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Process of Protein Transport by the Type III Secretion System

Partho Ghosh*

Department of Chemistry & Biochemistry, University of California—San Diego, La Jolla, California

INTRODUCTION

Many bacteria live in close relationships with host organisms in ways that may be beneficial, neutral, or detrimental to their hosts. The course of these relationships is often guided by proteins that are secreted by bacteria and that interact with specific host cell targets, with these interactions typically resulting in modulation of host cell behavior and response. A number of bacteria modulate host cell traits not only by secreting proteins into the extracellular environment but also by translocating them directly into the interior of host cells. One of the most widespread ways for translocating bacterial proteins into host cells is through the type III secretion system (TTSS). This system is found exclusively among gram-negative bacteria and is responsible for the transport of proteins across the inner bacterial membrane, the peptidoglycan layer, and the outer bacterial membrane, as well as across host cell barriers such as the plasma membrane and in some instances the plant cell wall, into the host cell interior.

In the great majority of cases, proteins delivered into host cells by the TTSS, termed effectors, contribute to a pathogenic relationship between bacterium and host. The TTSS has been

* Mailing address: Department of Chemistry & Biochemistry, 0375, 9500 Gilman Dr., University of California—San Diego, La Jolla, CA 92093-0314. Phone: (858) 822-1139. Fax (858) 534-7042. E-mail: pghosh@ucsd.edu.

TABLE 1. Proteins of the TTSS apparatus

Structure	TTSS protein	Sequence-related flagellar protein	Functionally related flagellar protein
Needle	*Yersinia* YscF, *Shigella* MxiH, *Salmonella* Prgl, *E. coli* EscF		FliC
Needle length determinant	*Yersinia* YscP, *Shigella* Spa32, *Salmonella* InvJ		FliK
Filament	*E. coli* EspA		
Pilus	*P. syringae* HrpA		
Inner membrane proteins	*Yersinia* YscV, *Salmonella* InvA, *E. coli* EscV	FlhA	
	Yersinia YscU, *Salmonella* SpaS, *E. coli* EscU	FlhB	
	Yersinia YscR, *Salmonella* SpaP, *E. coli* EscR	FliP	
	Yersinia YscS, *Salmonella* SpaQ, *E. coli* EscS	FliQ	
	Yersinia YscT, *Salmonella* SpaR, *E. coli* EscT	FliR	
ATPase	*Yersinia* YscN, *Salmonella* InvC, *E. coli* EscN, *P. syringae* HrcN	Flil	
ATPase-associated proteins	*Yersinia* YscQ, *Shigella* Spa33	FliN, FliM	
	Yersinia YscL *Yessinia* YscK, *Shigella* MxiK	FliH	
Inner membrane rings	*Salmonella* PrgH, *Shigella* MxiG		
	Salmonella PrgK, *Shigella* MxiJ, *Yersinia* YscJ	FliF	
Outer membrane rings (secretins)	*Yersinia* YscC, *Salmonella* InvG, *P. syringae* HrcC, *E. coli* EscC		

identified in many animal pathogens, such as *Yersinia* spp., *Salmonella* spp., *Shigella* spp., enteropathogenic and enterohemorrhagic *Escherichia coli* (e.g., O157:H7), *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Bordetella* spp., and *Chlamydia* spp. (26, 90, 102, 108, 116, 147, 150, 184, 204, 248, 253). Certain bacteria, such as *Salmonella enterica* serovar Typhimurium, *Yersinia pestis*, and *Y. enterocolitica*, have been discovered to encode more than one TTSS (80, 103, 211). The TTSS has also been identified in plant pathogens, such as *Pseudomonas solanacearum*, *P. syringae*, *Erwinia* spp., and *Xanthomonas* spp. (21, 74, 186, 236). The distribution of the TTSS is not limited exclusively to pathogens; certain endosymbiotic bacteria also encode TTSSs (45, 46, 75).

The arsenal of effectors translocated into host cells varies widely across bacteria, being tailored to fit the life-style demands of a particular bacterium within a particular host. In contrast, many components of the TTSS apparatus used to transport effectors are conserved in sequence among bacteria, and some of these components are also functionally interchangeable. For example, the *Yersinia* effector YopE is secreted into the extracellular medium by the TTSS of *S. enterica* serovar Typhimurium, *Xanthomonas campestris*, and *Erwinia chrysanthemi* (5, 195, 197). YopE can also be translocated into HeLa cells by *S. enterica* serovar Typhimurium, as determined by the characteristic rounding up of HeLa cells brought about by YopE action (195). Likewise, the *Yersinia* TTSS is capable of secreting the *P. syringae* effectors AvrB and AvrPto (5). The efficiency of secretion of these heterologous substrates by *Yersinia* was shown to be similar to that of the autologous *Yersinia* substrate YopE (5, 31). However, limits to functional complementarity are also observed. As evidence of this, the *S. enterica* serovar Typhimurium TTSS inner membrane protein InvA (Table 1) is complemented, at least partially, by its *S. flexneri* homolog MxiA but not by its *Yersinia* homolog YscV (also called LcrD) (93). Therefore, while functional generalizations across different TTSSs are often informative, it should be kept in mind that there are limits to these generalities.

This review deals with the process of transport of effectors. It first discusses proteins of the TTSS apparatus that effector proteins are likely to encounter in their transit from the bacterial cytosol to the host cell interior and then deals with features of effector proteins that are important for transport, including the involvement of specific chaperone proteins. A number of excellent reviews covering other aspects of the TTSS are available (8, 20, 36–40, 83, 84, 88, 89, 109, 182, 187, 216).

TYPE III SECRETION VERSUS TRANSLOCATION

The biological purpose of the TTSS is to translocate effector proteins from the bacterial cytosol into host cells. However, effector proteins can also be secreted under appropriate laboratory conditions into the extracellular medium rather than into host cells. For example, one of the two TTSSs of *Yersinia*, the Ysc TTSS, can be made to secrete effector proteins into the extracellular medium when bacteria are grown at 37°C in medium containing a low concentration of calcium (35, 154, 184). The TTSS of *Shigella* can be made to do the same when bacteria are grown in the presence of the dye Congo Red (175). Interestingly, conditions for secretion by the two *Y. enterocolitica* TTSSs, Ysc and Ysa, differ (251). The process of secretion provides a highly tractable way to address many questions regarding the TTSS. However, in a number of ways, secretion has been observed to be dissimilar from translocation (23), indicating fundamental differences in the architecture or regulation of the TTSS apparatus under secretion and translocation conditions. The nature of these differences is not well characterized. In addition, secretion of effectors has been observed to occur not only through the TTSS but also through the flagellar apparatus (135, 251). The *Yersinia* phospholipase YpIA is secreted by both TTSSs present in *Yersinia* (Ysc and Ysa systems) as well as by the *Yersinia* flagellar apparatus (251, 252), and certain internal deletions in the *Salmonella* TTSS effectors SopE and SptP have been found to lead to secretion of these TTSS proteins through the flagellar apparatus (135).

In contrast to secretion, translocation is strictly dependent on the TTSS and does not occur through the flagellar apparatus (135). Translocation is triggered by an unknown mechanism by bacterial contact with host cells (196) and is more experimentally difficult to study than secretion. One of the major breakthroughs in studying translocation was the use of a host cell-activated reporter, a ~400-residue portion of *Bordetella pertussis* calmodulin-activated adenylate cyclase (Cya) (214). The enzymatic function of Cya is activated only in mam-

malian cells, due to the dependence of this enzyme on calmodulin. Other reporters specific to mammalian cells have been used as well, including a short protein sequence that is phosphorylated by mammalian kinases (52) and one that specifically binds biarsenical fluorescent reagents, such as FIAsH (29). FIAsH is not strictly a mammalian cell-specific reporter, but it is taken up more efficiently by mammalian cells than by bacterial cells (99). A potential problem in these experiments is that fusion proteins or peptides are not always innocuous and noninterfering reporters but sometimes give rise to unintended consequences for protein folding or stability. To avoid this problem, a number of researchers have examined translocation of untagged effectors, using biochemical separation to discriminate between host cell internalized (i.e., translocated) and intrabacterial pools of effectors (87, 129, 136, 196). This has generally relied on the use of detergents that lyse mammalian cells but not bacteria and on the detection of translocated proteins in the soluble fraction of lysed cells by Western blot analysis or immunofluorescence. Biochemical separation also has its potential shortcomings, centered mainly around whether various detergents (e.g., digitonin) provide the intended discrimination between translocated and intrabacterial effectors (167). Although results sometimes depend on whether a native or tagged version of an effector is used, the combination of reporter fusion and biochemical methods has generally proven powerful and illuminated many features of the type III secretion process.

TYPE III SECRETION SYSTEM APPARATUS

The TTSS apparatus is composed of approximately 20 to 25 different proteins. About half of these proteins are conserved in most type III systems. Most of the conserved TTSS proteins are also similar in sequence to proteins that make up parts of the basal body of the bacterial flagellum (2, 90, 109). This suggests a shared evolutionary history between the two systems. Based on phylogenetic evidence, it has been hypothesized that the TTSS system arose early in evolution through duplication of certain flagellar genes (165, 202). The two systems then seem to have evolved independently, with little or no genetic exchange. However, another analysis contends that the TTSS is as ancient as the flagellar apparatus and that they share a common ancestor, rather than the TTSS having evolved from the flagellar apparatus (96). Both the flagellar and type III systems have protein secretion in common. While the flagellum functions as a rotary motor powered by transmembrane ionic potentials (145), its assembly requires secretion of flagellar subunits in a process powered by a flagellar ATPase (65, 112). Protein transport by the TTSS also depends on an ATPase (63, 221, 245) as well as a transmembrane ionic potential (241).

Bacterial Surface Structures

The most prominent and functionally suggestive architectural feature of the TTSS is a needle-like or, in the case of phytopathogens, pilus-like structure that projects from the bacterial surface (Fig. 1A). These structures invite obvious comparison with the bacterial flagellum, but neither needle nor pilus subunit is related in sequence to the flagellum subunit (flagellin, FliC; Table 1). Nevertheless, just as the flagellum is thought to serve as a conduit for protein transport during flagellar assembly, the widely held view is that TTSS needles and pili serve as conduits for protein translocation between the bacterium and host cell.

Needles. Needle-like structures have been visualized by electron microscopy for *Salmonella*, *Shigella*, and *Yersinia* (19, 107, 120, 123, 130, 131, 221). The needles are straight, apparently rigid, and hollow, looking very much like a pipeline through which proteins are translocated into host cells. Most notably, needles are much shorter than flagella. The length of the needle has been reported to be 80 nm in *Salmonella* (130), 45.4 ± 3.3 nm in *Shigella* (221, 222), and 58.0 ± 10 nm in *Yersinia* (120), compared to the often 10 to 15 μm of flagella (233). Although most studies report a tight size distribution for needle length, indicating regulation over this feature, one study observed *Yersinia* needles up to 210 nm in length along with more normally sized needles of 60 to 80 nm (107). *Salmonella* has been reported to have 10 to 100 needles per bacterium (130), and similarly *Yersinia* has been reported to have 50 to 100 needles per bacterium (107). The number of needles in *Yersinia* is dependent on the growth medium, and *Yersinia* needles are observed to be fairly uniformly dispersed over the surface of the bacterial cell (107).

The *Yersinia* needle is formed by the small protein YscF (9 kDa) (Table 1) (107). The *Shigella* and *Salmonella* needles are composed of MxiH (9 kDa) and PrgI (9 kDa) (Table 1), respectively, which are related in sequence to YscF (both with ~26% identity to YscF) (107, 123, 131). MxiH polymerizes to form a helical superstructure, with ~5.6 subunits per turn and a helical pitch of 24 Å, as revealed by electron micrographic reconstruction combined with X-ray fiber diffraction analysis (34). This arrangement is highly reminiscent of the bacterial flagellar filament and hook, which is surprising since MxiH has no appreciable sequence identity to proteins of the flagellar filament (FliC, flagellin, 52 kDa) or hook (FlgE, 42 kDa).

Overexpression of MxiH leads to needles as long as 1 μm. Disruption of the *Shigella* protein Spa32 (33 kDa) leads to even longer needles, longer than 5 μm (146, 221, 222). Spa32 is not found in most other TTSSs but appears to be distantly related to the *Salmonella* protein InvJ (36 kDa), whose deletion also leads to micron-length needles (131). The long-needle phenotype of Spa32 and InvJ mutants is much like that reported for the flagellar protein FliK (42 kDa; Table 1), which provides control over the length of the flagellar hook structure in the hook/basal-body complex (161). However, neither MxiH nor InvJ appears to be related in sequence to FliK. Knowledge of the three-dimensional structures is required to ascertain whether a deeper relationship exists among these functionally similar proteins.

