kb	28, 68

[a]tRNA gene
[b]apparently
[c]unstable in certain serotypes
[d]additional information received in personal communication with M. Stein
[e]Censini S, et al., 1996, submitted for publication

Table 2. Characteristics of several pathogenicity islands (continued)

Organism	Name	Location	Borders	Stable ?	Foreign origin G+C: %island/ %chromosome	Functions	Size	Ref.
	SPI 2	between *ydhE* and *pykF*, 31'		yes[b]	•G+C: 45%/52% •absent from *E. coli*; conserved among *Salmonella*	•type III secretion system	40 kb	43
	Salmonella induced filament gene A, *sifA*	*potB/ potC*	14 bp direct repeats	yes	•G+C: 41%/52% •direct repeats •absent in *E. coli*; conserved among *Salmonella*	•required for formation of structures associated with *Salmonella*-associated vacuoles within epithelial cells	1.6 kb	69[d]
Yersinia pestis	Ability to adsorb exogenous pigments, Pgm	*phoE*	2.2 kb direct repeats (=IS100)	no	•G+C: hemin storage region 47%/46-50%; yersiniabactin receptor/ iron-regulated protein region 56-60%/46-50% •direct repeats	•hemin and congo red binding •pesticin sensitivity •iron acquisition •growth at 37 C in defined medium	102 kb	70, 71
Helicobacter pylori	Cytotoxin-associated gene region, Cag	*glr*	•31 bp direct repeats, derived from glutamate racemase gene •IS605 on one end	see text	•G+C: 35%/38-45% •IS elements •not present in type II strains	•induction of IL-8 secretion •homologues to membrane-associated proteins: environmental sensors, translocases, permeases, pilus and flagella assembly proteins •IS elements	40 kb	e
Vibrio cholerae O139	*otnA otnB*	*rfb*	flanked by two different IS elements		•IS elements •not present in *Vibrio cholerae* O1 El Tor	•capsule and O antigen synthesis (by homology)	35 kb	93, 98
Listeria monocytogenes		between *prs* and *ldh*	No IS elements found	yes[b]	•not present in several nonpathogenic species	•escape from vacuole •intra-/inter-cellular spread	9.6 kb	99

[a]tRNA gene
[b]apparently
[c]unstable in certain serotypes
[d]additional information received in personal communication with M. Stein
[e]Censini S, et al., 1996, submitted for publication

cells to perform functions in addition to those in their usual repertoires. Studies in epithelial tissue culture systems show that these bacteria induce their own entry into normally nonphagocytic cells by using effector molecules secreted by their type III systems (28,40). During the course of disease, *Shigella spp.* enter and replicate in the mucosal epithelial cells of the large intestine, while *Salmonella spp.* gain entry into the peritoneal cavity by passing through the epitheloid-like M cells in the small intestine (41). In the murine model for typhoid fever, *S. typhimurium* that are defective in this secretion system are attenuated for infection when administered orally, but not intraperitoneally (42). Presumably this attenuation is a consequence of reduced entry into the M cells in the small intestine, a barrier that is bypassed by intraperitoneal delivery.

Although neither its effector molecules nor target host cells have been identified, a second type III secretion system, encoded by genes located in SPI II (Table 2), has been described in *S. typhimurium*, on the basis of sequence homology (43). The genes in SPI II, in contrast to those in SPI I, are required for systemic disease regardless of the route of infection (43,44). Presumably the factors encoded in SPI II act after the bacteria have crossed the epithelial barrier of the small intestine.

Although the functions and sites of action of the secretion systems differ among these enteric pathogens, effector molecules from one system can be secreted by other systems, provided the appropriate chaperones are present. Such heterologously expressed effector molecules can induce the same cellular response as when expressed from their native systems. For instance, an effector molecule from *Yersinia* that causes actin depolymerization has the same effect on tissue culture cells when secreted from *Salmonella* (45). Likewise, proteins of *Shigella* and *Salmonella* involved in bacterial uptake into cultured epithelial cells are functionally interchangeable (46).

