Review

Type III protein secretion mechanism in mammalian and plant pathogens

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Abstract

The type III protein secretion system (TTSS) is a complex organelle in the envelope of many Gram-negative bacteria; it delivers potentially hundreds of structurally diverse bacterial virulence proteins into plant and animal cells to modulate host cellular functions. Recent studies have revealed several basic features of this secretion system, including assembly of needle/pilus-like secretion structures, formation of putative translocation pores in the host membrane, recognition of N-terminal/5′ mRNA-based secretion signals, and requirement of small chaperone proteins for optimal delivery and/or expression of effector proteins. Although most of our knowledge about the TTSS is derived from studies of mammalian pathogenic bacteria, similar and unique features are learned from studies of plant pathogenic bacteria. Here, we summarize the most salient aspects of the TTSS, with special emphasis on recent findings.
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1. Introduction

Bacterial pathogens are responsible for numerous diseases in higher plants, animals, and humans, including such commonly known ones as human bubonic plague and diarrhea and plant wilt, blight, cankers, and leaf spots. These pathogens are very diverse in their taxonomic properties and hosts, and in the disease symptoms they cause. Thus, it was initially thought that each disease might be caused by a distinct molecular mechanism. However, in the past two decades, research into the molecular basis of bacterial pathogenesis has led to the conclusion that although different bacteria may use unique mechanisms to subvert hosts, a few strategies are common. One striking example is the discovery that many plant and animal bacterial pathogens contain members of a family of protein secretion systems classified as type III [1]. An interesting feature of the type III secretion systems (TTSS) is their ability to deliver bacterial virulence-associated "effector" proteins directly into host cells, which was first demonstrated for *Yersinia* effector proteins, Yops (for *Yersinia* outer protein), 10 years ago [2,3]. Prior to this remarkable demonstration, *Yersinia* was known to secrete Yops in culture [4,5]. In plant pathogenic bacteria, a cluster of hypersensitive response and pathogenicity (*hrp*) genes was identified in *Pseudomonas syringae* in 1986 [6]. The *hrp* genes are required for bacteria to trigger the hypersensitive response (HR, a plant defense response) in resistant plants and to cause disease in susceptible plants. But it was not until 1993–1994 that *hrp* genes were shown to be involved in the secretion of bacterial proteins (known as harpins) in culture, thus defining the secretion function for the enigmatic *hrp* genes [7–9]. In 1996, several groups showed that type III effectors of *Pseudomonas* and *Xanthomonas* function inside the plant cells, suggesting that the *hrp*-encoded TTSS, like the *Yersinia* TTSS, is also involved in the delivery of effector proteins directly into the host cytosol [10–14]. The intracellular action of type III effectors in plant cells has had a great impact on research in the field of plant–pathogen interactions that continues to this day. Recently, translocation of type III effectors in plant–path-

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ogen interactions has also been observed more directly by using an adenylate cyclase fusion-based assay and immunofluorescent microscopy [15,16].

The TTSS is not restricted to pathogenic bacteria; plant and insect symbiotic bacteria also use the TTSS to interact with their hosts [17–19]. Most bacteria appear to contain only one virulence-associated TTSS, but *Salmonella enterica*, *Yersinia enterocolitica* biotype 1B, *Y. pestis*, enterohemorrhagic *Escherichia coli* (EHEC), and *Burkholderia pseudomallei* have two TTSSs [20–22]. TTSS-encoding genes are almost always clustered in the chromosome or on the plasmid as part of a large pathogenicity island (PAI; Ref. [23]). These PAIs often have G+C contents different from the rest of the chromosome and are frequently flanked by remnants of mobile DNA elements, suggesting acquisition by horizontal transfer.

The central importance of the TTSS in mammalian and plant bacterial pathogenesis is underscored by the finding that a defect in this system often leads to a complete loss of bacterial pathogenicity [6,24–32]. Because of its importance in host–bacteria interactions, as well as its remarkable ability to deliver bacterial proteins directly into the host cell, type III secretion has received much attention in the past decade [33]. The literature on the TTSS is now so extensive that a comprehensive description of all aspects of all TTSSs could no longer fit into a short review. Our review thus will summarize only the most salient aspects of the type III secretion mechanism learned from selected bacterial models and will particularly highlight the recent studies. At appropriate places in this chapter, we will refer readers to many excellent reviews on specific and broad aspects of type III secretion. We apologize to authors whose work is not discussed in detail here.

2. Role of the TTSS in pathogen biology

TTSSs are used for different purposes in different bacteria. For example, intracellular pathogens such as species of *Salmonella*, *Shigella*, and *Chlamydia* use TTSSs for invasion of and/or multiplication within host cells [34–36]. *Yersinia* spp. use the TTSS to resist the uptake of bacteria by phagocytic cells in the later stages of pathogenesis [37,38]. The TTSS of *Pseudomonas aeruginosa* (most strains are extracellular pathogens) induces significant morphological changes in lung epithelial cells, alters the normal actin cytoskeleton, and induces apoptosis in infected macrophages, thereby inhibiting phagocytosis [39]. Pathogenic *E. coli* strains that cause diarrheal disease use the TTSS to deliver effector proteins that result in intimate bacterial attachment to and effacement of the microvilli from intestinal epithelial cells, eventually leading to the formation of attaching/effacing (A/E) lesions [40]. Plant pathogenic bacteria encounter a unique eukaryotic cell type that is enveloped by a cell wall. Plant pathogenic bacteria multiply predominantly in the intercellular space outside of the plant cell wall and are therefore extracellular pathogens [41]. The TTSS of *P. syringae* is used to counter plant defenses and possibly to release nutrients from the host cell [42,43].