In *Yersinia*, YscP (50 kDa) sets the length of the needle. Remarkably, it has been found that the number of residues in engineered variants of YscP correlates linearly with needle length; the metric is 1.9 Å of needle length per YscP residue (120). These data suggest a model in which YscP is tethered at one end to the base of the TTSS apparatus and at the other end to the growing end of the needle. The needle is thought to keep growing until YscP is fully stretched (120). YscP is not

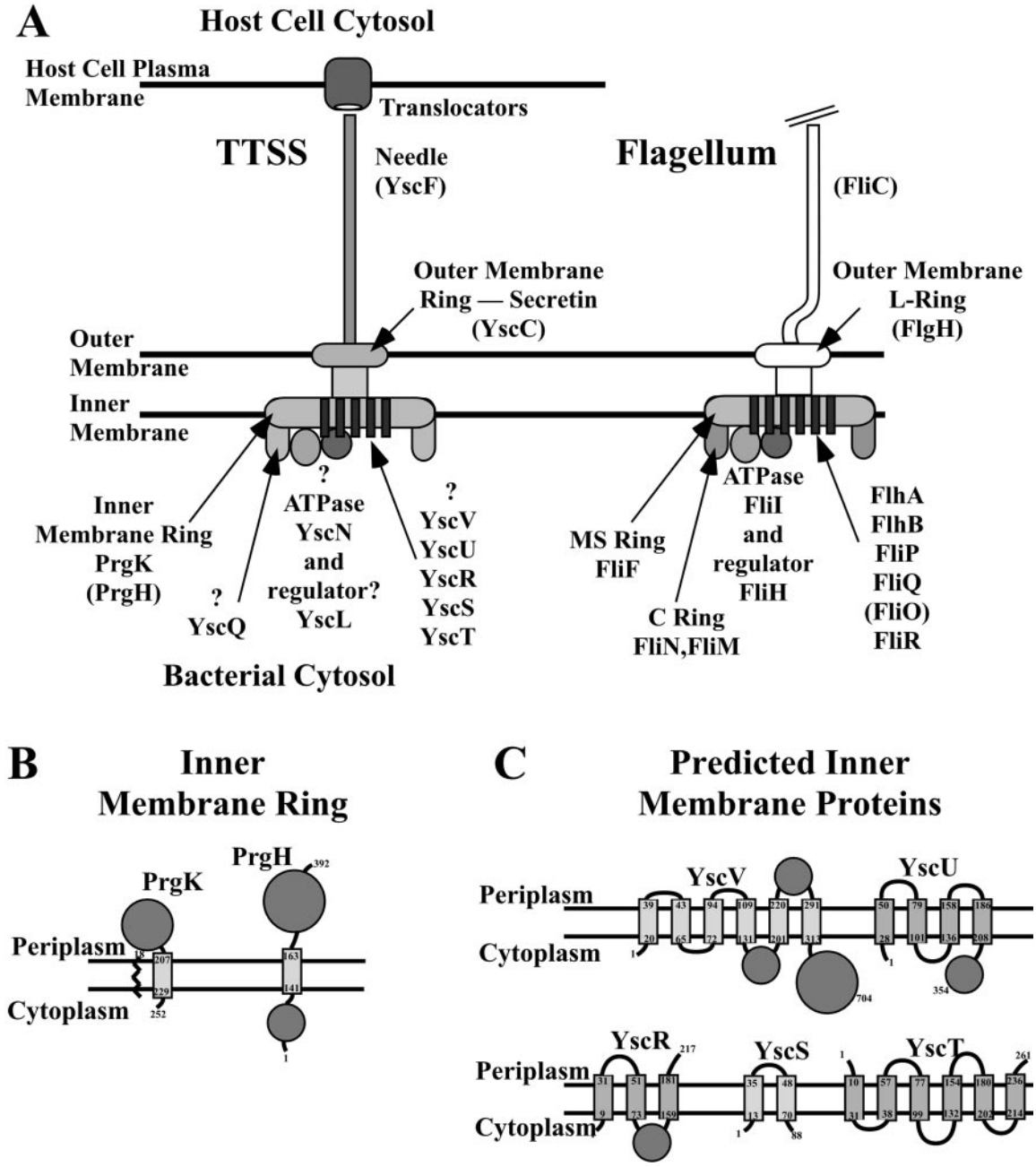

FIG. 1. The TTSS and the flagellum. (A) Schematic of the needle-containing TTSS apparatus (left), as found in bacterial pathogens of animals, and the flagellum (right). The TTSS needle is positioned on outer membrane and inner membrane rings, as is the flagellum. For the TTSS, proteins that are conserved in the flagellar apparatus are indicated without parentheses while those that are not conserved in the flagellar apparatus are enclosed in parentheses. Question marks indicate uncertain localization of proteins. *Yersinia* proteins are indicated for the TTSS, except for the inner membrane ring, for which *Salmonella* proteins are denoted. For the flagellar system, proteins that are conserved in the TTSS are indicated without parentheses and proteins that are not conserved in the TTSS are enclosed in parentheses. Effector proteins of the TTSS are thought to travel from the bacterial cytosol through the two rings and the needle and to cross into the host cell cytoplasm through pores formed in the host cell plasma membrane by TTSS translocator proteins. The TTSS needle is ~100-fold shorter than the flagellum. (B) Schematic of inner membrane ring components, as exemplified by *Salmonella* PrgK and PrgH. Putative transmembrane crossings are shown as rectangles, and cytoplasmic or periplasmic domains are shown as circles. The jagged line for PrgK indicates lipid acylation. (C) Schematic of predicted inner membrane proteins, with representation similar to that in panel B and exemplified by *Yersinia* proteins.

related in sequence to *Salmonella* InvJ, *Shigella* Spa32, or the flagellar protein FliK (131, 146, 221, 222).

While the external diameter of needles is estimated to be a spacious 60 to 130 Å (107, 130, 210, 221), electron micrographic reconstructions of negatively stained *S. flexneri* needles reveal an inner diameter of only 20 Å (19). Similar observations have been made on negatively stained *Y. enterocolitica* needles (107). Folded protein domains often have diameters of

20 to 30 Å, suggesting that if effector proteins were transported through the interior of the needle, they would need to be partially or fully unfolded, as discussed below.

The inner channel of the flagellum is also found to have a diameter of 20 Å, as deduced from an atomic model of the flagellar filament (203, 250). This model reveals that the inner surface of the flagellar channel consists mainly of polar residues, which may be advantageous for transport of unfolded proteins through the flagellum during its assembly. A polar surface would prevent association between the filament walls and exposed hydrophobic residues of potentially unfolded proteins in transit through the flagellum.

Filaments. A variation on the needle is seen in pathogenic *E. coli* strains, which have a long filament that appears to be attached to the end of the needle distal to the bacterial cell. The *E. coli* needle is composed of the protein EscF (8 kDa), which is related in sequence to YscF (23% identity), and the filament is composed of the protein EspA (~20 kDa) (244), which is not found in most TTSSs (Table 1). An EspA-related protein, SseB, has been identified in the SPI-2 TTSS of *S. enterica* serovar Typhimurium (106), although the existence of SseB filaments has not been demonstrated.

EspA filaments are helical tubes with an outer diameter of ~120 Å and an inner diameter of 25 Å, which is as constricting as the inner diameter of needles (48). The presence of a central channel in the filament and the observation that the filament is required for delivery of the EspB protein suggest that the filament serves as a continuation of the needle for protein transport (59, 124). Consistent with this hypothesis, the needle subunit EscF is found to bind the filament subunit EspA (48, 244). Filaments can be as long as ~700 nm but are most frequently between 40 and 140 nm (with an average of ~90 nm) (47, 210), suggesting that the length of the filament is not under tight control. The filament may need to be lengthy for pathogenic *E. coli* to attach to host intestinal epithelial cells through the thick, overlaying glycocalyx layer.

Pili. Rather than having a needle or filament, bacterial phytopathogens have a pilus-like structure termed the Hrp pilus. The Hrp pilus has an 80-Å outer diameter, similar to that of needles, but its length of ~2 μm is much greater than that of wild-type needles (192, 237). Additionally, pili appear to be flexible whereas needles appear to be rigid. The length of the Hrp pilus is probably an important attribute in traversing the thick (>100-nm) cell wall of plant cells to reach the cytoplasmic membrane. Unlike needles, which are dispersed over the entire bacterial surface, the Hrp pilus of *Ralstonia solanacearum* is seen to emanate only from one pole (237). In *P. syringae*, the small protein HrpA (11 kDa) is necessary and sufficient to form the Hrp pilus (Table 1) (191, 192). The sequence of HrpA varies among bacterial pathovars. For example, HrpA from *P. syringae* pv. tomato strain DC300 has only ~30% identity to HrpA from *P. syringae* pv. syringae or phaseolicola. HrpA from these pathovars have ~18 to 21% sequence identity to YscF, which is slightly but not substantially lower than the identity of MxiH, PrgI, or EscF to YscF. It will be interesting to see whether needle and pilus subunits are structurally related despite their low sequence identity (57).

Salmonella appendages. Surface appendages much larger than needles and filaments have been observed for invading *S. enterica* serovar Typhimurium (94). These appendages have diameters of ~60 nm and lengths of 0.3 to 1.0 μm. Unlike needles, the existence of these appendages does not depend on InvA, a conserved putative inner membrane TTSS protein; however, like needles, it does depend on certain conserved TTSS components, such as the TTSS ATPase InvC and the secretin InvG (Table 1) (94). The relationship of these surface appendages to TTSS needle-like structures is unclear.

Conduits for translocation? The tube-like morphology of type III needles, filaments, and pili suggests an obvious and appealing mechanism for protein transport involving transit through the central, hollow channel of these structures. Consistent with this model, effector secretion requires assembly of needles (131). Although there is no direct experimental evidence for protein transport through the center of the needle, evidence does exist for egress of effectors from the distal ends of Hrp pili (117, 138). Electron micrographic images of *P. syringae* in the process of secreting the effectors AvrPto and HrpZ demonstrate localization of these proteins to the distal tip of the Hrp pilus (117, 138). However, the resolution of the method used for visualization, involving immunolabeling with 10 to 20-nm gold particles, is insufficient to define the path of transport of these effectors. While the electron micrographic images are reasonably interpreted to mean that effectors reach the end of the pilus by traveling through its center, it should be kept in mind that other paths for effector transit remain formally possible.

Flagellar subunits are also thought to travel through the center of the flagellum. This model derives from two findings. First, flagellin is found to add to the distal end of the flagellum during growth, and second, flagellin subunits appear not to be released extracellularly before adding to the flagellum (65, 112). The first point was established directly through electron micrographic visualization of flagella belonging to bacteria grown in normal media and then pulsed with a radiolabeled amino acid (65). Flagella containing segments of unlabeled and labeled flagellin were observed to contain the radiolabel only at the distal end. The second point was established indirectly, through an experiment in which an *S. enterica* serovar Abortusequi mutant that produces curly flagella was grown together with a wild-type strain that produces normal flagella (112). While flagellin subunits of these two strains were known to copolymerize, it was found that, despite the two strains having been grown together, flagella were either all curly or all normal over their entire length. This result suggests that flagellin subunits are not secreted into the extracellular medium before adding to the distal ends of the flagellum, otherwise flagella containing mixed normal and curly segments should have been found. Again, these results are reasonably interpreted to mean that flagellin travels through the central channel of the flagellum to add to the distal end, but direct evidence is lacking. With knowledge of the structure of unassembled and assembled flagellin now available (203, 250), it may be possible to devise direct experiments to test this notion.

Inner Membrane Ring

The TTSS needle appears to be positioned on a pair of concentric membrane- embedded rings, with the larger of the concentric rings being located in the inner membrane and the smaller in the outer membrane (Fig. 1A). These two rings

appear to provide a continuous and direct path across the inner membrane, peptidoglycan layer, and outer membrane.

The identities of proteins that make up the inner membrane ring in *Salmonella* and *Shigella* are known, with these proteins having been partially purified from bacterial spheroplasts as parts of needle-containing protein complexes (19, 130, 131, 221). In *Salmonella* the inner membrane ring is formed by the proteins PrgK (28 kDa) and PrgH (44 kDa), and in *Shigella* it is formed by the proteins MxiJ (28 kDa) and MxiG (43 kDa) (Fig. 1B; Table 1).

PrgK and MxiJ are related in their N-terminal regions to the flagellar protein FliF (61 kDa). FliF forms part of the flagellar MS ring (220), which is localized to the inner membrane and functions as the passive core of the flagellar rotor. While it is estimated that 26 FliF monomers assemble to form the MS ring (118), the stoichiometries of PrgK and MxiJ are unknown. PrgK, MxiJ, and the related *Yersinia* protein YscJ (~25 kDa) are predicted to be lipoproteins, containing an N-terminal cysteine that is lipid acylated following cleavage of a signal sequence. These proteins are also predicted to have a ~200-residue periplasmic domain followed by a C-terminal transmembrane region (Fig. 1B).

In contrast to PrgK and MxiJ, PrgH and MxiG are not related to flagellar proteins or, indeed, broadly conserved across TTSSs, but they are related to one another in their C-terminal regions. The topologies predicted for PrgH and MxiG resemble the topology predicted for *Yersinia* YscD (47 kDa), with a small N-terminal cytoplasmic domain, a single transmembrane region, and a large periplasmic domain (Fig. 1B) (153, 183).

The diameter of the TTSS inner membrane ring is considerably wider than that of the needle. Outer and inner diameters of 210 and 120 Å, respectively, are observed for the *Salmonella* inner membrane ring (123), and the *Shigella* ring has been observed to have a ~260-Å outer diameter and a height of ~110 Å (19, 221). PrgH alone has been observed to form defined tetrameric structures, suggesting that PrgH oligomers may represent an early intermediate in the assembly of the inner membrane ring (123). However, the stability of the inner membrane ring is seen to require both PrgH and PrgK (218).

Predicted Inner Membrane Proteins

The MS ring of the flagellum contains at its center a number of integral membrane (FlhA, FlhB, FliO, FliP, FliQ, and FliR) and cytoplasmic (FliH and Flil) proteins. Except for FliO, these proteins are all conserved in the TTSS (Table 1). While these flagellar proteins have been shown by biochemical means to associate with the flagellar apparatus (72, 155, 158), the related TTSS proteins have not been found to copurify with needle assemblies, which probably reflects the need for gentler isolation procedures. These predicted inner membrane TTSS proteins have the potential to interact with effector proteins during transit and possibly to act as receptors that recognize secretion signals on effector proteins. No TTSS receptor has been identified, but such a receptor might be expected to be conserved in the flagellar system, since several proteins have been found to be substrates for both type III and flagellar secretion (135, 251, 252).