The observation that *Salmonella spp.* have two contact-dependent systems that function at distinct stages to cause disease raises several interesting questions. Why do *Salmonella spp.* need different type III secretion apparatuses when it is clear that effector molecules can be secreted from heterologous systems? Could one suffice if the two sets of effector molecules were expressed at appropriate times during the course of infection? Alternatively, do these two sets of effector molecules need to be delivered to different target cells in a specific manner, which is only possible with distinctly customized machinery? Answers to these questions will illuminate issues about both the course of salmonellosis and the basic mechanics of the secretion apparatus.

The Nuts and Bolts of Type III Secretion

The type III secretion apparatus in *Yersinia spp.* has been the most intensively investigated. However, this work has been done in three *Yersinia spp*; thus several proteins have been shown to be essential for effector molecule secretion in one species but have not yet been examined in others. Our analysis of these studies assumes that proteins essential in one will play a similar role in all (Table 1).

One essential feature of any secretion system is that energy must be provided to move molecules through the membrane (14). Only one protein in the system, YscN, has been shown to hydrolyze ATP and thus is a likely candidate for generating energy to drive secretion (47). YscN is predicted to be a cytoplasmic protein, closely associated with the inner membrane.

Several proteins essential to secretion including LcrD, YscD, R, S, T, and U, are known or predicted to reside in the inner membrane (10,12,48-50). At the outer membrane, only one protein, YscC (48), and two lipoproteins, YscJ and VirG (51,52), appear essential for proper secretion. The roles and subcellular locations are not known for several more essential proteins, YscE, F, G, I, K, and L (9,48,51). How all of these proteins interact with one another to form the secretion apparatus is not yet understood. It is clear, however, that correct assembly of the apparatus is required not only for secretion, but also for normal synthesis of effector molecules (47,48). If one component of the export machinery is missing, production of the effector molecules is altered. This

feedback regulation also occurs in systems that produce flagella (53). How it works in *Yersinia* is under investigation; thus far, only one protein, LcrQ, has been implicated (54).

Two proteins, YopB and YopD, are loosely associated with the outer membrane (55) and are crucial for efficient delivery of effector molecules into target cells. These two proteins use the type III secretion system to reach the bacterial cell surface. Without YopB, which has homology to pore-forming toxins (55), and YopD, effector molecules are secreted but not efficiently internalized by host cells; thus, their activities on host cells are severely abrogated (27,36). Presumably, YopB and YopD form a pore in the mammalian cell through which effector molecules pass.

Several proteins, called chaperones, play critical roles in secretion by binding to effector molecules in the bacterial cytoplasm. Chaperones have several proposed functions (56-58). Chaperone binding may stabilize and prevent proteins from folding into conformations that are impossible to secrete. Alternatively, as has been shown for *Shigella*, they may prevent effector molecules from improperly associating with one another before secretion (58). Lastly, chaperones may deliver molecules to the secretion apparatus.

In addition to the feedback regulation mentioned above, the synthesis of and secretion from the *Yersinia* type III system is regulated by two networks that respond to environmental cues (29,35). A temperature-sensing network induces synthesis of the apparatus at 37°C and includes VirF and YmoA (35). A host cell-sensing network increases both synthesis of and secretion from the type III system when *Yersinia* binds to target cells. This regulatory system is called the low-calcium response network—low calcium presumably mimics some signal generated by cell contact—and includes YopN, LcrG, and LcrQ (32,54,59,60). YopN localizes to the outer membrane, where it senses cell contact and transduces this signal to the cytoplasm by an unknown mechanism (60). The role of LcrG has not yet been elucidated. LcrQ functions as a repressor of the Yops. When *Yersinia* comes into close contact with host cells, LcrQ is secreted from the cytoplasm through the type III secretion system. This lowers the intracellular concentration of LcrQ and results in an increase in synthesis and secretion of the Yops (32). Flagella synthesis is controlled in a similar manner (61).

Many of the structural components of the *Yersinia* system have homologues in *Shigella*, *Salmonella*, and *EPEC* (Table 1). A comparison of proteins found in each system shows that certain core structural components are present in all type III apparatuses, whereas others may exist in only one or a subset. These differences may be due to particular functions of each system.