3. Phylogenetic relationship between TTSSs

Phylogenetic analysis suggests that various TTSSs can be organized into five groups: (i) the Ysc group, including the plasmid-borne *Yersinia* Ysc TTSS, the *P. aeruginosa* Psc TTSS, the *Bordetella* Bsc TTSS, the *Rhizobium* Rsc TTSS, and the *Chlamydia* TTSS; (ii) the Hrp1 group, including *P. syringae* and *Erwinia* TTSSs; (iii) the Hrp2 group, including *Xanthomonas* and *Ralstonia* TTSSs and one of the two *Burkholderia* TTSSs; (iv) the Inv/Mxi/Spa group, including the TTSS encoded by *Salmonella* pathogenicity island 1 (SPI-1), the *Shigella* TTSS, the *Y. enterocolitica* Ysa TTSS, TTSS2 of EHEC, the other *Burkholderia* TTSS, and the *Sodalis* TTSS; and (v) the Esa/Ssa group, including the TTSS of enteropathogenic *E. coli* (EPEC), TTSS1 of EHEC, the *Salmonella* SPI-2 TTSS, and the chromosomal TTSS of *Y. pestis* (Ref. [21]; Fig. 1). Interestingly, there does not seem to be any similarity between the TTSS-based phylogenetic tree and the rRNA-based phylogenetic tree, the latter of which indicates the evolutionary relationship among species. This discord further supports the idea that TTSS-associated PAIs have been transferred horizontally among phylogenetically unrelated bacteria.

Many core components of virulence-associated TTSSs share strong sequence similarities with those involved in flagellar assembly in Gram-negative bacteria (Table 1). Furthermore, there are striking similarities in the morphology, assembly, and mechanisms of secretion and regulation between virulence-associated TTSSs and flagella, as will be discussed here and in the chapter by Macnab (also see Ref. [44]). It has been widely assumed that virulence-associated TTSSs have evolved from a TTSS involved in the assembly of flagella. This belief is intuitive because flagella are ancient structures and are almost ubiquitously distributed among bacteria, whereas, to date, virulence-associated TTSSs are found only in bacteria that have an intimate interaction with eukaryotic hosts, which have evolved more recently. Furthermore, under special conditions or mutant backgrounds, both virulence-associated TTSSs and the flagellar TTSS can secrete the same proteins, phospholipase YplA in *Yersinia* and SptP in *Salmonella* [45,46]. In a recent analysis, Gophna et al. [47] examined sequences of four of the conserved proteins that comprise the TTSS apparatus of plant and animal pathogens and compared the sequence divergence to the flagellar homologues. Their analysis does not support the idea that the virulence-associated TTSS evolved from an ancestral flagellar TTSS. That is, there is no evidence from the molecular evolutionary data indicating that the virulence-associated TTSS components were derived from an ancestral flagellar apparatus. For example, a molecular phylogeny of homologues of the HrcV reveals a
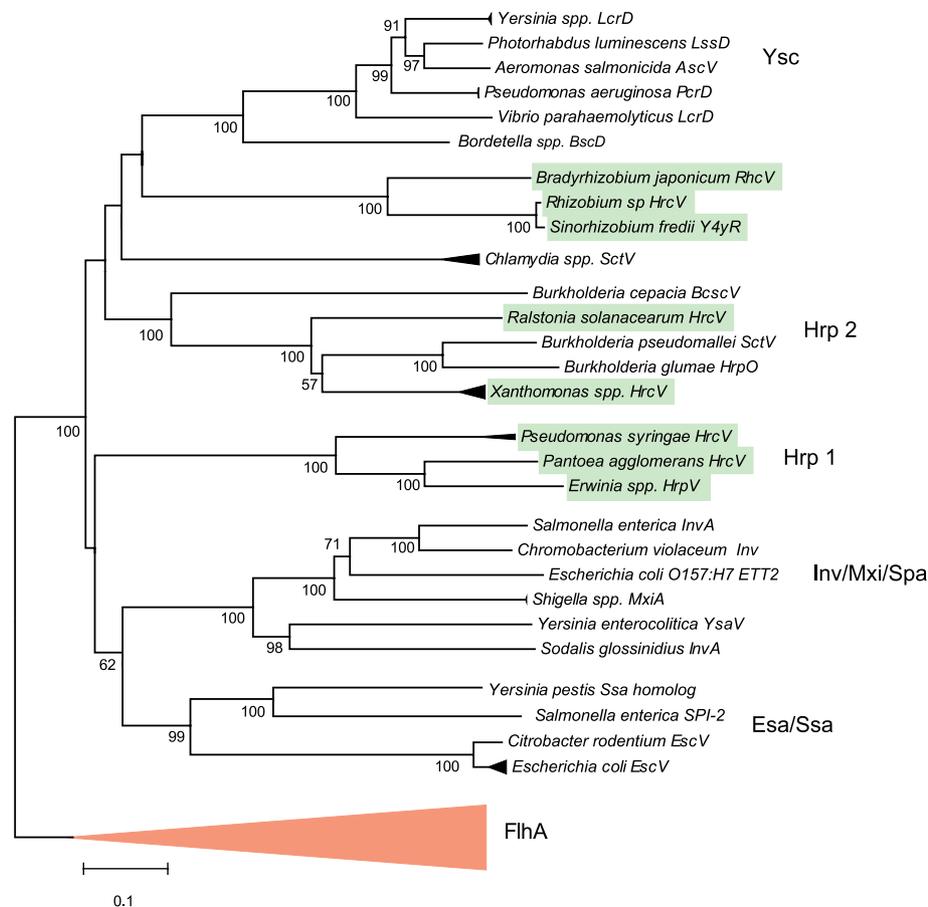
Fig. 1. Phylogenetic tree based on aligned amino acid sequences of HrcV/FlhA homologues. The numbers at each node are bootstrap confidence values from 500 replicate neighbor-joining trees. Distances are calculated based on differences at the amino acid level using a Poisson correction as in Gophna et al. [47]. Plant pathogens are highlighted in green. The five TTSS groups are indicated on the right.

deep divergence of the lineages of the virulence-associated component of the TTSS (Fig. 1). The virulence associated HrcV fall into a single monophyletic branch that is separate from and equally diverse as the flagella FlhA homologues. The same pattern is seen in comparisons of the other conserved components of flagellar and virulence TTSSs [47]. Although there is clear homology between the conserved proteins of flagellar and virulence-associated TTSSs, the evolutionary results support the hypothesis that both flagellar and virulence TTSSs are ancient and diverged independently from a common ancestral system. There is also no indication from the gene phylogenies as to whether the ancestral system functioned in virulence or motility.