YscV and YscU. Among conserved integral inner membrane proteins, *Yersinia* YscV (78 kDa, also called LcrD) (181) is one of the few predicted to have a large cytoplasmic domain that could serve as a possible receptor site for interaction with translocation substrates (Fig. 1C; Table 1). YscV influences effector secretion, with low-level but not high-level expression of YscV promoting secretion (133). YscV is related in sequence to the *Salmonella* inner membrane flagellar protein FlhA (75 kDa). By using Hidden Markov Models (152), both YscV and FlhA are predicted to have seven transmembrane regions and an N-terminus located in the cytosol. However, this assignment places the ~400-residue C-terminal domain of these proteins in the periplasmic space and contradicts experimental data supporting a cytoplasmic location of the FlhA C-terminal domain (158). A model containing six (or possibly eight) transmembrane crossings is more likely (Fig. 1C).

FlhA associates with the MS ring protein FliF (122) and the flagellar inner membrane protein FlhB (42 kDa) (257). The *Yersinia* protein YscU (40 kDa) is related to FlhB, and YscU and YscU-like proteins represent a second candidate for a signal receptor since they also have predicted, sizeable cytoplasmic domains (Fig. 1C; Table 1). YscU, like FlhB, is predicted to have four transmembrane regions followed by a ~150-residue cytoplasmic domain, with topology experiments using alkaline phosphatase confirming this prediction (4). FlhB controls the choice between secretion of flagellar rod-hook proteins and secretion of flagellar filament proteins (86). Genetic evidence suggests that YscU has a similar function in the TTSS (60).

YscU appears to control the choice between secretion of the needle subunit YscF and secretion of effectors (60). Overexpression of the cytoplasmic domain of YscU in wild-type *Yersinia* increases effector secretion (133). Control of YscU over secretion substrates occurs in conjunction with the TTSS protein YscP, which is involved in controlling needle length (Table 1) (60, 120, 177). Deletion of YscP leads to enhanced secretion of YscF, with this phenotype being suppressed by mutations in the cytoplasmic domain of YscU (60). For FlhB, the C-terminal cytoplasmic domain is seen to undergo proteolytic (possibly autocatalytic) cleavage, enabling the switch between secretion substrates (156). The cleaved fragments remain associated (156), perhaps in a different conformation from the uncleaved C-terminal domain. The C-terminal cytoplasmic domain of YscU has also been observed to undergo proteolytic cleavage, but this cleavage is unnecessary for secretion of effectors (133).

The FlhA and FlhB cytoplasmic domains associate both homotypically and heterotypically (257). These domains also bind the flagellar ATPase Flil and FliH, the negative regulator of Flil (Table 1). In addition, the cytoplasmic domain of FlhA binds the soluble flagellar protein FliJ (85). FliJ appears to have chaperone-like function in preventing the aggregation of flagellar rod-hook and filament proteins (158). FliJ is related to *Yersinia* YscO (19 kDa), which plays a role in effector secretion (176). YscO also partitions into both soluble and membrane-bound fractions, suggesting a role in shuttling proteins between the bacterial cytosol and the membrane-embedded TTSS apparatus (176).

YscR, YscS, and YscT. *Yersinia* YscR (24 kDa), YscS (10 kDa), and YscT (28 kDa) are conserved across TTSSs and are also related to the flagellar proteins, FliP, FliQ, and FliR, respectively (Fig. 1C; Table 1) (14, 76). Little is known about the function of these proteins, which are predicted to be inner

membrane proteins with multiple transmembrane regions and no appreciable cytoplasmic or periplasmic regions. An exception is the ~80-residue cytoplasmic segment predicted for YscR. The *E. coli* YscR-related protein EscR has been found by yeast two-hybrid analysis to interact with itself, with the YscS-related protein EscS, and with the YscU-related protein EscU (43). The genes for *fliR* and *flhB* are fused in *Clostridium*, and a similar fusion construct engineered in *Salmonella* has been found to be functional (234). This result provides indirect evidence for association between FliR and FlhB in the MS ring and suggests that YscR may interact with YscU in the inner membrane protein assembly of the TTSS.

ATPase. The ATPase FliI (49 kDa) is an essential component of the flagellar apparatus, providing energy for assembly, and is peripherally associated with the inner membrane (145). ATPases related to FliI have been identified in the TTSS and are essential to protein secretion (Fig. 1A; Table 1) (63, 221, 245). These ATPases are also related to the β-subunit of the F_0F_1-ATP synthase. The mechanism by which energy from ATP hydrolysis is transduced to drive protein transport in the flagellar system or the TTSS is not known.

In the presence of ATP, FliI forms a sixfold symmetric, ring-like structure with an external diameter of 100 Å and a central cavity of 25 to 30 Å (33). The ATPase activity of FliI displays positive cooperativity, and both oligomerization and positive cooperativity are enhanced by phospholipids (33). The TTSS ATPase HrcN (48 kDa) from *P. syringae* pv. phaseolicola forms both monomers and oligomers, with the oligomeric form most probably being hexameric (185). As with FliI, oligomerization of HrcN is favored by its association with the membrane, and the oligomeric form of HrcN has significantly more ATPase activity than the monomeric form does. Hexameric association of secretion-related ATPases has been visualized by X-ray crystallography for ATPases involved in type IV bacterial secretion and in bacterial conjugation (95, 205, 249).

Both *Yersinia* and *Shigella* TTSS ATPases are parts of multiprotein complexes: The *Yersinia* ATPase YscN (48 kDa) (245) appears to be part of a complex containing at least YscQ and YscL, as detected by yeast two- and three-hybrid experiments (Table 1) (114). Similarly, the *Shigella* TTSS ATPase Spa47 is found by yeast two-hybrid and immunoprecipitation experiments to interact with Spa33 and MxiK, which are the *Shigella* versions of YscQ and YscK (119).

YscQ (34 kDa) is related at its C-terminal end to the flagellar proteins FliN (15 kDa) and FliM (38 kDa), which are structural components of the flagellar C-ring, a cup- like structure attached to the cytoplasmic surface of the flagellar MS ring (58). The X-ray crystal structure of a ~80-residue C-terminal fragment of $HrcQ_B$ (15 kDa), the *P. syringae* homolog of YscQ, has been determined (71). Unlike FliM and FliN, which are estimated to have stoichiometries of 37 and 111 (255, 256), respectively, the C-terminal fragment of $HrcQ_B$ is found to form a dimer of dimers. It is hypothesized that the interfaces found in the tetramer might correspond to sites of association of $HrcQ_B$ with the highly related protein $HrcQ_A$ (26 kDa) in forming a C-ring-like structure.

YscL (24 kDa) (153) is related to the flagellar protein FliH (26 kDa), the negative regulator of the ATPase FliI. FliH and FliI form a heterotrimeric $FliH_2$-FliI complex (12, 157), and both proteins interact with C-terminal cytoplasmic domains of FlhA and FlhB (257).

Outer Membrane Ring

The smaller of the two coaxial ring-like structures of the TTSS apparatus is embedded in the bacterial outer membrane and is formed by members of the secretin protein family (Fig. 1A; Table 1) (92). This contrasts with the flagellar system, in which the outer membrane ring (L-ring) is formed by a lipoprotein, FlgH (25 kDa), that is unrelated to secretins (208). Secretins are responsible for the transport of large macromolecules, such as filamentous phage, across the bacterial outer membrane (139). They are also involved in the type II secretion of proteins across the bacterial outer membrane and in the assembly of type IV bacterial pili (139).

Secretins associate as oligomers, as seen by the 14-subunit multimer formed by the secretin pIV (46 kDa), which is involved in phage extrusion (170). The pIV multimer forms three discernible rings and is 120 Å long, with an outer diameter of 135 Å and an inner diameter varying from 60 to 88 Å (170). The channel in the pIV multimer appears to be blocked, raising the possibility that secretins are gated (170). The secretin PulD from *Klebsiella oxytoca*, which is involved in type II secretion of pullulanase, has also been found to be oligomeric but is reported to have 12-fold rather than 14-fold symmetry (168, 169).

The TTSS secretins YscC (67 kDa) from *Yersinia* and InvG (62 kDa) from *Salmonella* also form ring-shaped structures. YscC rings have external and internal diameters of ~200 and ~50 Å, respectively (125), and InvG rings have external and internal diameters of ~150 and ~70 Å, respectively (41). Ysc rings have 13-fold symmetry (25). The localization of InvG to the outer membrane is promoted by the TTSS outer membrane lipoprotein InvH (16 kDa) (41, 218). However, InvH does not form a complex with InvG and is not required for type III secretion. InvH is not related to flagellar proteins and is not widely conserved across TTSSs. The *Yersinia* lipoprotein YscW (15 kDa) also plays a role in proper localization of the secretin YscC to the outer membrane (125), but YscW is not related to InvH and is not widely conserved across TTSSs. Mutation of the *P. syringae* pv. syringae 61 secretin HrcC leads to accumulation of the effector HrpZ in the periplasm, consistent with the role of TTSS secretins in promoting the transport of effectors across the outer membrane (30).

TRANSLOCATOR PROTEINS

Effectors appear to gain entry into host cells through pores formed in host cell membranes by type III-secreted proteins termed translocators (Fig. 1A). Translocators are typified by *Yersinia* YopB (42 kDa) and YopD (33 kDa) (101), which are widely conserved across TTSSs and required for translocation of effectors into host cells (82, 101, 167, 194, 214). Direct evidence for entry of effectors through a proteinaceous pore formed by these translocators is not in hand, but indirect evidence supporting this hypothesis is available. In addition, complicating analysis of translocator function is the fact that YopD plays a regulatory in addition to a pore-forming role (6, 243).

As would be expected for pore-forming proteins, YopB and

YopD form ion channels in planar lipid bilayers, with these channels having a conductance of 105 pS (223). Ion channels are observed in the absence of YopD, but channels formed by YopB alone have ill-defined conductance characteristics (223). Pores formed by YopB and YopD have a defined size, being permeant to Lucifer yellow CH (443 Da) but not to Texas Red-X phalloidin (1490 Da), indicating a narrow pore size (163). Pores formed in mammalian membranes by enteropathogenic *E. coli* have been visualized (111). These pores have an inner diameter of ~30 to 50 Å and are presumably formed by the YopB-related protein EspB and the YopD-related protein EspD (111). EspB binds EspA, the filament component, suggesting that a continuous path exists between the filament and the host cell for polarized and direct translocation (104). EspA also binds host cell membranes in the absence of EspB, indicating that the filament may make initial contact with host cells before EspB inserts into membranes. Pores formed by *E. coli* are highly asymmetric, rising about 150 to 200 Å above the membrane plane, although it is uncertain whether this represents the external or cytoplasmic face of the pores. The YopB- and YopD-related proteins from *P. aeruginosa*, PopB and PopD, respectively, also form similar pores, with outer and inner diameters of ~80 and ~40 Å, respectively (207). The *P. aeruginosa* pores are not as asymmetrically disposed as the *E. coli* pores (207).

The YopB-related proteins SipB from *Salmonella* and IpaB from *Shigella* integrate into the mammalian cell plasma membrane without causing membrane disruption or lysis (105, 110). SipB forms trimers and hexamers, whereas IpaB forms only trimers (105, 110). Trimerization appears to occur through a sequence that has the potential to form a triple-stranded coiled coil (105). Labeling studies in combination with liposome protection experiments suggest that IpaB and SipB each have two transmembrane crossings (151).

In contrast to the hypothesis that translocation across the host cell membrane occurs through translocator pores, it has been suggested that the TTSS needle directly punctures the host cell membrane to deliver effectors (107). This is based on electron micrographic visualization of *Yersinia* needles that appear to be inserted into membranes of red blood cells that had been incubated with *Yersinia* (107). However, in another study the *Yersinia* needle was found to be insufficient to form pores in macrophage membranes, with this activity requiring at least YopB and YopD (148).

TARGETING EFFECTORS TO THE TYPE III SECRETION SYSTEM APPARATUS

Of the thousands of bacterial proteins, only a few are substrates for type III secretion into the extracellular space and even fewer are substrates for type III translocation into host cells. For example, only six effectors (YopE, YopH, YopM, YopO, YopP, and YopT) are translocated by *Yersinia* into host cells. How are these proteins targeted to the TTSS, or, to state it another way, what is the signal sequence?

In contrast to the easily discernible signal sequences of proteins secreted by the sec-dependent pathway (166), no consensus signal sequence has been identified for proteins secreted or translocated by the TTSS. What is certain for type III-secreted proteins is the location of the signal sequence. What remains controversial is the molecular composition—mRNA or protein—of this signal.

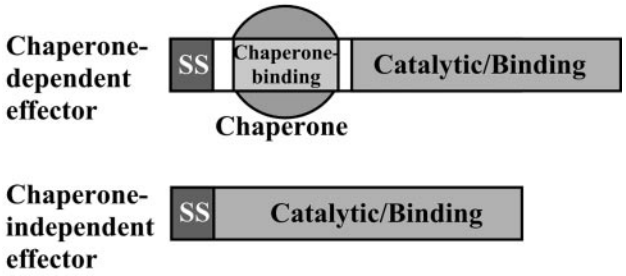

FIG. 2. Effectors and secreted proteins of the TTSS have a secretion signal (SS) encoded in their first ~15 mRNA codons, amino acids, or both. Physiologically significant translocation of many effectors depends on the action of TTSS chaperones, which generally bind to a chaperone-binding region that follows the secretion signal; the chaperone is shown as a circle. Effector activities, either catalytic or host cell target binding, are encoded by domains that usually follow the chaperone-binding region. Some effectors apparently have no cognate chaperones and are translocated independently of chaperone action.