Several studies have examined whether structural components from different bacteria are interchangable. In general, core constituents from *Shigella* and SPI I of *Salmonella*, which both facilitate bacterial uptake by epithelial cells, are interchangable with one another, but not with those in the *Yersinia* system (7,62). These results may be due to the observation that factors from *Shigella* and SPI I of *Salmonella* are predicted by sequence homology to be more structurally similar to each other than to those in *Yersinia* (7). Alternatively, some of the regulatory cues for secretion and assembly may be different for *Yersinia* than for *Salmonella* and *Shigella*.

Most proteins in the type III secretion systems, including effector proteins, regulatory proteins, structural proteins, and chaperones (Table 1), are encoded by genes that belong to several large operons, which are clustered together (7,9,12,63). These operons are on plasmids in some species, and on the chromosome in others (Table 1). In some cases, such as in *Shigella* and SPI I of *Salmonella*, the order of the genes within operons and the arrangement of the operons with respect to each other are conserved (7). These observations suggest that type III systems were inherited en masse, and likewise, could be transmitted to other bacteria en masse. One can speculate that the acquisition of a type III secretion system could allow a bacterium to adapt to different environments or hosts. For instance, a new pathogen could perhaps arise if a skin-commensal bacterium were to acquire the means to penetrate and survive in the skin-

associated lymphoid tissue by obtaining a type III secretion system from an enteropathogen.

Where Do Type III Secretion Systems Come From?

It seems plausible that the original type III secretion system for virulence factors evolved from those for flagellar assembly (22,53). The bacterial flagellum exists in a wide range of eubacteria and some archaebacteria, which indicates that it probably emerged well before gram-negative bacteria, the hosts of the type III virulence factor secretion systems identified thus far.

Attempts to establish any of the known type III secretion systems as the progenitor have been fruitless. On the basis of degrees of homology among different type III systems and well-established evolutionary relationships between the bacteria, each organism can be ruled out as the source (64). For example, *Shigella* emerged from *E. coli* after *Salmonella* and *E. coli* diverged from a common ancestor; thus, *Shigella* cannot have provided the type III systems conserved in the *Salmonella spp*. Conversely, as *Shigella* type III apparatus sequences have a G+C content well below that of *Salmonella* and of the bulk of the *Shigella* chromosome, *Salmonella* could not have been the source of the *Shigella* genes (Table 2). Thus, the ability to secrete effector molecules by this mechanism seems to have been introduced independently into each of these bacteria.

Examination of homologous genes in the epithelial cell invasion loci of *Salmonella* and *Shigella* shows that some are highly conserved, while others display much lower levels of homology (65). Li and colleagues have found a relationship between evolutionary rate of change and subcellular location: genes encoding several secreted proteins are hypervariable in relation to genes encoding several proteins located in the bacterial inner membrane (65). In principle, hypervariability could reflect antigenic variation or adaptations to diverse host environments; however, neither of these explanations appears to pertain to the particular proteins examined (28,65).

Type III systems sometimes provide much of what distinguishes particular organisms from closely related, often nonpathogenic, species. As described above, many operons encoding type III secretion machinery are clustered. DNA sequence analysis has shown that these loci are often distinguishable from the bulk of the genomic DNA. The loci that are chromosomally located represent "pathogenicity islands" (66).

History and Definition of "Pathogenicity Islands"

The phrase "pathogenicity island" was first used to describe two large, unstable pieces of chromosomal DNA, unique to uropathogenic *E. coli*, that encode a number of genes required for virulence (3,4). Since its conception, the term has evolved to include regions of chromosomal DNA essential for pathogenicity that do not appear to "belong" (Table 2). Not all pathogenicity islands are genetically unstable, but each one shows an indication of foreign origin. These pieces of DNA are often missing in closely related, nonvirulent bacteria. Many pathogenicity islands differ from the bulk of the genome in G+C content and codon usage, and their borders are often marked by repeated sequences or insertion elements, which suggests that some kind of recombination event delivered them to the chromosome. Several encode multiple proteins that collaborate to confer a single, complex virulence property to the bacterial host.