4. Type III secretion mechanism

Type III secretion involves the delivery of effector proteins from the bacterial cytoplasm to the host cell interior, passing both bacterial and host membranes/cell walls. In this section, we first discuss the mechanism of type III "secretion" across the bacterial cell wall. In particular, we will highlight recent studies on type III

secretion-associated supramolecular structures, secretion signals, and chaperone proteins. We will then discuss the mechanism of type III "translocation" across the host membrane/cell wall. It is important to note that in vivo, type III secretion and translocation may occur as a single step though a continuous secretion channel.

4.1. Type III secretion genes

In each bacterium examined, ~ 20 genes are associated with optimal type III secretion [48]. Nine are common and share high-level sequence similarity [48]. Additional sequence similarities may be present in a few other type III secretion genes across all TTSSs, but homology is usually very low and may not be detectable by standard BLAST programs. However, there are significant gene synteny and homology within each of the five groups of TTSSs mentioned above.

For historical reasons, the same type III secretion genes were given different names in different bacteria. Some efforts have been made to standardize the nomenclature. For example, the nine highly conserved type III secretion genes have been named *hrc* (for *hrp* gene conserved) in

Table 1
Nomenclature of type III secretion-associated proteins frequently discussed in this review[a]

Yersinia spp.	Plant pathogens	Pseudomonas aeruginosa	Salmonella SPI-1	Shigella flexneri	EPEC	Flagellar proteins	Localization (excluding flagellar proteins)[b]
Secretion components							
LcrD	HrcV	PcrD	InvA	MxiA	EsaV	FlhA	Inner membrane
YscN	HrcN	PscN	InvC	Spa47	EscN	FliI	Cytoplasm/inner membrane
YscQ	HrcQ	PscQ	SpaO	Spa33	EscQ	FliN/FliY	Secreted (SpaO), secretion
YscR	HrcR	PscR	SpaP	Spa24	EscR	FliP	Inner membrane
YscS	HrcS	PscS	SpaQ	Spa9	EscS	FliQ	Inner membrane
YscT	HrcT	PscT	SpaR	Spa29	EscT	FliR	Inner membrane
YscU	HrcU	PscU	SpaS	Spa40	EscU	FlhB	Inner membrane
YscJ	HrcJ	PscJ	PrgK	MxiJ	EscJ	FliF	Inner membrane/needle base
YscC	HrcC	PscC	InvG	MxiD	EscC		Outer membrane/needle base
YscF		PcsF	PrgI	MxiH	EscF	FlgE	Needle
YscP		PscP	InvJ	Spa32		FliK	Secreted, needle length control
			PrgH	MxiG			Needle base
YscI			PrgJ	MxiI			Needle, cap protein?
Translocation components							
YopB	HrpK/HrpF/PopF2[c]	PopB	SipB	IpaB	EspB, EspD		Host membrane
YopD		PopD			EspB		Host membrane
LcrV		PcrV					Host membrane
			SipC	IpaC			Host membrane
			SipD	IpaD			Host membrane
Other components							
SycD/LcrH		PcrH	SicA	IpgC	CesD		Chaperone of translocators
			InvF	MxiE			Transcriptional activator

[a] Proteins on the same line share sequence similarities.

[b] Localization is inferred from studies using one or more bacteria.

[c] HrpK, HrpF, and PopF2 sequences are from *P. syringae*, *Xanthomonas campestris*, and *R. solanacearum*, respectively.

plant pathogenic bacteria, with suffix letters corresponding to those of the *ysc* genes (Ref. [49]; Table 1). Thus, *hrcC* in plant pathogenic bacteria is equivalent to *yscC* in *Yersinia*. Eight of the nine *hrc* genes share significant sequence similarity with the components of the flagellar assembly machinery in Gram-negative bacteria (Ref. [49]; Table 1). As will be discussed in more detail later, both in this chapter and in the chapter by Macnab, there is a close mechanistic and morphological relationship between the flagellar assembly machinery and virulence-associated TTSSs.

4.2. Type III secretion supramolecular structures

A landmark achievement in the field of type III protein secretion is the isolation of at least part of the type III secretion structure from *S. enterica* serovar *typhimurium* [50]. This structure resembles the flagellar basal body, consisting of two outer rings that interact with the outer membrane, two inner rings that interact with the cytoplasmic membrane, and an extracellular needle-like extension that is 8 nm in diameter and 80 nm in length (Refs. [50,51]; Fig. 2). A similar type III secretion structure has been found in *Shigella*, EPEC, and *Y. enterocolitica*, although exact measurements may vary somewhat in different bacteria [52–56].

The needle complex is characterized most extensively in *Salmonella* and *Shigella*, both of which produce the Inv/

Mxi/Spa group of TTSSs [51–53,57–60]. To date, five proteins have been found to be associated with the needle complex: MxiD/InvG (*Shigella*/*Salmonella*), MxiG/PrgH, MxiJ/PrgK, MxiH/PrgI, and MxiI/PrgJ.

MxiD/InvG are the outer membrane secretin family of proteins, equivalent to HrcC in plant pathogens (Table 1). MxiJ/PrgK are inner membrane proteins, their homologs are present in all TTSSs (HrcJ in plant pathogens) and also in flagella (FliF). MxiG/PrgH are also inner membrane proteins, but no homologues are detectable in other TTSSs. MxiH/PrgI are major needle structural subunits that are present in all mammalian pathogens. MxiI/PrgJ are minor components of the needle; their precise function is not known. They could serve as capping or scaffold proteins assisting the assembly of the needle, much in the same way as the flagellum FlgD hook cap protein assists the polymerization of the hook protein FlgE [61].