The type III secretion signal has been localized to the first ~15 mRNA codons or amino acids of secreted or translocated proteins. Fusion of the first 15 codons of the effector YopE to the N terminus of Cya (398 amino acids) or neomycin phosphotransferase II (NPT) (262 amino acids) is sufficient to drive type III secretion of these fusion proteins (Fig. 2) (7, 206, 213). This signal, however, is not sufficient to drive translocation, at least in a wild-type genetic background (23, 213). The first ~15 codons or residues of other effectors besides YopE drive the secretion of fusion partners as well. These include the TTSS effectors *Yersinia* YopH, *P. syringae* AvrB and AvrPto, and *X. campestris* AvrBs2, as well as the secreted but not translocated proteins *Yersinia* YopN and YopQ and *Salmonella* InvJ (5, 7, 115, 160, 188, 189, 200, 213). In addition to Cya and NPT, fusion partners for these studies have included the α-peptide of β-galactosidase, *E. coli* alkaline phosphatase, green fluorescent protein (GFP) ubiquitin, and dihydrofolate reductase (DHFR) (73, 115, 135, 137, 153). However, it has also been found that not all heterologous proteins are competent substrates for secretion by the TTSS (73, 137).

Nucleic Acid Signal

The controversy over the molecular composition of the signal sequence stems from its apparent mutability. For example, it has been observed that the secretion signals in YopE, AvrB, AvrPto, AvrBs2, YopN, and YopQ retain functionality despite frameshift mutagenesis (5, 7, 160). These frameshifted mutants have drastically altered protein sequences at their N termini (usually just their first 15 amino acids, with the remaining sequence restored in frame) but an mRNA sequences that is essentially unchanged. These results are consistent with a signal sequence that is composed of mRNA. It has been shown that upstream, untranslated mRNA is not involved in this putative mRNA signal (188).

The most compelling evidence for an mRNA signal derives from experiments examining the secretion signal of the *Yersinia* type III-secreted but not translocated protein YopQ (189). A single synonymous base change in codon 3 of YopQ

was found to destroy the ability of YopQ codons 1 to 10 to promote the secretion of an NPT fusion (189). The synonymous base change preserves the identity of the encoded amino acid, as was confirmed through protein sequencing. This clearly suggests a signal sequence composed of mRNA. However, it was also found that frameshifting destroys the secretion signal in YopQ codons 1 to 10, which suggests a protein-based signal. How might this work? The authors reconcile these findings by positing that the secretion signal does indeed reside in the mRNA, which explains the effect of synonymous base changes, but that the signal also requires the interaction of specific tRNAs with the mRNA, which accounts for the frame being important (189). Complicating the picture a bit further, it was also found that codons 11 to 15 of YopQ act to suppress mutations introduced into codons 1 to 10. The suppressor activity of codons 11 to 15 was found to be sensitive to synonymous base changes but not to frameshifting. It is not known yet whether these results extend to other type III secretion substrates, and no consensus RNA sequence or secondary structure is apparent as a secretion signal.

Protein Signal

Support for a protein-based signal sequence comes from experiments in which the functional integrity of the signal is seen to be maintained despite dramatic changes in the mRNA sequence. The secretion signal in YopE was found to be unaffected by mutations that leave the protein sequence intact but alter 17 of 27 nucleotides in codons 2 to 10 (142). Similarly, the secretion signal in codons 4 to 7 of *Salmonella* InvJ was found to be unaffected by extensive mutations that leave the protein sequence intact but alter the mRNA sequence vastly (200). It should be noted that the signal does not always survive frameshifting. Introduction of a +1 or +2 frameshift at codon 10 of the *Salmonella* effector SptP, which results in a small change in the mRNA but a drastic change in the protein sequence for residues 11 to 35, was found to destroy secretion (135). Nevertheless, in most cases the integrity of the signal withstands frameshifting (5, 7, 160). This suggests that if the signal were protein based, it would have to be highly degenerate.

The degeneracy of a putatively protein-based signal has been confirmed in studies using synthetic secretion signals for YopE. A sequence encoding alternating serines and isoleucines at residues 2 through 8 of YopE (i.e., SISISISI replacing the wild-type KISSFIS) is sufficient to act as a secretion signal (142). These studies also provide some of the best evidence for a protein-based signal (143). Seven cases of single amino acid substitutions in YopE synthetic signals were found that affect secretion efficiency, with six of these substitutions increasing the amphipathic character of the sequence. For example, the YopE sequence ISIISSS (residues 2 to 8) was found to be unable to confer secretion, but substitution of residue 4 with Ser (changing the sequence to ISSISSS) restored secretion (143). Since these substitutions alter the mRNA only slightly, these results favor the idea of a protein-based signal.

As with the mRNA hypothesis, no clear consensus sequence is apparent at the amino acid level. Systematic variation of YopE amino acids 2 to 8 with serines or isoleucines (128 different sequences) reveals that sequences containing four or five serine residues have the greatest likelihood of promoting secretion (143). Multiple linear-regression analysis indicates that the most favorable secretion sequence in YopE residues 2 to 8 is Ile-X-Ser-Ser-Ile-Ser-Ser (143). Analysis of predicted N-terminal secretion signals of effectors from *Yersinia*, *Salmonella*, *Pseudomonas*, enteropathogenic *E. coli*, *Shigella*, and *Xanthomonas* demonstrates enrichment in Ile, Ser, Asn, and Thr (143). Cys and Trp are absent in this region (143). Analysis of the first 50 amino acids of effectors from *P. syringae* reveals a statistically significant enrichment of Ser and a paucity of Asp, Leu, and Lys (100). Lastly, analysis of the first 50 amino acids of *S. enterica* effectors reveals a similar statistically significant enrichment of Ser and a paucity of Asp (100).

Structures of N-Terminal Putative Secretion Signals

Despite the degeneracy of putative protein-based secretion signals, are there common features in the three-dimensional structures of these N-terminal segments? The X-ray crystal structures of the N-terminal putative secretion signal regions of the *Yersinia* effectors YopH and YopM are known (Fig. 3). Residues 1 to 17 of YopH constitute the putative secretion signal and form an amphipathic α-helix (α1) in the structure of a fragment of YopH (YopH-N, residues 1 to 130 of 468 total) (68, 212). The α1 helix is not an independent structural element but, instead, wraps against the globular body of YopH-N (Fig. 3A). The existence of structure at the N terminus of YopH may represent a special case, however, in that this region has multiple functions. The N terminus of YopH serves not only as a putative secretion signal but also as part of a phosphotyrosine binding site (17, 159). This latter function increases the efficiency of the tyrosine phosphatase activity of YopH by aiding in substrate recognition (17, 55). Alternative functions for the putative N-terminal secretion signal regions of other effectors have not been described.

In contrast to the amphipathic α-helix in YopH, the putative secretion signal in YopE shows no requirement for sequences that can form amphipathic α-helices (143). Indeed, the Ser-Ile repeat found to confer secretion for YopE has the correct periodicity to form an amphipathic β-strand rather than an α-helix. In further contrast to YopH, the putative secretion signal in YopM, which has been mapped to within the first 40 residues, is mostly flexible and disordered, with only residues 34 to 40 being structured (Fig. 3B) (22, 66). Therefore, the putative N-terminal secretion signal is not defined by any particular structural element, at least in its isolated state. It is possible that it is induced to adopt a specific structure in association with a receptor.

Structural evidence is also available for a potential secretion signal in the flagellar protein FlgM, which is secreted through the flagellar basal-body/hook and filament. As with the TTSS, no clear signal sequence has been identified for flagellar proteins. Residues 7 to 25 of FlgM are essential for transport and are found by nuclear magnetic resonance (NMR) spectroscopy to be unfolded in free FlgM or when FlgM is bound to the protein σ^{28} (51). While the unfolded nature of these protein segments may be useful in transport through needles or flagella, it is unlikely that the lack of structure serves as a signal for a putative receptor (51). An unstructured element, by definition, adopts a very large number of conformations. It is

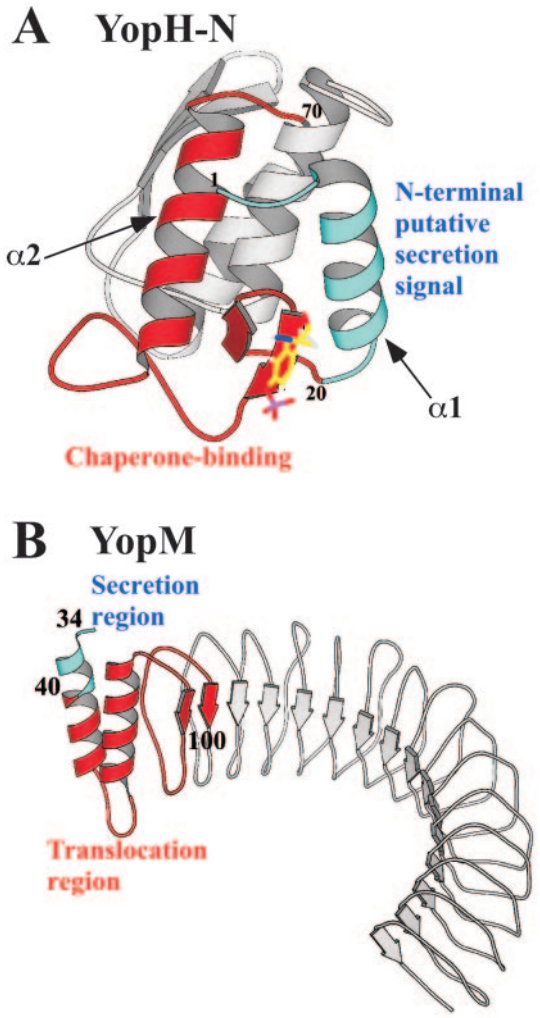

FIG. 3. Structures of N-terminal putative secretion signals. (A) Ribbon representation of YopH-N (PDB 1HUF, 1K46, 1MOV). Residues 1 to 20, the N-terminal putative secretion signal, are in blue, and residues 20 to 70, the chaperone-binding region, are in red. A bound phosphotyrosine is shown in bonds representation. This and other molecular figures were made using Molscript (127). (B) Ribbon representation of YopM (PDB 1JL5). The N-terminal putative secretion signal is encoded within residues 1 to 40, with residues 1 to 33 being disordered and residues 34 to 40 forming part of an α-helix (blue). Residues 41 to 100 (red) promote translocation.

difficult to envision the physical properties of a receptor that can recognize a multitude of conformations with enough specificity and affinity to achieve discrimination between substrates and nonsubstrates.

TYPE III SECRETION SYSTEM CHAPERONES

Translocation for many effectors not only depends on the secretion signal (mRNA or protein) but also relies heavily on the activity of specific TTSS chaperone proteins (213, 239, 240). A large family of TTSS chaperones exists, and these proteins have no counterparts in the flagellar system. There are proteins termed chaperones in the flagellar system (e.g., FliS, FliT, and FlgN), but no sequence or structural homology appears to exist between TTSS and flagellar chaperones (15, 67, 70, 144, 215, 229).

TTSS chaperones function in dedicated fashion, with an individual TTSS chaperone usually promoting translocation of just a single cognate effector. For example, the *Yersinia* TTSS chaperone SycE (15 kDa) promotes the translocation of YopE (23 kDa) but not of other effectors (239). Likewise, the *Yersinia* TTSS chaperones SycH (16 kDa) and SycT (15 kDa) are required for translocation of their cognate effectors, YopH (51 kDa) and YopT (36 kDa), respectively (Fig. 2) (113, 239). SycE, SycH, and SycT have only ~14% sequence identity, which is representative of sequence conservation across the large family of TTSS chaperones (15). Despite the low sequence conservation, TTSS chaperones are remarkably similar in structure (Fig. 4), suggesting a common mechanism of action (15, 70, 144, 215, 229, 235).

TTSS chaperones associate physically with their cognate effectors in the bacterial cytosol and remain in the bacterial cytosol following translocation of effectors into host cells (32, 87, 196). While the effector YopE is detected both intrabacterially and in infected HeLa cells by using immunofluorescence and Western blotting (87, 196), its cognate chaperone SycE is detected exclusively within *Yersinia* and is not found in infected HeLa cells (87). Rather than the weak association anticipated for a complex that must dissociate prior to effector translocation, SycE and YopE are found to bind very tightly. The dissociation constant measured for SycE binding to refolded, detergent-solubilized YopE is reported to be 0.3 nM (32). How this tight interaction is broken apart to allow effector translocation is not known, but a role in this process for the TTSS ATPase seems likely. The TTSS ATPase EscN from enteropathogenic *E. coli* interacts individually with the effector Tir and with Tir's cognate chaperone CesT, as determined by affinity chromatography and immunoprecipitation using tagged proteins (91). The flagellar ATPase FliI also interacts with the FlgN- FlgK chaperone-cargo complex but not with uncomplexed FlgN or FlgK (225). The functional consequences of these interactions are at present unclear.