The definition of pathogenicity islands includes chromosomal location. As such, the plasmid-borne type III gene clusters of *Yersinia* and *Shigella* do not qualify (Table 1). This seems somewhat arbitrary. Indeed, phages and a number of plasmids can easily insert into and excise from the chromosome. Similarly, many transposable elements replicate and function equally as well in the chromosome as on an extrachromosomal element. It seems to us that a block of apparently foreign genes found uniquely in pathogenic members of a genus and required for virulence is a more useful and relevent defining feature of a pathogenicity island than location. Thus, it makes sense to include the loci encoding type III secretion systems, regardless of whether they reside on a plasmid or chromosome. In the

discussion below, however, we adhere to the established definition that includes chromosomal location.

Pathogenicity Islands Contain Virulence Genes and Regulatory Elements

Pathogenicity islands also contain virulence genes other than those encoding type III secretion systems; a common theme appears to be inclusion of genes for secreted or cell surface-localized proteins such as hemolysins, fimbriae, and hemin-binding factors (Table 2). In fact, the similarities between pathogenicity islands extend further: examination of the large ones shows that many also contain genes that encode a secretion system and environmental sensors. They also can include proteins that regulate expression of genes that lie outside the pathogenicity island. For example, pathogenicity island II (Pai II) of uropathogenic *E. coli* contains genes that encode transcriptional activators of S-fimbrial genes that reside at a chromosomal locus remote from either of the known pathogenicity islands in this species (67).

Pathogenicity Islands Can Exist in Various Structural Types and Numbers Within a Bacterium

A single bacterial strain can harbor more than one pathogenicity island. *Salmonella* contains at least five: the gene clusters encoding the two type III secretion systems described above, *sifA* (see below), and two groups of genes that are activated by the two-component regulator, PhoP/PhoQ. These loci vary in size and complexity and reside at distinct chromosomal locations (43,68,69, and S. Miller, pers. comm.).

Pathogenicity islands themselves can be composed of distinct segments. For example, an unstable 102-kb region of DNA that encodes several traits important for virulence of *Y. pestis* appears to consist of several regions (70-72). One contains the hemin storage genes and has a G+C content similar to that of the bulk of the chromosome (R. Perry, pers. comm.); the other contains genes encoding the Yersiniabactin receptor and iron-regulated proteins and has a significantly higher G+C content (R. Perry, pers. comm) (73). Although the 102 kb region often deletes entirely, the two regions can also act independently. In some strains the chromosomal region containing the hemin storage genes spontaneously deletes from the chromosome at a significant frequency, while the Yersiniabactin receptor/iron-regulated protein region appears stable (72). Furthermore, only the Yersiniabactin receptor/iron-regulated segment is present in *Y. enterocolitica* (70).

Even more complex pathogenicity islands are harbored by strains of *Helicobacter pylori*, the causative agent of gastritis and peptic ulcer disease in humans. Strains of *H. pylori* have been divided into two classes: type I strains express the cytotoxin-associated gene A (CagA) antigen and induce secretion of the neutrophil attractant IL-8 by epithelial cells in vitro, while type II strains lack both of these properties. Patients with duodenitis, duodenal ulcers, and gastric tumors are most often infected by type I strains. Likewise, type I strains are more likely than type II strains to cause gastric injuries in murine model systems. Analysis of the chromosomal region that contains the *cagA* gene has shown that it is a pathogenicity island of approximately 40 kb of DNA, missing in type II strains, and that mutations in this region abolish IL-8 induction in gastric epithelial cell lines (Censini, S et al. A pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated factors. 1996; submitted for publication).

Different type I strains display considerable heterogeneity in the cag region (Censini, S et al. A pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated factors. 1996; submitted for publication). In some isolates, the cag region is interrupted by one or more insertion sequences. In a small number of strains, there is an additional 20-kb sequence that is also present in type II strains. Partial deletions of the cag region have been detected as well. Thus, the cag pathogenicity island appears to be undergoing dynamic changes in natural *Helicobacter* populations. Further study of the cag region may elucidate details of pathogenicity island

acquisition and help correlate regions of the pathogenicity island with disease symptoms in the murine model system.

Since its establishment, the definition of pathogenicity islands has evolved to include genetic regions that are neither large nor complex; single genes of apparently foreign origin can also be inserted into chromosomal DNA. *S. typhimurium* has recently been shown to contain such a gene, called *sifA*, which is required for formation of distinctive structures associated with *Salmonella*-containing vacuoles within epithelial cells and contributes to pathogenicity in the murine typhoid fever model system (Table 2) (69).