The major needle subunits MxiH/PrgI and the minor cap or scaffolding proteins MxiI/PrgJ appear to be distantly related to each other [52]. Interestingly, proteins with sequence similarities to MxiH/PrgI or MxiI/PrgJ do not appear to be present in plant pathogens. The absence of the needle structural proteins in plant pathogenic bacteria led to speculation by Kubori et al. [57] that the structure of the needle may represent the major difference between TTSSs of plant and animal pathogenic bacteria. The extracellular location of the needle makes it a prime candidate for
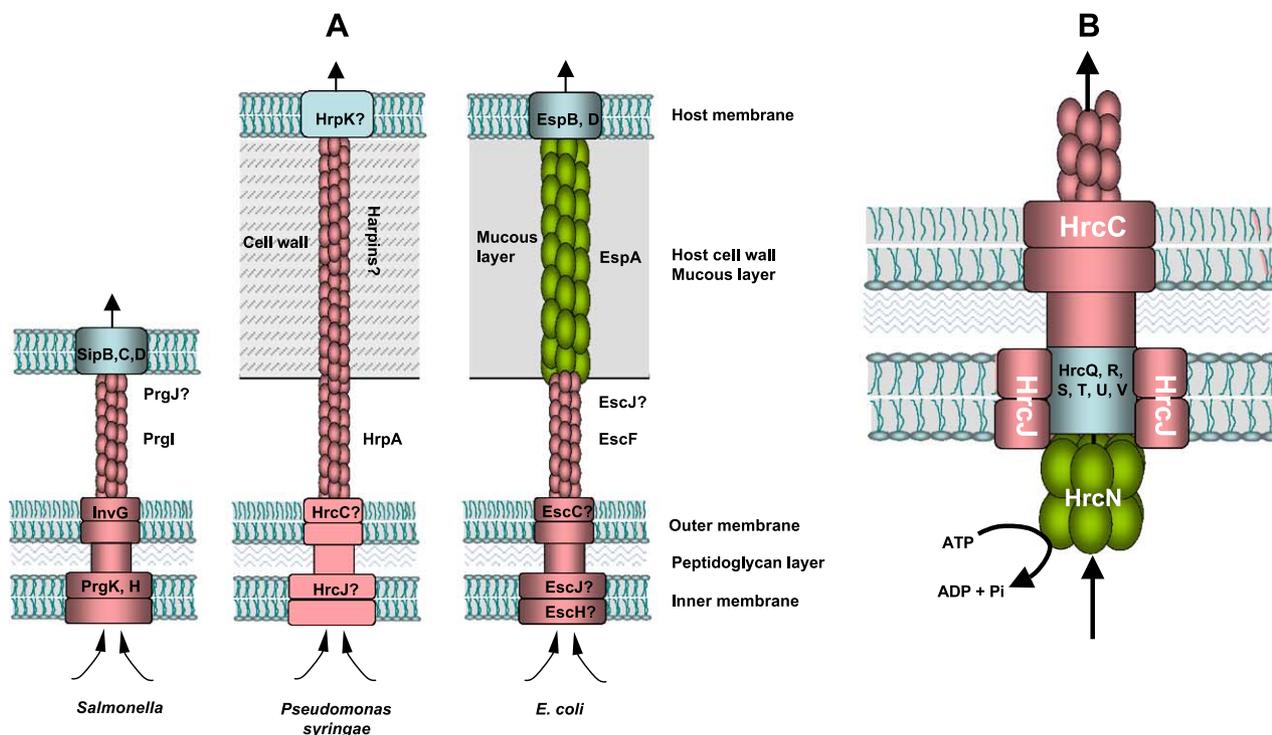
Fig. 2. (A) A schematic of type III secretion structures in *Salmonella* (representative of *Shigella* and *Yersinia* structures), *P. syringae* (representative of plant pathogenic bacteria), and enteropathogenic *E. coli* (EPEC). Various type III secretion structures are believed to be composed of a needle-like complex (in pink), which is anchored in the bacterial cell wall and has been visualized in and purified from several mammalian pathogens; and a translocation complex (translocon; in light blue) in the host membrane, which has been visualized only at low resolution in EPEC and *P. aeruginosa*. EPEC also assembles the EspA filament (in green), which extends from the tip of the needle and may help to penetrate the host mucous barrier and a thick glycocalyx in order to reach the underlying intestinal enterocytes. *P. syringae* presumably uses the Hrp pilus (made up of the NrpA protein) and possibly harpins to penetrate the host cell wall. (B) A more detailed diagram of the hypothetical base substructure in the bacterial cell wall. Protein names are for plant pathogens. Note the hypothetical two-deck hexamer rings of the ATPase HrcN (in green) and other mostly inner membrane components (in light blue) of the type III secretion machinery.

controlling specificity in delivering effector proteins across the plant and animal surface barriers, which are structurally very different. Plant cells have a cell wall, whereas animal cells do not.

A recent study revealed remarkable similarities between the architectures of the flagellar filament and the type III secretion needle extension [62]. X-ray fiber diffraction- and electron microscopy-based 3-D reconstruction of the *Shigella* needle showed that the MxiH subunits are arranged in a helical fashion to form a cylindrical filament with an approximate outer diameter of 7 nm traversed by a central channel of ~ 2 nm in diameter [62]. This 3-D structure bears essentially the same architecture (~ 5.6 units/turn) and inner channel diameter as the flagellar filament [63–65]. This architectural similarity is striking considering the marked differences among the molecular sizes of the structural proteins MxiH (~ 9 kDa) and flagellin (~ 51 kDa). It thus appears that the size of the structural subunit is proportional only to the outer diameter of the filament, but the inner diameter of the TTSS-associated filament remains constant (~ 2 nm).