Structures

The X-ray crystallographic structures of the TTSS chaperones SycE (15 kDa), *S. enterica* serovar Typhimurium SicP (13 kDa) and SigE (13 kDa), and enterohemorrhagic *E. coli* CesT (18 kDa) have been determined (Fig. 4) (15, 70, 144, 215, 229). These proteins have an average of 10% sequence identity but have a structurally conserved αβα sandwich fold that is unique to TTSS chaperones. The chaperones are homodimeric, with large surface areas buried at the dimer interface (~2,190 Å^2 in SigE, ~2,100 Å^2 in SicP, and ~2,400 Å^2 in SycE), indicative of constitutive dimerization (15, 70, 229). CesT forms a domain-swapped dimer that is likely to be an artifact of high protein concentrations used for crystallization, as discussed below. The domain-swapped CesT dimer is related to the other chaperone dimers by a swap of the first helix and strand of CesT (144). The *Yersinia* chaperone SycT has also been found by X-ray crystallography to resemble these other chaperones (Z. Xu and J. Dixon, unpublished data). Likewise, the structure of the *S. flexneri* chaperone Spa15 has been determined (235), revealing that it is homodimeric and has a protein fold observed for

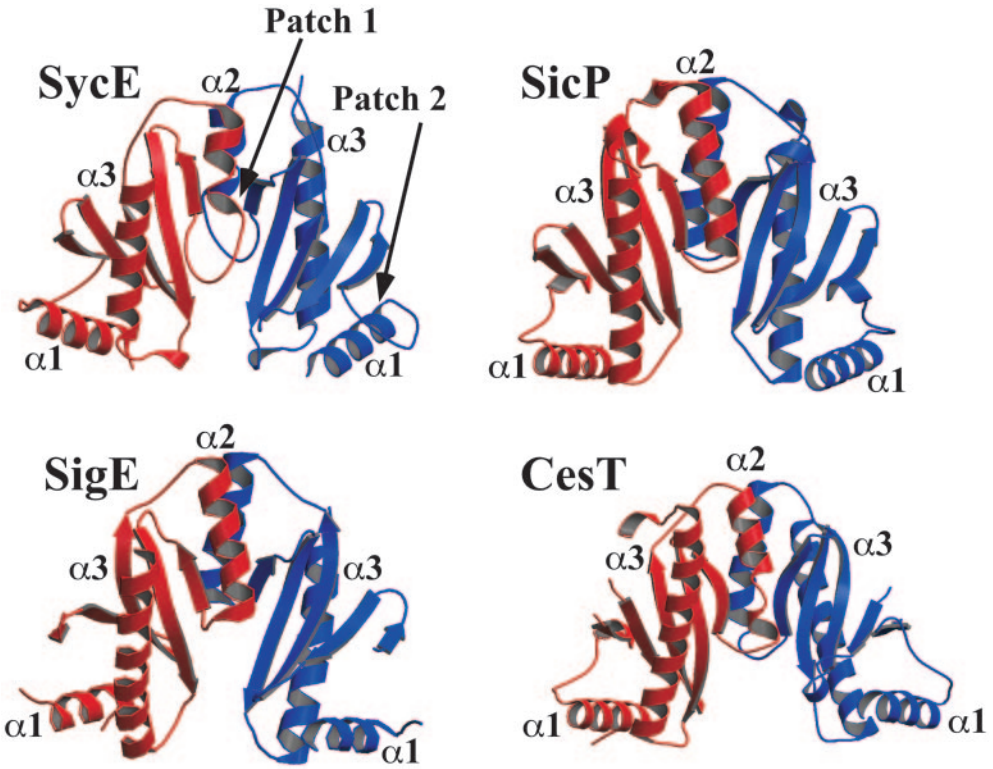

FIG. 4. Ribbon representation of the TTSS chaperones *Yersinia* SycE, *Salmonella* SicP, *Salmonella* SigE, and *E. coli* CesT. Chaperones are dimers, and monomer subunits are shown in red and blue. The α1, α2, and α3 helices are indicated, and the locations of patch 1 and patch 2 are indicated for SycE. Domain swapping is corrected for in an approximate way for CesT. PDB identifications are as follows: SycE, 1JYA, 1KSZ, 1N5B; SicP, 1JYO; SigE, 1K3S; and CesT, 1K3E.

these other TTSS chaperones. However, Spa15 is the least like these other chaperones, in that Spa15 monomers are rotated ~30° with respect to one another, in contrast to the roughly parallel orientation of monomers seen for the SycE, SicP, SigE, and CesT dimers (Fig. 4).

The specificity for homodimerization in TTSS chaperones appears to be conferred by the α2 helix, with the α2 helix from one monomer pairing with the α2 helix from a second monomer (Fig. 4). This helix contains residues that are unique to particular chaperones and are not broadly conserved, consistent with a role for this helix in guiding homodimerization specificity. An exception to chaperone homodimerization comes from the *Yersinia* chaperones SycN and YscB (54). These chaperones are not involved in the translocation of effectors but, rather, in the secretion of YopN, which is not translocated and whose function is necessary to prevent premature release of effectors. SycN and YscB associate in simultaneous fashion with YopN, consistent with the existence of a possible SycN-YscB heterodimeric chaperone, although the stoichiometry of the complex is not yet known (54).

Most significantly for function, TTSS chaperones have hydrophobic surface patches that are formed by residues broadly conserved among chaperones (15, 144, 215). These patches act as effector-binding sites, as shown through direct structural examination (16, 215). There are two different hydrophobic patches, called patch 1 (or helix-binding groove) and patch 2 (15, 16, 215). Patch 1 is found at the dimer interface and includes residues from the α2 helix and adjacent β-strands (β3 and β5) (Fig. 4). Patch 2 is formed by the amphipathic α1 and α3 helices, which are found at the N and C termini, respectively, of TTSS chaperones. The α1 and α3 helices are positioned roughly perpendicular to one another and are positioned on the central β-sheet of TTSS chaperones, with the β1 and β2 strands of the sheet also contributing hydrophobic residues to patch 2. Since the chaperone is a dimer, patch 1 and 2 occur as pairs, giving rise to a total of four exposed hydrophobic patches. As the broad sequence conservation of these hydrophobic patches suggests, these sites confer overall binding energy but little in specificity to effector association (16, 215). For example, one of the patch 1 regions in SycE forms a binding site for a tyrosine, leucine, and alanine of YopE, and the other forms a binding site for two phenylalanines of YopE (16).

Most but not all chaperones are negatively charged, but the functional implication of this is unclear. The structures reveal that the negative charge is conserved in character but the primary sequence position of negatively charged residues is not strictly conserved (15). There is no obvious candidate for a positively charged TTSS protein that would interact electrostatically with chaperones.

Effector versus Translocator Chaperones

The term "chaperone" has been used widely in the TTSS literature. Recent evidence indicates that two different protein families are encapsulated by this term. The first family is com-

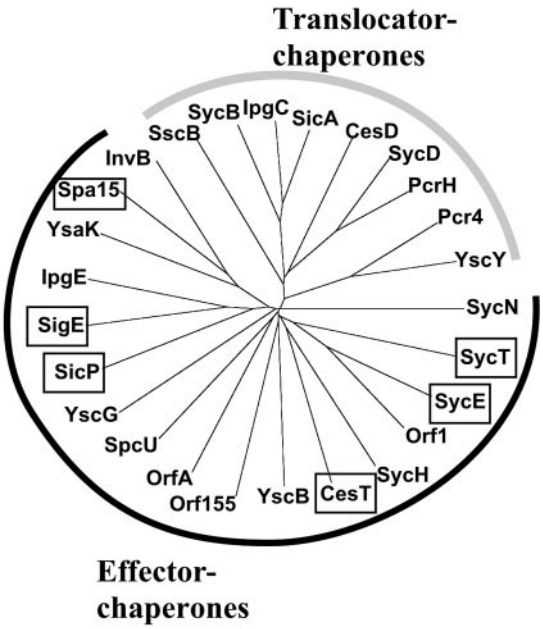

FIG. 5. Phylogenetic analysis of TTSS chaperones, which indicates a basis for distinguishing between effector chaperones and translocator chaperones. The analysis was carried out using Clustal W (226). Boxed chaperones have been characterized to be structurally similar. GenBank accession numbers: *Y. enterocolitica* SycN, NP_863520; *Y. enterocolitica* SycT, NP_863508; *Y. pestis* SycE, AAC62588; *P. aeruginosa* Orf1, AAA66490; *Y. enterocolitica* SycH, NP_863547; *E. coli* CesT, P58233; *Y. enterocolitica* YscB, NP_863534; *Y. enterocolitica* Orf155, NP_052379; *E. amylovara* OrfA, AAF63399; *P. aeruginosa* SpcU, AAP82960; *Y. enterocolitica* YscG, NP_863539; *S. enterica* serovar Typhimurium SicP, AAF63399; *S. enterica* serovar Typhimurium SigE, NP_460063; *S. flexneri* IpgE, AAP78997; *Y. enterocolitica* YsaK, AAK84111; *S. enterica flexneri* Spa15, AAP79011; *S. enterica* serovar Typhimurium InvB, NP_461816; *S. enterica* serovar Typhimurium SscB, NP_460368; *Y. enterocolitica* SycB, AAM47500; *S. flexneri* IpgC, AAP78992; *S. enterica* serovar Typhimurium SicA, NP_461807; *E. coli* CesD, NP_312603; *Y. enterocolitica* SycD, NP_863513; *P. aeruginosa* PcrH, AAO91772; *P. aeruginosa* Pcr4, AAC45943; *Y. enterocolitica* YscY, NP_863518.

posed of chaperones that promote the translocation of effectors and is typified by SycE. The second is composed of proteins that promote the secretion of translocator proteins (e.g., YopB and YopD) rather than effectors and is typified by *Yersinia* SycD (19 kDa).

Translocator chaperones. No structure of a SycD-like translocator chaperone exists, but these proteins are predicted to have a tetratricopeptide-like repeat fold within their central region, which is unrelated to the fold found in the SycE-like family of effector-chaperones (Fig. 4) (172). A hydrophobic groove is proposed to exist in the tetratricopeptide-like repeat-like domain of SycD that binds an amphipathic α-helix of the translocator YopD (172, 224). YopB does not appear to have a discrete, single SycD-binding region, since no single deletion in YopB has been found to abrogate association with SycD (164). Phylogenetic analysis is consistent with effector chaperones of the SycE class being distinct from translocator chaperones of the SycD class (Fig. 5). Furthermore, the mechanism of action of effector chaperones is likely to differ from that of translocator chaperones. Indeed, proteins belonging to the SycD-like family of translocator chaperones play regulatory in addition to secretory roles (6, 27–29, 49, 50, 81, 128, 149, 179, 232, 242).

Promiscuous effector chaperones. Most effector chaperones are highly specific and bind just a single cognate protein or two target proteins with similar sequences, as in the case of SycH. SycH binds regions of the effector YopH and the negative regulatory protein LcrQ that are related in sequence and promotes the transport of both proteins (27, 28, 179, 212, 242). In contrast to highly specific effector chaperones, promiscuous effector chaperones have been uncovered; this has led to the suggestion that promiscuous chaperones may form a subfamily of effector chaperones. The *S. flexneri* effector chaperone Spa15 binds not only the effector IpaA but also the secreted proteins IpgB1 and OspC3, as assessed by yeast two-hybrid assays and coprecipitations (Fig. 5) (171, 174). Intriguingly, the chaperone-binding regions of IpaA, IpgB1, and OspC3 have no obvious sequence relationship, suggesting that Spa15 is a promiscuous effector chaperone. Likewise, the *Salmonella* chaperone InvB, which is related in sequence to Spa15, binds the effectors SipA (24), SopA (62), and SopE (as well as the related SopE2) (61, 134), with these effectors having no obvious sequence similarity to one another. InvB also promotes secretion of these effectors, as well as translocation of SipA (24), SopE, and SopE2 (61, 134). Based on sequence, the Spa15-InvB family of promiscuous effector chaperones also includes *Yersinia* YsaK (Fig. 5), whose cognate effector has not yet been identified.

The *E. coli* chaperone CesT also binds at least two effectors, Tir and Map (Fig. 4) (1, 42, 64). CesT has been reported to bind within the N-terminal 100 residues of the effector Tir (1), although another report suggests that additional portions of Tir are required for association, based on yeast two-hybrid assays (64). CesT is also reported to bind within the N-terminal 101 residues of the effector Map (42). Since Tir and Map are not related in sequence, CesT may also be considered a promiscuous chaperone. The basis for promiscuity is unknown for CesT and Spa15 and awaits structural studies of chaperone-effector complexes for these or other promiscuous chaperones.

The effector chaperone family also appears to include members that bind target proteins that are not secreted or translocated. YscG (13 kDa) belongs to the SycE class of effector chaperones but has been identified to bind YscE (~7 kDa), a protein that is not transported out of *Yersinia* (53).

For simplicity, in this review the term "chaperone" will continue to refer to the SycE class of effector chaperones and not to the SycD class of translocator chaperones.

Mode of Effector Binding by Chaperones

The structures of fragments of two chaperone-effector complexes have been determined, revealing the mode of effector binding by TTSS chaperones. These structures are of the chaperone-binding (Cb) regions of the *Salmonella* effector SptP and *Yersinia* effector YopE bound to their respective chaperones, SicP and SycE (Fig. 6) (16, 215). The chaperone-binding region of YopE comprises residues 23 to 78, as defined by the structure. This region is distinct and separable from the RhoGAP catalytic domain of YopE, which is composed of residues 100 to 219 (16, 69), and from the N-terminal putative secretion signal (codons or residues 1 to 15) (Fig. 2). The

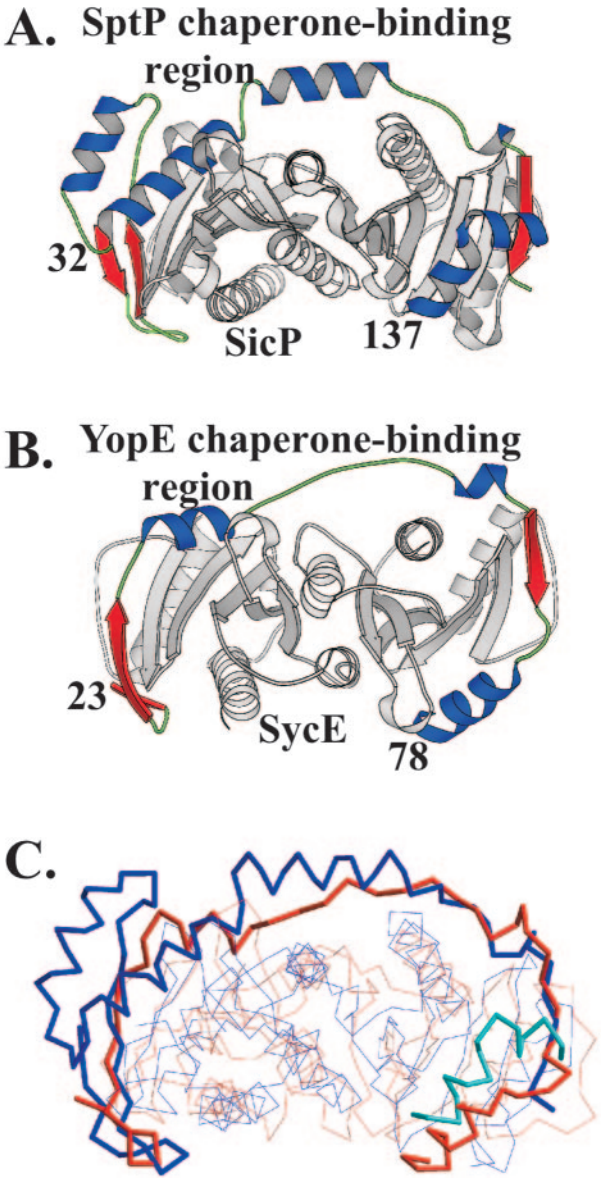

FIG. 6. Chaperone-effector fragment complexes. (A) Ribbon representation of the SicP-SptP fragment (PDB 1JYO). The chaperone SicP is in gray, and the chaperone-binding region of the effector SptP is in red (β-strands), blue (α-helices), and green (coils). Domain swapping in this complex has been corrected for in an approximate way. (B) Ribbon representation of the SycE-YopE fragment (PDB 1L2W). The chaperone SycE is in gray, and the chaperone-binding region of the effector YopE is colored as in panel A. (C) Overlay of Cα traces of the SicP-SptP fragment (blue) and SycE-YopE fragment (red). Chaperones are shown in thin lines, and effectors are shown in thick lines. Two different SptP molecules are shown (in dark and light blue) to correct for domain swapping. This panel has been reproduced from Birtalan et al. (16) with permission from Elsevier.