Possible Origins of Pathogenicity Island DNA and Mechanisms of Transfer and Insertion

While the sources of pathogenicity islands are unknown, their presence in a wide variety of organisms (Table 2) indicates that bacteria can acquire DNA despite multiple barriers to chromosomal gene transfer between species. The existence of "foreign" genomic DNA is particularly intriguing as sequence divergence is a major limitation to such transfers because it severely limits the potential for homologous recombination (74).

Although the identity of the vectors that transport pathogenicity islands from donor to recipient organisms is unknown, any number of mobile genetic elements are candidates. Clear evidence showing an extrachromosomal stage of a pathogenicity island is lacking; however, it is intriguing that the G+C contents of the *Helicobacter* pathogenicity island and plasmid are similar to each other and distinct from the chromosome (Censini, S et al. A pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated factors. 1996; submitted for publication). Phages, plasmids, transposons, integrons, and even free DNA carry genes from one organism to another (74). Indeed, many phages and plasmids contain virulence genes, and often these loci seem alien to the bacterial species in which they reside (75). Furthermore, the animal host environment may be particularly conducive to DNA transfer events. For example, the phage that encodes cholera toxin infects *V. cholerae* more efficiently within the gastrointestinal tract of a mammalian host than under laboratory conditions (76).

Pathogenicity islands insert into the chromosome by an unknown mechanism; however, the existence of insertion elements and repeated DNA motifs at the boundaries of several pathogenicity islands suggest that recombination events are involved. Recombination has recently been shown to be the major factor governing the divergence of a group of *E. coli* strains and is a significant driving force for evolution (74,77). Although the genetic material comprising pathogenicity islands may be introduced into a new host organism in a single step, the events that generate known pathogenicity islands are unlikely to be simple insertions, because DNA rearrangements and alterations are common in the flanking chromosomal regions.

The identification of several tRNA genes as insertion sites for pathogenicity islands is also notable, although the significance of this remains obscure (Table 2). tRNA genes serve as integration sites for a variety of prokaryotic genetic elements, including several phages and transmissible plasmids (78-80). Perhaps the conserved portion of tRNA genes is a useful landmark for mobile genetic elements that inhabit a variety of prokaryotic hosts; in addition, the regions of dyad symmetry characteristic of all tRNA genes could serve as binding sites for enzymes involved in recombination.

Relative Advantages of Instability and Stability

Some pathogenicity islands can excise from the chromosome and are apparently lost from the host bacterium (Table 2) (3,4,70). Such instability may provide an adaptive advantage. Virulence properties may be dispensable at certain stages of infection, and the coordinated loss of these characteristics could be beneficial to the bacterium. Indeed, expression of particular genes at inappropriate times can be detrimental to bacterial pathogens (81). Natural selection of strains with deleted

virulence regions can occur in specific environments: diabetic patients are more susceptible to uropathogenic *E. coli* strains not exhibiting virulence phenotypes (4).

On the other hand, particular virulence traits could provide a continual adaptive advantage, resulting in stable pathogenicity islands. The "foreign nature" of pathogenicity islands may reflect this benefit; foreign DNA may be actively maintained in the population because of its limited ability to recombine with related organisms (75). It is not clear whether stable islands exist because of a divergence of sequences at the borders (for example, repeated elements that are no longer recognizable as such), an integration mechanism completely different from that of the unstable islands, or because of a lack of excision machinery.

Deletion of pathogenicity islands can affect gene expression by altering the chromosomal site of insertion and by removing the genes contained in the island. The locus of enterocyte effacement (LEE) comprising the type III secretion apparatus in *EPEC* and pathogenicity island I (Pai I) of uropathogenic *E. coli* both insert at the selenocysteine tRNA (*selC*) gene (82,83). The presence of Pai I does not interfere with *selC* expression. However, excision from the chromosome appears to occur by a recombination event between the repeated sequences in *selC* and the distal end of Pai I. This recombination event results in deletion of part of the tRNA gene and inhibits anaerobic growth due to the cell's inability to produce formate dehydrogenase, which contains selenocysteine (84). Similarly, in Pai II deletion strains, the *leuX* tRNA gene at the insertion site is disrupted, which interferes with its ability to act as a global regulator of several virulence factors that lie outside the pathogenicity island (84).