The TTSS needle may be the equivalent of the innermost D0 domain of the flagellar filament if viewed in cross section [62]. The D0 domain is composed of the N- and C-terminal chains of flagellin, forming an α-helical coiled coil. The D0 domain constitutes the center tube of ~ 7 nm in diameter with a central channel of ~ 2 nm. The D0 domain is successively enveloped by the D1, D2, and D3 domains, making the outermost diameter of the *Salmonella* flagellum up to 25 nm [66,67]. α-Helical coiled coil is a common motif for type III secretion-associated proteins, including needle structural subunits [52,53,57,68,69]. Another very interesting finding is that the inner surface of the flagellar channel consists of mainly polar amino acids [65]. The polar nature of the inner surface may be well suited for fast diffusion of unfolded proteins, because unfolded proteins should have many hydrophobic side chains exposed, which would be trapped on a channel surface of hydrophobic nature. It remains to be seen whether the hydrophilic nature of the inner channel is a common feature of virulence TTSS-associated filaments.

4.3. Assembly of the needle complex and control of needle length

The assembly pathway of the needle complex has begun to be elucidated, mostly using the *Salmonella* SPI-1 TTSS as a model (Fig. 3; Refs. [51,59,69]). There appear to be
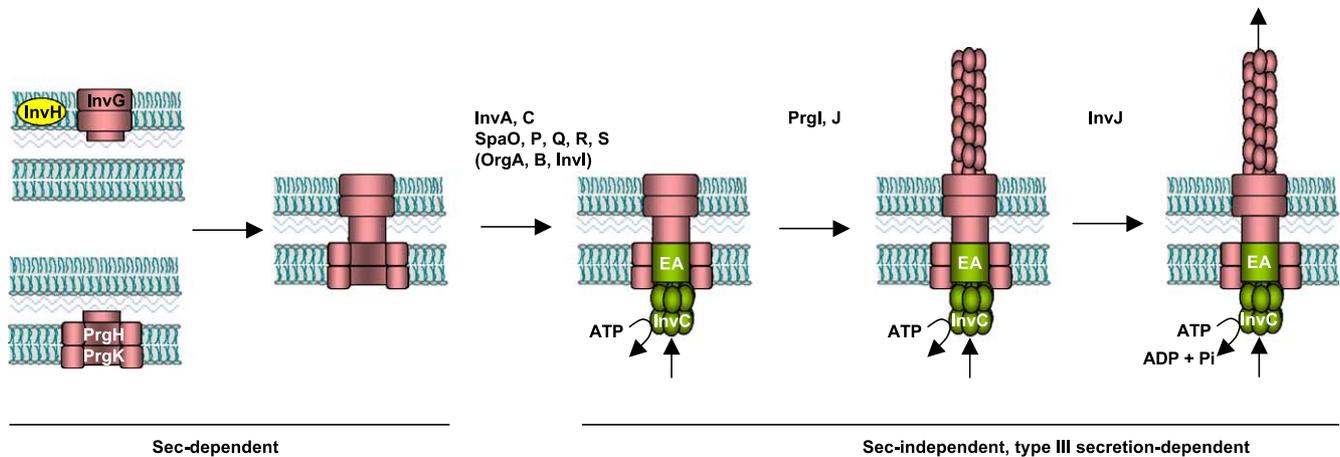
Fig. 3. A schematic diagram of the assembly pathway of the type III needle complex in *Salmonella* revealed by mutational and electron microscopic analyses. The InvG outer rings and PrgH/PrgK inner rings are assembled first, in a Sec-machinery-dependent manner, to make a base substructure (in pink). The availability of the base substructure allows subsequent incorporation of several mostly inner-membrane-localized components within the cavity of the PrgK/PrgH ring structure to make an active export apparatus (EA; in green). The export apparatus, however, has not been purified with the needle complex. The export apparatus first secretes PrgI (the major needle structural protein) and PrgJ (a minor protein), in a Sec machinery-independent manner, to assemble the needle extension. When the needle reaches a fixed length (e.g., ~ 80 nm), InvJ switches substrate specificity, allowing the export apparatus to secrete other proteins (e.g., translocators and intracellular effectors).

several genetically separable steps leading to the construction of a bacterial envelope-embedded base substructure and a largely extracellular needle extension. There is also strict genetic control of the length of the needle extension.

Assembly likely begins with the two inner membrane proteins (PrgH and PrgK) forming the inner membrane rings shown in Fig. 3. Both PrgH and PrgK are necessary for this step, which requires no other needle components. The PrgH–PrgK ring is probably equivalent to the FliF-based MS ring of the flagellum [70]; it is hollow in the center, where it could encase other structurally undefined components of the type III secretion machinery, as suggested for the flagellar MS ring [71]. The secretin family protein InvG alone can independently form the outer membrane ring, and somehow the inner membrane and outer membrane ring structures come together to form a base substructure without the needle extension. The presence of all three membrane proteins (PrgH, PrgK, and InvG) is necessary to make the most stable ring structures in the bacterial cell wall.

Assembly of the InvG outer membrane ring as well as the entire needle complex is enhanced in the presence of a secretin-specific pilot protein, InvH in *Salmonella*. InvH, however, is not part of the needle structure [59,72,73]. PrgH, PrgK, InvG and InvH are all believed to be exported through the Sec-dependent machinery in bacteria because they all carry N-terminal signal peptide sequences, although the N-terminus of PrgH is apparently not removed [50]. Thus, the first step of needle complex assembly appears to be largely dependent on the Sec machinery. The efficiency of this step is also influenced by the protein–peptidoglycan interaction within the bacterial envelope [74].