YopE Cb region is not widely conserved but is found in two related effectors, *P. aeruginosa* ExoS/ExoT and *A. salmonicida* AexT. The chaperone-binding region of SptP encompasses residues 35 to 139 and is followed by two catalytic domains, a RhoGAP domain in residues 161 to 295 and a protein tyrosine phosphatase domain in residues 300 to 543 (217). The chaperone-binding region of SptP appears to be unique.

Stoichiometry. One SycE chaperone dimer binds to one molecule of YopE, both in solution studies of intact SycE-YopE complexes and in the crystal structure of the truncated SycE-YopE(Cb) complex (16). An identical 2:1 chaperone-to-effector stoichiometry is observed for the association of the *E. coli* chaperone CesT with the effector Tir (144). On the other hand, the SicP-SptP(Cb) chaperone-effector complex is observed in the crystal lattice to form a 4:2 complex. The SicP-SptP(Cb) complex is domain swapped in crystals through a short α-helix found at the C terminus of truncated SptP (Fig. 6). Besides SicP-SptP(Cb), the aforementioned N-terminal domain of YopH (YopH-N) and the chaperone CesT are also domain swapped in crystal structures (68, 144, 212). Is there functional relevance to these domain-swapped structures?

Domain swapping occurs by the exchange of like structural elements by identical protein molecules, leading to the formation of intertwined oligomers (140, 162, 198). The oligomers have the same structure as the original monomer, except at the position where the exchange occurs. The phenomenon was first described for diphtheria toxin but had been observed earlier for RNase A and λ cro repressor (3, 10, 13, 44). Most often, domain swapping requires a high protein concentration, with dissociation constants for domain swapping measured to be in the millimolar range for the suppressor of cyclin- dependent kinase 1 (suc1) and for RNase A (173, 209). In addition, a large kinetic barrier to domain swapping is generally found, with interconversion between unswapped and domain-swapped forms occurring on the order of days to months (199). About 40 cases of domain swapping have been characterized structurally (140), and only in a few cases has biological relevance been demonstrated. These few cases include domain swapping in RNase A, suc1, and Hsp33 (97, 98, 209, 238). In most cases, domain swapping appears to arise as an artifact of high protein concentration, the use of truncated protein fragments, or both.

For TTSS proteins, the functional relevance of domain swapping has yet to be demonstrated. Domain swapping in CesT does not change its homodimeric state but does create a different sort of dimer arrangement from that observed for SycE, SigE, and SicP (15, 144, 215). Notably, the dimer arrangement for CesT differs from the dimer arrangement seen in either the free or complexed forms of the other chaperones, suggesting that the domain swapping in CesT is artifactual. For YopH-N, domain swapping creates a dimeric form whereas YopH-N is observed to be monomeric in solution by NMR spectroscopy (121). Intact YopH has also been found to be monomeric by equilibrium sedimentation ultracentrifugation (254). These data suggest that the dimeric, domain-swapped dimer form of YopH-N is also likely to be artifactual. In SicP-SptP(Cb), domain swapping alters the stoichiometry of the complex from 2:1 to 4:2. Both 2:1 and 4:2 complexes of the truncated SicP-SptP(Cb) complex are observed by size exclusion chromatography, but it is not known whether intact SicP-SptP complexes are capable of forming 4:2 complexes. It is possible that truncation of SptP (by generating an unnatural C terminus), combined with high protein concentrations leads to the formation of a 4:2 complex from the more physiologically relevant 2:1 complex.

Extended conformation in effectors. The chaperone-binding regions of SptP and YopE associate with their respective chaperones, SicP and SycE, in similar highly extended conforma-

tions (Fig. 6) (16, 215). The general structural similarity is especially striking since these effectors have no sequence similarity in their chaperone-binding regions. The chaperones themselves have about 10% sequence identity. While the structure of free SicP is not known, a comparison between free and complexed SycE reveals no conformational changes (root-mean-square deviation in Cα positions, 0.9 Å), indicating that chaperones function as static binding platforms (15, 16).

The chaperone-binding regions of SptP and YopE wrap more than three-quarters of the way around the chaperones and lack independent tertiary structure. Just over half of the chaperone-binding region of these proteins forms secondary structure, with the remaining portion forming a random coil. The chaperone-binding region of SptP (residues 35 to 139) is about twice as long as that of YopE (residues 23 to 78); and the extra length in SptP is taken up by a helical insertion and an overall greater helicity (215). Otherwise, SptP and YopE take similar three-dimensional paths along the surface of their chaperones, involving similar contacts at the two hydrophobic patch 1 sites (or helix-binding groove) and the two hydrophobic patch 2 sites. Short α-helices in the chaperone-binding regions make hydrophobic contacts to the two patch 1 sites, and short β-strands make hydrophobic contacts to the two patch 2 sites. The short β-strands of the effector also extend β-sheets in the chaperone by forming hydrogen bonds to the sheet. In this regard, it would be appropriate to label patch 2 as the "strand-binding groove."

Hydrophobic contacts formed at the patches are most probably involved in conferring affinity, whereas specificity appears to arise from the large number of hydrogen bonds between the chaperones and effectors (16). While the hydrophobic residues of the chaperones in patches 1 and 2 are generally conserved, the residues forming hydrogen bonds are not conserved and are specific to individual chaperones. For YopE, mutations in residues involved in hydrophobic interactions with the chaperone have large effects on translocation efficiency, as assessed using a YopE-Cya fusion protein (206). By comparison, mutation of YopE residues forming hydrogen bonds to the chaperone were found to have smaller effects on translocation efficiency (206). A total of 6,200 $Å^2$ of surface area is buried in the domain-swapped SptP-SicP(Cb) complex, while a smaller buried surface area of ~4,700 $Å^2$ is observed in the SycE-YopE(Cb) complex. The extent of the buried surface areas agrees with the tight binding between chaperone and effector noted for SycE-YopE (32).

YopE Chaperone-Binding Site and Translocation

The chaperone-binding region of YopE has been the subject of numerous studies. Surprisingly, only the first half of the chaperone-binding region of YopE appears to be required for translocation. Fusion of the first 49 or 50 residues of YopE to Cya was found to be sufficient to drive translocation of the fusion protein (206, 213), although substantially higher translocation levels were found when the fusion included the first 75 YopE residues (206). This larger 75-residue region includes almost the entire chaperone-binding region of YopE, as defined structurally (Fig. 6) (16). Similarly, while the N-terminal 53 residues of YopE were found to be sufficient to drive translocation when fused to GFP, the N-terminal 138 residues of YopE were seen to drive translocation with much greater efficiency (115).

Are these first 50 YopE residues sufficient to bind SycE? Experimental results are contradictory on this point. Using gel overlay assays, it has been shown that YopE lacking residues 50 to 77 retains the ability to bind SycE (23) and that YopE residues 17 to 52 fused to DHFR are able to confer SycE binding (73). Similar results have been demonstrated in solution, with a YopE-Cya fusion containing just the first 50 YopE residues shown to bind SycE, as determined by a calmodulin-agarose pull-down assay (246). However, other groups using the same or similar assays in solution find that the first 50 YopE residues are insufficient to confer SycE binding. Also using a calmodulin-agarose pull-down assay, Schesser et al. found that the first 49 residues of YopE were insufficient to bind SycE and that the first 75 residues were necessary and sufficient (206). Likewise, the first 50 YopE residues were insufficient but the first 100 YopE residues were sufficient to confer SycE binding in a glutathione- agarose bead pull-down assay examining binding between a YopE-DHFR fusion protein and a glutathione *S*-transferase (GST)–SycE fusion protein (31). The crystal structure of SycE-YopE(Cb) clearly supports the notion that YopE residues 23 to 78 are involved in binding, although residues 63 to 78 of YopE have a higher crystallographic B-factor than do residues 23 to 62, possibly indicating greater mobility and less tight binding in the C-terminal portion of the chaperone-binding region (16). Quantitative rather than qualitative affinity assays are needed to sort out these contradictory results, but it would be highly surprising to find the translocation activity of the YopE chaperone-binding region to be independent of chaperone binding.

Chaperones and Secretion

Surprisingly, chaperones are found in many cases not to be required for secretion (206, 246). A strain of *Yersinia* lacking SycE, the cognate chaperone of the effector YopE, is able to secrete YopE (31). In this SycE-deficient strain, the total level of YopE is about half of that in a wild-type strain (31), but the total percentage of secreted YopE is about the same in wild-type and SycE-deficient strains (31). Therefore, loss of SycE decreases the steady-state expression level of YopE but does not affect the efficiency of secretion. Additionally, short N-terminal regions of YopE (N-terminal 11 or 15 residues) or YopH (N-terminal 17 residues) are sufficient to drive the secretion of Cya or NPT (31, 206, 214). These N-terminal regions do not include the chaperone-binding regions of YopE or YopH, and so the secretion of these fusion proteins appears to occur independently of chaperone action. Additionally, the *P. aeruginosa* effector ExoU is secreted in the absence of its cognate chaperone SpcU, albeit at low levels (77). Similarly, low levels of SycE-independent type III secretion of YopE are seen when YopE is expressed in *X. campestris* pv. vesicatoria (197).

In contrast to the above examples of chaperone-independent secretion, type III secretion of effectors in *Salmonella* is dependent on chaperones. Secretion of the *Salmonella* effectors SptP and SopE through the TTSS requires the presence of the chaperone-binding domain of these proteins, presumably re-

flecting the requirement for chaperone action (135). Interestingly, variants of SptP and SopE lacking their chaperone-binding regions are still secreted, but this occurs through the flagellar apparatus rather than through the TTSS. Both the chaperone-binding regions and N-terminal putative secretion signals of SptP and SopE are required for secretion through the TTSS.

Might this scenario of effectors being secreted through the flagellum operate in *Yersinia*, thereby explaining why secretion does not depend tightly on chaperone action? It seems unlikely, since the TTSS and the flagellar system in *Yersinia* are reciprocally regulated (18), and experimental conditions used to trigger secretion through the *Yersinia* TTSS discourage secretion through the flagellum (251). However, the notion of whether disruption of chaperone binding diverts the secretion of effectors toward the flagellar pathway in *Yersinia* needs to be tested explicitly.

It should be noted that under certain circumstances secretion in *Yersinia* is observed to depend on chaperone action. This is the case for YopE fusion proteins that include the YopE chaperone-binding region. Secretion of a YopE-Cya fusion protein containing the N-terminal 130 residues of YopE, which includes the chaperone-binding region, was found to require SycE (246). However, secretion of a fusion protein containing just the N-terminal 40 residues of YopE, which lacks most of the chaperone-binding region, was found to be independent of SycE (246). It is possible that addition of the Cya fusion partner to YopE influences the behavior of the chaperone-binding region, such that SycE is required for secretion of YopE-Cya but not of native YopE (31, 246).

Chaperones and Translocation

The functional importance of chaperone action is most clearly seen for the process of effector translocation into host cells as opposed to secretion into the extracellular medium. Chaperones greatly enhance the efficiency of effector translocation, although the requirement for chaperone action does not appear to be absolute. Deletion of the chaperone SycE does not entirely eliminate translocation of YopE and, instead, results in levels of translocated YopE that are ~40-fold lower than wild-type levels (87). The impairment in YopE translocation caused by deletion of SycE gives rise to an avirulent strain of *Yersinia*, which has a 450-fold-higher 50% lethal dose (LD_{50}) than a wild-type strain when introduced into mice by an intraperitoneal route (78, 193). This increase in the LD_{50} is similar to that resulting from deletion of YopE itself or loss of the virulence plasmid encoding the TTSS, indicating a crucial role in physiology for chaperone action (79, 193). Similarly, deletion of SycH results in avirulence in mouse models of disease (56), paralleling the avirulence resulting from deletion of YopH (193).