Bacteria may be able to have the best of both the stable and the unstable worlds. The phenotypic loss of the enteroinvasive *E. coli* and *Shigella flexneri* virulence plasmids is sometimes due to plasmid insertion into a specific site on the chromosome (85,86). After integration, excision also can be detected; strains containing precisely excised plasmids regain virulence, while those with imprecisely excised plasmids remain noninvasive. Several of the *Y. pestis* plasmids exhibit similar behavior (87-89). Integration, which simultaneously maintains these plasmids in the bacterial genome while downregulating their genes, may represent a sophisticated adaptation to the requirements of different environments or may represent? stages in the bacterial life cycle. Furthermore, integration and excision remind us that strict definitions of "chromosomal" versus "plasmid-borne" do not always reflect biological reality.

Foreign DNA Is a Significant Determinant in Recently Emerged Pathogens

Horizontal gene transfer has been invoked to explain the origin of enterohemorrhagic *E. coli* (*EHEC*), which causes hemorrhagic colitis and hemolytic uremic syndrome (90). Like *EPEC*, *EHEC* induces striking morphologic changes–called attaching and effacing (AE) lesions–in host cells of the small intestine; however, unlike *EPEC*, *EHEC* contains Shiga-like toxins. After analyzing the genetic relationships between many *E. coli* strains, Whittam and colleagues proposed that *EHEC* arose from an *EPEC*-like progenitor strain, which then acquired the prophage-encoded Shiga-like toxins, thus becoming a new pathogen that expresses both sets of traits (90).

The new epidemic *Vibrio cholerae* O139 strain may have emerged after acquisition of a pathogenicity island (91). Although it appears that *V. cholerae* O139 arose from a strain of the same serotype (O1) that is causing the ongoing cholera pandemic (O1 El Tor) (92), *V. cholerae* O139 contains an additional piece of DNA that replaces part of the O antigen gene cluster of O1 strains (93). The inserted DNA contains open reading frames homologous to proteins involved in capsule and O antigen synthesis, two factors that distinguish O139 and O1 El Tor, and are thought to mediate activities important for pathogenesis and evasion of immunity.

Benefiting From Information About Type III Secretion and Pathogenicity Islands

Pathogenic bacteria continue to exhibit impressive genetic flexibility and exchange and use these abilities to adapt to varied

types of lifestyles within host organisms. It should be possible to use the information from studies of pathogenicity islands and type III secretion systems in the ongoing characterization of bacterial infections. When a novel pathogen is isolated, it may be worthwhile to identify chromosomal regions specific to it by comparing the gross genomic structure with that of related organisms, which may provide a shortcut to the identification of virulence genes. Likewise, simple molecular techniques can determine whether bacteria contain type III secretion systems, because genes encoding particular components are highly conserved; perhaps this procedure should be part of our standard investigative arsenal as well.

Our knowledge of type III secretion systems may yield therapeutic benefits. The contact-dependent systems appear to reside in pathogenic and not in commensal bacteria. If this observation reflects a general truth, antibiotics that target type III systems may specifically attack intruding bacteria and spare the normal flora; therefore, these antibiotics might produce minimal side effects. In addition, type III secretion systems will provide new targets for therapeutic drugs that might not kill the bacterium but would inhibit the disease process. We also may be able to exploit this secretion system, by using appropriately attenuated bacteria, to prime immunity. Chimeric proteins—fusions between effector and other proteins—can be secreted in large quantities by the type III secretion machinery and be internalized by host cells; furthermore, these proteins can elicit an antibody response in mice (38,94).

The virulence traits of pathogenic microorganisms at the genetic and molecular level remind us that bacterial pathogenicity does not arise by slow adaptive evolution but by "quantum leaps" (95); therefore, microbes can acquire complete systems that radically expand their capabilities to exploit and flourish in different host environments. History teaches us that infectious diseases may change in severity and form, but they will not simply disappear. Microrganisms are, after all, survivors, and there is no escaping our destiny—to be consumed by them in one way or another. However, the more we learn about the microbial tactics of survival, the longer we forestall this destiny.

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