After the Sec machinery-dependent assembly of the base substructure, the next step is the TTSS-dependent (Sec-independent) assembly of the needle extension. Many of the TTSS components are believed to be encased within the base of the PrgH/K ring structure in the inner membrane, and presumably secrete needle structural proteins into the central channel within the basal substructure (Fig. 3). Six of the nine most conserved TTSS proteins play an essential role at this stage and are either confirmed or predicted to be inner membrane proteins (Table 1; Refs. [38,48]). Recently, yeast two-hybrid experiments with EPEC TTSS-associated proteins suggested multiple putative physical interactions among some of the conserved inner membrane components [75]. However, none of these proteins was found in the purified needle complex, possibly because detergents were used in the purification and/or because these inner membrane proteins have only transient interactions with the needle complex. Curiously, in vivo visualization shows a large "bulb" beneath the *Shigella* needle complex [52]. The relationship between this bulb-like structure and the secretion machinery is not clear.

In addition to these highly conserved and essential inner membrane secretion proteins, all TTSSs have a cytoplasmic/inner membrane ATPase (HrcN in plant pathogens) that bears sequence similarity to the catalytic β subunit of the mitochondrial F1 ATPase. The F1 ATPase is a heterohexamer consisting of alternating α and β subunits with a central channel [76]. The α subunit equivalent is not found in TTSSs, so it is likely that the HrcN ATPase is a homo-oligomer and, in association with other TTSS components, could function as an ATP-powered secretion pump at the base of the needle complex. Indeed, using hydrodynamic, cross-linking, and ultrastructural analyses, Pozidis et al. [77] found that the *P. syringae* HrcN ATPase activity is activated by homo-oligomerization and is associated peripherally at the plasma membrane. The dodecamer oligomer has the highest ATPase activity. When viewed by electron micros-

copy, the dodecamer appears as an organized round particle with an outer diameter of 13 nm [77]. The dodecameric HrcN ATPase may form double hexameric stacks, as was found for other dodecameric traffic ATPases (Fig. 2; Refs. [78,79]).

A defining feature of the needle assembly is its fixed length, mirroring the assembly of the hook portion of the flagellum. There is clear evidence that the length of the needle is controlled by homologous proteins InvJ in *Salmonella* and Spa32 in *Shigella* [57–59,80]. Mutations in *invJ* or *spa32* genes result in greatly elongated needles, which resemble pili. Interestingly, the flagellar hook length is also fixed and is controlled by FliK [57,80]. In the *fliK* mutant, hook length is greatly elongated, forming "poly-hooks" [81]. It was recently suggested that the fixed flagellar hook length is determined by an absolute amount of hook monomers (FlgE) that can be accumulated to fill the cytoplasmic (C) ring, which may function as a "mea-suring cup", at the base of the flagellum. The FlgE hook monomers are secreted en bloc to form the flagellar hook of a defined length. When the C ring is empty, which may be sensed by FliK, the type III secretion machinery terminates hook protein secretion and initiates secretion of terminal protein flagellin [82]. FliK, along with the hook and filament proteins, binds to the cytoplasmic domain of FlhB [83,84]. Therefore, upon the completion of hook assembly, it is possible that FliK switches the substrate specificity of the flagellar TTSS by altering the conforma-tion of FlhB to promote the export of flagellin, the primary filament subunit.

Is a similar mechanism at work in InvJ/Spa32-mediated control of needle length? The *invJ* and *spa32* mutants do not secrete other proteins, suggesting that InvJ and Spa32, like FliK, are involved in substrate switching. The primary defect of the *invJ* and *spa32* mutants may be their inability to arrest PrgI/MxiH export and switch from secretion of needle components to secretion of other substrate proteins. In *Yersinia*, the InvJ/Spa32 equivalent is YscP. The *yscP* mutant secretes an increased amount of needle protein YscF to the bacterial cell surface prior to eukaryotic cell contact, presumably resulting in an elongated needle, although this has not been shown. In contrast, the *yscP* mutant secretes only low levels of the translocator proteins, YopB and YopD, and Yop effectors. Interestingly, mutations in the cytoplasmic domain of the inner membrane protein YscU (an FlhB homolog) can restore a level of Yop effector secretion to the *yscP* mutant higher than that to the corresponding isogenic wild-type strain, while the amount of YscF present on the bacterial cell surface is reduced [85]. These results suggest that YscP and YscU function analo-gously to FliK and FlhB to control the substrate specificity that enters the *Yersinia* TTSS. However, instead of func-tioning as a measuring cup, as suggested for FliK, YscP functions as a molecular ruler to determine the length of the needle, because an alteration of the PscP protein length results in a corresponding change in the needle length [86].

InvJ [87], Spa32 [58], YscP [88] and FliK [89] are all secreted. The biological significance of their secretion in their role as regulators of needle/hook length is not yet understood.

4.4. Other TTSS-associated filamentous extensions in mammalian pathogens

In addition to the needle extension, several other extra-cellular filaments are assembled in a type III secretion-dependent manner. The first surface appendage reported to be assembled by a TTSS is the invasome of *S. typhimurium* [90,91]. The invasome (50 nm in diameter and up to 2 μm in length) is formed transiently upon contact of bacteria with host cells. The protein composition of the invasome is not known, but its dependency on type III secretion suggests that it could be made up of TTSS proteins. There is, however, a report questioning the dependence of invasomes (or perhaps invasome-like appendages) on type III secretion [92].

The EPEC EspA filament is another type III surface appendage assembled by a mammalian pathogenic bacteri-um (Fig. 2; Refs. [93,94]). Morphologically, this filament appears to be similar to the *Salmonella* invasome (12–50 nm in diameter and up to 2 μm in length). The EspA appendage is required for translocation of other proteins (e.g., EspB) into the host cell, but is not required for secretion of effector proteins across the bacterial envelope [93]. Recent studies showed that the EspA filament is an extension of the needle [55,56] and EspA interacts with EscF (the needle structural protein in *E. coli*) [95]. It is hypothesized that the long extension of the EspA filament penetrates the host mucous barrier and a thick glycocalyx in order to reach the underlying intestinal enterocytes [55].