An exception to the crucial role of chaperones in directing effector translocation is seen in a *Yersinia* strain that is missing almost all effectors (23). This strain lacks the effectors YopH, YopO, YopP, YopE, and YopM (ΔHOPEM) and is able to efficiently translocate a YopE-Cya fusion protein containing just the N-terminal 15 YopE residues. This fusion protein contains only the putative N-terminal signal sequence and not the chaperone-binding region of YopE (23). The authors also show that a strain with the effectors YopT, YopH, and YopE deleted (ΔTHE) is capable of translocating a version of YopE in which the chaperone-binding region (residues 17 to 77) is deleted (23). These results indicate that chaperones are not required for translocation when other effectors are missing but are required in a wild-type background. The implications of these results are discussed below.

Temporal Phases of Effector Transport

Intriguingly, loss of SycE has an additional effect on YopE translocation besides the approximately 2-fold-lower levels of expression and ~40-fold lower levels of translocation (31, 87). This additional effect is a temporal one, in which translocation of YopE is delayed (87). Various pieces of evidence suggest that there are two temporal phases to effector secretion and translocation, with an early phase occurring immediately on activation of the TTSS and a later phase following activation. It appears that at least some effectors are synthesized prior to activation and await translocation as fully translated effectors or chaperone-effector complexes. Host cell contact or other stimuli then lead to the translocation of effectors from these presynthesized pools in a posttranslational manner. It appears that chaperone action is required in the early phase of translocation but perhaps not in the later, postactivation phase.

Evidence for this model is derived mainly from experiments on YopE secretion. By arresting protein synthesis in *Yersinia* with chloramphenicol and then shifting bacteria to conditions that induce secretion, it was observed that YopE was secreted by wild-type but not by SycE-deficient *Yersinia* strains (142). Similarly, a pulse-chase labeling study using [^{35}S]methionine showed that YopE is secreted posttranslationally by wild-type but not by SycE-deficient *Yersinia* strains (32). It was also observed that YopE is secreted immediately after induction of the TTSS in wild-type *Yersinia* strains but that secretion is significantly delayed in SycE-deficient *Yersinia* strains (142). These results suggest that SycE is required for an early phase of posttranslational secretion of YopE but not for a late phase of YopE secretion. Similarly, the *Shigella* chaperone Spa15 is also required for posttranslational secretion of its cognate effector IpaA (171). In a Spa15-deficient strain, secretion of IpaA is significantly delayed, consistent with the two phases of secretion observed for YopE. It is tempting to speculate that the late phase of secretion occurs cotranslationally, but the existence of cotranslational type III secretion is controversial. Evidence both supporting and disputing cotranslational secretion of *Yersinia* YopQ has been reported (9, 230).

Indirect evidence suggests that, like secretion, translocation occurs in two temporal phases, an early chaperone-dependent process and a later chaperone-independent phase. The cytotoxic response induced by YopE is significantly delayed in a SycE-deficient strain compared to a wild-type strain (87, 219). This suggests that translocation of the 40-fold-lower levels of YopE in the absence of SycE occurs by a postactivation, chaperone-independent mechanism (87). The effect of chaperone action on the temporal nature of effector translocation has not been explored in other cases. Nevertheless, it has been shown that YopH is translocated into host cells within the first minute of host cell contact, as seen by blockage of calcium transients in human neutrophils due to YopH action (11). It will be

interesting to see whether this immediate effect depends on chaperone action and whether a late-phase action of YopH is detectable in the absence of chaperone action.

MODELS OF CHAPERONE ACTION

How do chaperones promote the translocation of their cognate effectors? In considering this question, two important points are worth bearing in mind.

The first point is that not all effectors appear to have cognate chaperones. Therefore a class of effectors is apparently translocated in a chaperone-independent manner (Fig. 2). In *Yersinia*, the apparently chaperone-independent effectors are YopM (46 kDa), YopO (81 kDa), and YopP (33 kDa). Although it is difficult to rule out the possibility that these effectors have undiscovered chaperones, the likely chaperone candidates for these effectors have been ruled out by the creation of a mini-virulence plasmid version of the 70-kb *Y. enterocolitica* virulence plasmid (231). This miniplasmid excludes all known effectors and chaperones (i.e., SycE, SycH, and SycT), as well as so-called orphan chaperones (e.g., ORF155 and ORF91B), which lack identified cognate effectors (231). Both YopM and YopP are translocated by *Yersinia* strains carrying the mini-virulence rather than the full virulence plasmid (231). Of course, this does not exclude the possibility that YopM and YopP have chaperones encoded in the portion of the TTSS retained in the mini-virulence plasmid or in the *Y. enterocolitica* chromosome. The portion of the TTSS retained in the miniplasmid encodes the SycE-like chaperones YscB and YscG, but these two chaperones have been shown to function with proteins other than YopM and YopP (53, 54). It would also be highly unusual in *Yersinia* to find a chromosomally encoded chaperone. Therefore, although not definitive, the weight of evidence favors the notion that YopM, YopO, and YopP are translocated independently of chaperone action.

The second point is that a number of experiments indicate that chaperones act locally, just on the chaperone-binding regions of effectors, rather than globally on the entire effector. For example, the chaperones SycE and SicP protect only the chaperone-binding region of their respective effectors and not distal regions, such as catalytic domains, from proteolysis (16, 215). Additionally, chaperones have no effect on the catalytic or binding activities of domains of effectors distal to the chaperone-binding region (16, 144). Lastly, SycE, complexed with the chaperone-binding region of YopE, constitutes a domain that is independently folded and separate from the catalytic RhoGAP domain of YopE (16). Therefore, models of chaperone action need to account for the nature and consequence of local action on the chaperone-binding region of effectors.

Passive Protection

A simple model for chaperone action is passive protection. In this model, the chaperone-binding region would have a function separate from binding the chaperone, for example, a role in the host cell. The chaperone would then fulfill a protective function in the bacterial cytosol, shielding the chaperone-binding region from possible aggregation, proteolytic destruction, or premature activity. These protective functions would be fulfilled simply through passive binding of the chaperone-binding region.

The clearest example of a chaperone-binding region having a host cell function is for YopH, the protein tyrosine phosphatase whose chaperone-binding region, in addition to binding the cognate chaperone SycH, binds phosphotyrosines of substrate proteins in host cells (17, 159, 254). Disruption of phosphotyrosine binding but not chaperone binding in the YopH chaperone-binding region results in *Yersinia* with a 10-fold lower LD_{50} in mice injected with bacteria intraperitoneally, compared with a 20-fold lower LD_{50} when YopH is entirely absent (55). The chaperone-binding domain of YopH is well structured and not prone to aggregation in the absence of its chaperone SycH (68, 121, 212, 254). Since YopH does not need to be shielded from potential substrates in the bacterial cytosol or to be disaggregated, it seems unlikely that SycH functions by providing a passive protective function to the chaperone-binding region of YopH.

The protective model of chaperone action has been suggested for SycE, since the chaperone-binding region of YopE in its free state is highly prone to aggregation. Furthermore, SycE prevents YopE aggregation and consequent degradation. The half-life of intrabacterial YopE is diminished approximately 2.5-fold in a SycE-deficient strain compared to that in a wild-type strain, and the steady-state level of YopE is approximately 2-fold lower in a SycE-deficient strain (31, 32, 87). The distribution between soluble and insoluble forms of YopE changes dramatically between wild-type and SycE-deficient strains (32). In a wild-type strain, 95% of YopE is soluble, as assessed by ultracentrifugation (32). The remaining 5% of YopE is hypothesized to be associated with the membrane-embedded TTSS apparatus in the process of being secreted (32). In contrast, 100% of YopE is insoluble in a SycE-deficient strain. One-third of this insoluble YopE appears to be peripherally associated with membranes (extractable with 1 M potassium acetate), and the remaining two-thirds may be either membrane embedded or aggregated. Heterologous expression of YopE in *E. coli* in the absence of SycE leads to YopE aggregation as well, but coexpression of YopE with SycE results in the formation of heterotrimeric complexes that can be concentrated in vitro to a remarkable 5 mM (16).

The most aggregation-prone part of YopE appears to be the last half of the chaperone-binding region, residues 50 to 78. This segment appears to have a profound effect in inhibiting secretion in the absence of SycE in some experiments but no apparent effect in others (23, 31). Most interestingly for the protective model of chaperone action, YopE residues 50 to 78 have also been reported to have a host cell function, in that they favor membrane localization (126). When fused to GFP, YopE residues 50 to 78 direct 37% of the fusion protein to a host cell membrane fraction, as compared to 5% for unfused GFP (126). However, YopE residues 50 to 78 are also dispensable for host cell functions. This region is not required for promoting actin reorganization, rounding up of cells, or cytotoxic action (23, 126). These results describing a nonessential role for YopE residues 50 to 78 cast doubt on the passive-protection model for SycE. It is unlikely that chaperones exist only to disaggregate aggregation-prone portions of effectors that have no essential function except for conferring chaperone binding.

It may be that the aggregation-prone behavior of YopE residues 50 to 78 and other chaperone-binding regions is an epiphenomenon. It is possible that the function of chaperone-binding regions relies entirely on their chaperone-bound states and that these regions are under no selective pressure to have a defined structure in their free states. Nevertheless, the question arises of how YopE and other aggregation-prone effectors are able to remain soluble in mammalian cells. The number of YopE molecules required to achieve cytotoxicity is not known. But for the *P. aeruginosa* effector ExoU, it has been demonstrated that half-maximal cytotoxicity requires translocation of 300 to 600 ExoU molecules (180). In a bacterial cell, 300 to 600 molecules correspond to a concentration in the tens of millimolar range, whereas in a typical mammalian cell, 300 to 600 molecules correspond to a concentration in the tens of micromolar range. The dilution effect in going from bacterial to mammalian cells may be enough to prevent aggregation of certain effectors.

Lastly, the chaperone-binding region of the *Salmonella* effector SopE has been implicated in functioning as a target for ubiquitination in host cells (129). The SopE chaperone-binding region has not been shown to be directly ubiquitinated, but it seems reasonable that if this were the case, this region would probably lack ordered structure and would need to be protected in the bacterial cytosol. Replacement of the SopE chaperone-binding region with that from the *Salmonella* effector SptP results in stabilization of SopE (129), suggesting that the SptP chaperone-binding region is a less efficient target for ubiquitination. The chaperone-binding region of SptP has not been implicated in host cell function, and it is not clear why it would need passive protection. Therefore, while it is possible that passive protection is one function of certain chaperones, for example for InvB in possibly protecting the region of SopE that later gets ubiquitinated (61, 134), the requirement for protection does not appear to be general, suggesting that chaperones have functions in translocation besides passive protection.

Secretion Competency

An alternative to the passive-protection model is an active unfolding model, in which the chaperone is posited to maintain an unfolded or partially folded secretion-competent state in the effector. The extended nature of the chaperone-binding regions of SptP and YopE bound to their respective chaperones immediately raises this possibility (16, 215). The reasoning is that the TTSS needle appears to have a limiting inner diameter of only about 20 Å, as seen by electron microscopy (19, 107), and folded proteins generally have dimensions greater than 20 Å. For example, the smallest dimension of the RhoGAP domain of YopE is 25 Å (69).

No structure of an intact chaperone-effector complex exists to indicate whether the entire effector is unfolded, but biochemical evidence suggests that it is not. First, the *E. coli* effector Tir has as much affinity for the protein intimin in its free state as in its chaperone-bound state (CesT-Tir) (178). Second, the *Salmonella* effector SigD has as much inositol phosphatase activity in its free state as in its chaperone-bound state (SigE-SigD) (144). Lastly, YopE has as much RhoGAP activity in its free state (using a GST-YopE fusion protein for reasons of biochemical tractability) as in its chaperone-bound state (SycE-YopE) (16). These results argue that the chaperone does not unfold the effector globally or maintain the effector in a globally unfolded state, reinforcing the notion that chaperones act locally and not globally.

While the catalytic or binding domains of effectors are not unfolded in chaperone-effector complexes, it is possible that chaperones maintain just the chaperone-binding regions of effectors in an unfolded state. The notion that unfolding of the chaperone-binding region is necessary for interaction with the chaperone would seem to be the case for YopH. While the structure of the SycH-YopH complex is unknown, it is reasonable to assume that the chaperone-binding region of YopH will adopt the extended structure seen for the chaperone-binding regions of SptP and YopE in complex with their respective chaperones (16, 215). In its free state, the YopH chaperone-binding region (residues 20 to 70) does not form an independent structural unit but, instead, contributes a coil, a β-hairpin, and an α-helix (α2) that pack against the body of a compact globular structure, which is composed of a four-helix bundle surrounded by two β-hairpins (Fig. 3) (68, 121, 212). Therefore, it is likely that chaperone binding requires unfolding of the N-terminal domain of YopH.

Is the requirement for unfolding of the chaperone-binding region to allow chaperone binding general? It may be that YopH represents a special case, in that its chaperone-binding region plays a clearly defined alternative role in binding phosphotyrosines in host cells (17, 159). Unfortunately, the structures of the chaperone-binding regions of SptP and YopE are known only in their chaperone-bound states and not in their free states. However, an indirect argument has been made that the chaperone-binding region of YopE is likely to lack ordered structure in the free state. This comes from the observation that partially purified YopE is able to bind SycE in vitro without a prior refolding step, suggesting that the chaperone-binding region does not need to be unfolded in order to adopt the extended structure required for chaperone-binding (16).

It is entirely possible that, for YopE and perhaps other effectors, the chaperone may, rather than unfolding the chaperone-binding region, confer structure on an otherwise disordered region. A disorder-to-order mode of binding has been observed previously and is well characterized for binding of a cyclin/cyclin-dependent kinase (CDK) inhibitor to a cyclin-CDK complex. A region of the cyclin-CDK inhibitor $p27^{Kip1}$ binds phosphorylated cyclin A-Cdk2 in a highly extended fashion (201). This region of $p27^{Kip1}$ lacks a hydrophobic core and forms only short stretches of secondary structure (201), highly reminiscent of the extended nature of chaperone-binding regions bound to chaperones. The buried surface area of 5,752 $Å^2$ for the $p27^{Kip1}$-cyclin A-Cdk2 complex is also similar to that seen for chaperone-effector complexes. Most interestingly, free $p27^{Kip1}$ is disordered in its free state but ordered in its bound state, as shown directly by NMR spectroscopy (132).

Even if chaperones were to unfold chaperone-binding regions, what would this accomplish? One argument is that it may prime the unfolding of the rest of the effector. Since the chaperone-binding region of YopH forms an integral part of a structured domain, this is a possibility for SycH binding to YopH, but it is not clear whether unfolding of the N-terminal YopH domain would be transmitted to the C-terminal catalytic domain. For YopE, it seems unlikely that unfolding of the

chaperone-binding region would be transmitted to its catalytic domain, since these two portions form independently folded domains (16). Therefore, unfolding of the chaperone-binding domain would seem to provide no advantage in the unfolding of other domains of effectors, either by the chaperone itself or by other bacterial components.

The secretion competency model also raises the question of how effectors that apparently lack chaperones, such as YopM, YopO, and YopP, are able to be translocated (231). The translocation signal of YopM is located in its N-terminal 100 residues, with the N-terminal 40 residues being sufficient for translocation and the N-terminal 100 residues conferring more efficient translocation, as assessed using YopM-Cya fusions (Fig. 3) (22). This region in YopM, starting from residue 34, forms an integral part of its structure (66). Residues 34 to 100 form a two-α-helix element and slightly more than one leucine-rich repeat (66). It is not clear how this region would act in promoting translocation, but no special features of protein sequence or structure appear to distinguish chaperone-dependent effectors from those that are apparently chaperone independent (Fig. 2).

Protein Unfolding in Transport

Are proteins in an unfolded state when being transported by the TTSS? Recent experiments using DHFR fusions to YopE provide initial support for this hypothesis (73, 137). Most globular proteins would need to be in an extended and nonnative state if they are transported through the narrow channel in the center of the needle. This has led to the idea that secretion should be enhanced by lowering the stability of an effector. The effector would then be unfolded or maintained in an unfolded state more easily. In support of this model, the N-terminal putative secretion signal (residues 1 to 16) of YopE is incompetent to promote the secretion of fused wild-type DHFR but is competent to promote the secretion of a DHFR mutant with lowered stability (73). Similar results have been shown for wild-type and a destabilized mutant of ubiquitin (137). It is not known whether Cya, NPT, or other heterologous proteins that are capable of being secreted through the action of the N-terminal putative secretion signal of YopE are less stable than DHFR or ubiquitin. Denaturation experiments with YopH and with the RhoGAP effector domain of YopE reveal the stabilities of these proteins or domains to be ~6 and ~7 kcal/mol, respectively (16, 254). These values do not seem unusually low and are similar to the ~5 to 6 kcal/mol measured for DHFR (228), making it difficult to explain why YopE and YopH are observed to be secreted but DHFR is not. Significantly, YopE residues 1 to 18 are sufficient to promote the secretion of GFP (115). GFP is a very stable protein, maintaining native structure in 6 M urea (190) and having an apparent melting temperature of ~80°C (227). The ability of GFP to serve as a type III secretion substrate indicates that the relationship between protein stability and secretion is not simple.

The role of the chaperone in these stability experiments is perplexing. It appears that SycE is necessary for secretion of wild-type DHFR but not of destabilized DHFR, suggesting that SycE promotes the unfolding of DHFR (73). However, the authors of this study found that SycE promotes the secretion of DHFR fusion constructs containing the N-terminal 52 YopE residues (YopE52), which constitute only half of the chaperone-binding region, but not of DHFR fusion constructs containing the N-terminal 80 residues of YopE, which constitute the entire chaperone-binding region. Indeed, DHFR fusions containing the entirety of YopE are also not secreted. Fusion proteins containing the N-terminal 80 residues of YopE or all of YopE prevent the secretion of other effectors by presumably blocking the secretion apparatus (73). The authors argue that the shorter chaperone-binding region in fusion constructs containing just the N-terminal 52 YopE residues brings SycE closer to DHFR and therefore promotes unfolding more efficiently. It is difficult to determine why this issue of spacing is not a problem for native YopE or other fusion partners, such as Cya.

A classic question with DHFR fusions is whether the addition of folate analogs, such as aminopterin, blocks secretion. This is because binding of folate analogs by DHFR confers great stability to the protein and acts as an indicator of whether DHFR needs to be unfolded for transport. Using a construct containing the N-terminal 52 residues of YopE fused to DHFR, it was found that the addition of aminopterin does not prevent secretion of this fusion protein (73). Aminopterin appears to gain access to the bacterial cytosol to bind the fusion protein, since its application results in inhibition of bacterial growth, requiring the use of an aminopterin-resistant DHFR in the experiment. The inability of aminopterin to block secretion suggests that the fusion protein, which presumably has SycE bound to it, either is unfolded in vivo prior to secretion or does not need to be unfolded for secretion. The former view would fit the in vivo results obtained with DHFR and ubiquitin stability mutants and would also lend credence to an unfolding role for SycE (73, 137), but it would contrast with in vitro findings that catalytic effector domains in chaperone-effector complexes are folded (16, 144).

Chaperone-Effectors as Targeting Motifs

Another possibility for the mode of chaperone action involves targeting. Chaperones, in complex with their effectors, may act as three-dimensional targeting motifs that specify interactions with the TTSS apparatus or other bacterial components required for translocation (16, 141). The *E. coli* effector Tir and its cognate chaperone CesT interact individually with the type III ATPase EscN (91). It is not clear whether interaction with type III ATPases is a general feature of chaperones and effectors. While no direct evidence exists for the targeting model, numerous pieces of indirect evidence support this model.

First, it has been found that chaperones in *Salmonella* control the pathway of effector secretion (135). When the chaperone-binding regions of the *Salmonella* effectors SptP and SopE are deleted, the resulting proteins are secreted through the flagellar apparatus instead of through the TTSS. This contrasts with the observation that intact effectors containing the chaperone-binding regions are secreted through the TTSS apparatus and not through the flagellar apparatus (135). For both type III and flagellar secretion of these proteins, the N-terminal putative signal sequence was seen to be required. In addition, both the N-terminal putative signal sequence and the chaperone-binding region of SopE were required for host cell

translocation, as assessed indirectly through effects on bacterial internalization. As expected, translocation occurred only through the TTSS and not through the flagellar apparatus. These experiments suggest that the chaperone-binding region, complexed with the chaperone, is required to target effectors to the TTSS apparatus or other bacterial components in a process that also requires the putative N-terminal secretion signal. In the absence of the chaperone-binding region, the putative N-terminal secretion signal appears to be recognized by the flagellar apparatus, directing secretion through that system. These experiments also call into question the secretion competency model of chaperone action, since the flagellum and TTSS needle have similar inner diameters and it is difficult to see why secretion through one would require maintenance of an unfolded state by the chaperone but secretion through the other would not.

Second, a dominant negative form of a chaperone has been identified. A GST-SycE fusion protein was found to be unable to promote secretion or translocation of YopE, although this fusion protein is capable of binding YopE (32). This GST-SycE fusion also interfered with normal SycE-dependent secretion or translocation of YopE, therefore acting in dominant negative fashion (32). These results indicate that association between SycE and YopE by itself is not sufficient for translocation and suggest a targeting role for the chaperone-effector complex. To explain the dominant-negative phenotype of GST-SycE, it was proposed that chaperone-effector complexes containing GST-SycE interact nonproductively with the TTSS apparatus and compete with native chaperone-effector complexes.

Third, secretion that occurs via chaperone action rather than by means of the N-terminal putative secretion signal has been observed for YopE (Fig. 2). A YopE variant missing its first 15 residues and fused to NPT was found to be secreted, albeit at six- to sevenfold-lower levels than a fusion protein containing all of YopE (31). This secretion in the absence of the N-terminal putative secretion signal was found to require the action of SycE (31). Furthermore, a variant of YopE, with its N-terminal putative secretion signal disabled by a +1 frameshift, was found to drive secretion of NPT, but only in a strain expressing SycE (31). Similar results were seen when the N-terminal putative secretion signal of YopE alone (without a fusion partner) was disrupted by +1, −1, +2, or −2 frameshifts (142). These mutant forms of YopE were not secreted in the absence of SycE but were secreted in its presence. However, very curiously, if the secretion signal was disabled through deletion rather than frameshifting, SycE was unable to direct the secretion of YopE but was able to direct the secretion of a YopE-NPT fusion (31, 142). Additionally, if the putative N-terminal secretion signal in YopE was disabled by replacement with a synthetic sequence containing serines and isoleucines in residues 2 to 8, SycE was unable to direct secretion (142). Taken together, these results suggest that the chaperone-effector complex specifies interactions with bacterial components required for type III secretion and that this targeting motif complements certain types of disablement of the N-terminal putative secretion signal.

Interestingly, YopE variants with the N-terminal putative secretion signal disabled are still translocated into mammalian cells in a SycE-dependent fashion, suggesting that the putative chaperone-effector targeting signal is sufficient for translocation in this case (142). These conclusions are at odds with those found for *Salmonella* SptP and SopE, which require both N-terminal putative secretion signals and chaperone-binding regions for type III secretion and translocation (135).

Lastly, it has been found that overexpression of YopE or YopO results in decreased translocation of YopE-Cya fusions that contain just the N-terminal 15 YopE residues but not of those that contain the N-terminal 130 residues (23). The former construct contains solely the N-terminal putative secretion signal, whereas the latter contains both this region and the chaperone-binding region. Therefore, it appears that the N-terminal putative secretion signal is unable to confer competition for the TTSS apparatus against overexpressed effectors and that chaperone action is required for this, consistent with a targeting role for the chaperone-effector complex. It has also been found that the N-terminal putative secretion signal is insufficient for YopE translocation by wild-type *Y. enterocolitica* but is sufficient for YopE translocation by *Y. enterocolitica* lacking the effectors YopH, YopO, YopP, YopE, and YopM (ΔHOPEM) (23). This is consistent with the view that chaperone-effector complexes are required for competitive access to the TTSS apparatus or other bacterial components involved in translocation.

The mechanism by which the chaperone-effector complex may operate in targeting is not known, but there are general similarities between the structures of SicP-SptP(Cb) and SycE-YopE(Cb) that are suggestive (16). Structural conservation of these two chaperone-effector complexes despite their high sequence divergence and the existence of functional complementarity in the TTSS suggest that a possible three-dimensional target motif has been maintained in these complexes through selective pressure. A thorough test of this idea will require identification of discrete portions of chaperone-effector complexes involved in targeting, the identity of these targets, and analysis of the functional consequence of interactions between chaperone- effector complexes and these targets. Without this sort of information, it will be difficult to determine which is more meaningful, the structural similarities or differences observed for chaperone-effector complexes.

Hierarchy of transport. Why would two signals, one putatively at the N terminus and one specified by the structure of the chaperone-effector complex, be necessary? Is it possible that a combination of signals modulates the success of association of effectors with the TTSS apparatus, perhaps resulting in differential efficiencies of translocation or a temporal hierarchy to translocation (141)? The initial burst of translocation that follows host cell contact may come primarily from chaperone-effector complexes that outcompete effectors lacking cognate chaperones. This would occur in a posttranslational, chaperone-dependent manner. As pools of chaperone-effector complexes are depleted through translocation, effectors lacking cognate chaperones may be translocated. This later phase of translocation may be chaperone independent and possibly cotranslational. In this way, *Yersinia* may coordinate an immediate antiphagocytic response through initial translocation of YopE, YopH, and YopT and then modulate longer-term responses; such as inhibition of host inflammatory responses, through later translocation of effectors such as YopP.

This model is highly speculative, and indeed temporal control of the *Salmonella* effectors SptP and SopE has been shown to rely on host mechanisms rather than chaperone action (129). However, evidence for hierarchy of secretion in chaperone-dependent effectors has been demonstrated for YopE and YopH. It has been observed that overexpression of the chaperone SycH and the negative regulator LcrQ, which presumably exists as SycH-LcrQ complexes, blocks the secretion of YopE (247). In contrast, deletion of LcrQ results in early secretion of YopE. These results support a model in which a staged order of secretion occurs. The initial event would entail the secretion of LcrQ from SycH-LcrQ pools, which would then free SycH to bind to and direct the secretion of YopH. After YopH is secreted, YopE would then be secreted. Supporting this model, deletion of YopH also results in early secretion of YopE. Overexpression of YopH, however, has little effect on YopE secretion, suggesting that the temporal ordering of YopH followed by YopE may be set by SycH-YopH complexes having greater affinity than SycE-YopE complexes for bacterial components required for type III secretion.

CONCLUSION AND FUTURE DIRECTIONS

The TTSS is crucial to determining the outcome of interactions between many gram-negative bacterial pathogens and host organisms. Much progress has been made in identifying components of the TTSS apparatus and in characterizing the phenotypes of mutations in genes encoding these components. However, it has also been realized that the TTSS is immensely complex, with many proteins serving multiple and disparate functions. Fundamental aspects of how proteins are recognized for secretion or translocation and transported into host cells are unknown and form active areas of research. These processes are beginning to be dissected biochemically and structurally, leading to the formulation of mechanistic hypotheses. For example, the structural basis for interaction between TTSS chaperones and effectors has been elucidated, and this provides detailed functional models whose predictions need to be tested through further study. In the future, quantitative analysis of the interactions of TTSS components will be required to disentangle contradictory data and competing hypotheses. Receptors for signals that target effectors to the TTSS for transport need to be identified, and various TTSS processes need to be reconstituted in vitro. These efforts are essential not only to a basic understanding of this highly complex secretory system but also to the design of antimicrobial strategies that broadly target bacteria utilizing the TTSS.

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