The 3-D reconstruction of electron microscopic images of the EspA filament revealed an outer diameter of ∼ 12 nm and an inner channel of ∼ 2.5 nm. Like that of the type III secretion needle, the 3-D image of the EspA filament bears the same architecture (∼ 5.6 units/turn) and inner channel diameter as the flagellar filament [63–65,95].

4.5. TTSS-associated filamentous extensions in plant pathogens

The type III secretion-associated needle complex has yet to be discovered in any plant pathogenic bacteria. However, the TTSSs of plant pathogens and symbionts assemble a surface filament morphologically resembling bacterial pili (Fig. 2; Ref. [96]). These TTSS pili are named Hrp pili because their assembly is dependent on the *hrp* type III secretion genes. Hrp pili are much longer (several μm) than the needle extension, but have the same diameter (∼ 8 nm) as the needle extension [97–101].

The TTSS-associated pili were first discovered in *P. syringae* [97], before the first needle complex from *Salmo-nella* was reported [50]. The major subunits of the *hrp-*

dependent pili, like the needle structural proteins, are all small proteins (of 6 to 11 kDa), but their sequences are surprisingly hypervariable, even within pathovars of *P. syringae*. For example, the major subunit of the Hrp pilus of *P. s.* pv. *tomato* is the 11-kDa HrpA protein, which shares only 30% identity with the HrpA protein of *P. s.* pv. *syringae* [102]. In comparison, other structural components of the TTSS are much more similar among different pathovars of *P. syringae*. For example, HrcC proteins, which are members of the outer membrane secretin family, of *P. s.* pv. *tomato* and *syringae* share 80% identity at the amino acid level [102]. The hypervariability of the structural proteins of Hrp pili extends beyond *P. syringae* pathovars. For instance, the major subunit of the *Erwinia amylovora* Hrp pilus is a 6.5-kDa protein that shares only 30% identity with the C-terminal portion of the *P. s.* pv. *tomato* DC3000 HrpA protein [99]. The structural protein (7-kDa HrpY) of the *Ralstonia solanacearum* Hrp pilus shares no detectable similarity with the HrpA protein of *P. syringae* or *E. amylovora* [98]. Despite the hypervariability of the primary sequences of the pilus structural proteins, the predicted secondary structures of these proteins are remarkably similar, consisting almost exclusively of α-helices [100]. Only the N-termini (the first 50 aa) of the HrpA proteins of *P. syringae* pathovars are predicted to contain β-strands, which, however, are dispensable for function [103]. The hypervariability of the primary sequences of the pilus subunit may reflect the evolutionary adaptation of extracellular pili to avoid recognition by the plant defense surveillance system.

To date, no other proteins have been found to be structural components of Hrp pili besides the HrpA (in *P. syringae* and *E. amylovora*) and HrpY (in *R. solanacearum*) proteins. Purified HrpA protein is able to form a pilus-like structure in vitro [104]. At this point, however, one cannot rule out the existence of minor subunits for the Hrp pilus. In *P. syringae*, for example, there is a predicted type III-secreted protein, HolPtoY, that has sequence similarity to HrpA at the C-terminus [105,106]. In *R. solanacearum*, there is also a HrpY-related protein (AL646081). These proteins may function analogously to the putative capping/scaffold proteins PrgJ/MxiI in *Salmonella*/*Shigella*. As discussed above, PrgJ and MxiI are minor subunits of the needle complex, share sequence similarities with their cognate major needle subunits, and function primarily to catalyze the polymerization of PrgI and MxiH into needles.

Genetic evidence suggests a strict requirement of the Hrp pilus for bacterial interactions with plants. The *hrpA* mutants of *P. syringae* and *E. amylovora* and the *hrpY* mutant of *R. solanacearum* do not cause disease in susceptible plants nor elicit the defense-associated HR in resistant plants [97–99]. Initial speculations included a role of the Hrp pilus in attaching bacteria to plant cells or in transporting proteins [107,108]. Because intimate contact between bacteria and host cells is important for type III protein secretion, as demonstrated for *Yersinia* [109], an attachment

function of the Hrp pilus is an attractive hypothesis. However, an exclusive attachment function for the Hrp pilus has not gained any experimental support. In fact, mutational analysis showed that the Hrp pilus of *R. solanacearum* is not required for adhering bacteria to infection sites in tomato [98].

Although the attachment function of the Hrp pilus remains unresolved, accumulating evidence suggests that the Hrp pilus plays an essential role in protein delivery. Genetic analysis revealed that the *hrpA* genes of *P. syringae* and *E. amylovora* and the *hrpY* gene of *R. solanacearum* were all required for extracellular secretion of effector proteins in culture [98,99,110]. A recent comprehensive mutational study of type III secretion genes in *R. solanacearum* showed a close correlation between Hrp pilus assembly and secretion of effector proteins [111]. The *hrpA* deletion mutation in *Pst* DC3000, but not in *R. solanacearum* or *E. amylovora*, also down-regulated the expression of all examined TTSS-associated genes [110]. The exact mechanism by which the *hrpA* deletion affects the accumulation of the TTSS transcripts remains to be determined.

In addition to the strong genetic evidence discussed above, recent in situ immunogold labeling experiments support a direct involvement of the Hrp pilus in type III protein secretion in *P. syringae* and *E. amylovora* [99,112]. In these experiments, bacteria were grown in a manner to preserve and visualize Hrp pili during active type III secretion. It was found that all the examined type III-secreted proteins, including HrpZ, HrpW, and AvrPto of *P. syringae* and HrpN and DspE of *E. amylovora*, were localized along the entire length of the Hrp pilus. The co-localization of type III-secreted proteins to Hrp pili is highly specific because these proteins are not present randomly in the extracellular space or along flagella, except after shaking or prolonged incubation, which presumably results in the diffusion of secreted proteins away from Hrp pili [99]. Several type III-secreted proteins (Nops) in *Sinorhizobium fredii* were also found to be associated with type III pili [101].

The Hrp pilus-specific localization is a very important observation because it demonstrates that type III secretion occurs only at specific sites where the Hrp pilus is assembled. Moreover, the deposition of type III-secreted proteins along the entire length of the Hrp pilus suggests that secretion of effector proteins occurs while the Hrp pilus is being constructed. This observation is consistent with the fact that the expression of type III secretion/Hrp pilus assembly genes (*hrp/hrc*) and effector genes (e.g., *avrPto*) is coordinately regulated in *P. syringae* and *E. amylovora*, owing to the presence of a common "hrp box" regulatory motif in the promoters of these genes [113].

There are two alternative models to explain the localization of secreted proteins along the Hrp pilus during active type III secretion [99,112]. In the "conduit" model, effector proteins exit from the tip of the Hrp pilus. As the Hrp pilus secretes effector proteins and continues to grow, the secreted

proteins are left behind, marking the trail of Hrp pilus growth. In the "guiding filament" or "conveyer" model, the Hrp pilus carries type III effector proteins with it as it grows out of the type III secretion basal body.

To distinguish the two type III secretion models, two research groups made a critical modification of the in situ immunogold labeling method by incorporating a step to uncouple the synthesis of effector proteins from Hrp pilus assembly [114,115]. Specifically, the expression of the effector proteins AvrPto and HrpZ was placed under the control of heterologous promoters so that assembly of the Hrp pilus could be induced first before synthesis and secretion of AvrPto or HrpZ. The authors then addressed the question of whether the newly synthesized AvrPto or HrpZ is extruded from the tip (as in the conduit model) or the base (as in the guiding filament/conveyor model) of the Hrp pilus. These experiments showed that effector proteins exit from the tip of the Hrp pilus, a prediction of the conduit model [114,115]. Demonstration of the conduit function of the Hrp pilus provides direct support for a shared mechanism in type III secretion and flagellar assembly based on similarities between type III secretion genes and flagellar assembly genes. Flagellar assembly is thought to involve export of partially folded flagellin through an internal channel of a growing flagellum filament before being assembled at the tip [116].

The polarity of Hrp pilus assembly has also been determined. As mentioned before, eight *hrc* genes are homologous to genes involved in flagellar assembly [49]. Therefore, a simple prediction is that Hrp pilus assembly is mechanistically similar to flagellar assembly. This speculation has been confirmed experimentally. By conditionally expressing the FLAG-tagged HrpA protein in wild-type *Pst* DC3000 cells, Li et al. [115] showed that the newly made HrpA proteins are added at the tip of a growing Hrp pilus.

What is the relationship between the Hrp pilus and other TTSS-associated surface filaments? The Hrp pilus is likely the functional equivalent of the needle. The drastic difference in length between the Hrp pilus and the needle may reflect the adaptation of these appendages to overcome the various surface barriers of recipient plant and mammalian cells. For instance, the TTSS of plant pathogens must penetrate a thick (>100 nm) cell wall enveloping the plant cell, which is absent in mammalian cells. This speculation is supported by the following observations: First, the diameters of the Hrp pilus and the needle are very similar (about 8 nm). As discussed before, in the *invJ/spa32* mutants of *Salmonella* and *Shigella* or when the needle structural protein MxiH is overexpressed, the needle can be very long, resembling the Hrp pilus [53,57,58]. Second, the needle structural proteins—MxiH in *Shigella*, PrgI in *Salmonella*, YscF in *Yersinia*, PscF in *P. aeruginosa*, and EscF in EPEC—like Hrp pilus structural proteins, are all small in size, ranging from 7 to 9 kDa [50–56,97–99]. Third, mutations in the structural genes of Hrp pili and needles block secretion of effector proteins across the bacterial

envelope into culture medium [50–56,97–99]. An alternative but less likely possibility is that the Hrp pilus is an extension of the needle and may be the equivalent of the EPEC EspA filament, which extends beyond the needle [55,56]. However, in contrast to the Hrp pilus, the EspA filament is not required for type III secretion across the bacterial envelope, but is required only for translocation of type III effectors into the host cell [93,94].

4.6. Type III secretion signals

Proteins that traverse TTSSs do not carry the typical cleavable signal peptides at the N-termini that are present, for example, in those that traverse the Sec machinery (see the chapter by Economou). Initial work in *Yersinia* spp. suggested that Yop proteins possess noncleavable secretion signals at the N-termini (Fig. 4). It was shown that the first 15 and 17 residues are sufficient for secretion of YopE and YopH fusion proteins, respectively, across the bacterial envelope in culture [117,118]. However, no discernible amino acid or peptide similarities can be found at the N-terminal regions of YopE and YopH or other Yop proteins. How can different N-terminal sequences serve as secretion signals for the same secretion pathway? Anderson and Schneewind [119,120] challenged the N-terminal signal theory by showing that frameshift mutations that completely change the first 15 amino acid sequences of YopE and YopN do not affect secretion of the YopE–neomycin phosphotransferase (Npt) or YopN–Npt fusion proteins in culture [119]. They also showed that the secretion signals for the Ysc/Yop secretion system are the 5' regions of the *yopE* and *yopN* mRNA [119]. Analysis of predicted RNA structures of the YopE and YopN secretion signals reveals the presence of a stem loop that buries the AUG translational start codon in a base-pair duplex [119]. It was proposed that this stem loop structure may be recognized by a component of the *Yersinia* TTSS [119]. The mRNA-based recognition mechanism, which assumes co-translational secretion, has become controversial [121,122]. Results from Wolf-Watz and colleagues show that the mutations in YopE resulting in an altered mRNA structure do not abolish its type III secretion [123]. Furthermore, by replacing amino acids 2–8 of YopE with all permutations of synthetic serine/isoleucine sequences, they show that amphipathic N-terminal sequences, containing four or five serine residues, are more likely than hydrophobic or hydrophilic sequences to target YopE for secretion [124]. This finding suggests that it is the amphipathic peptide sequence, not mRNA, that serves as a type III secretion signal.

The amphipathic nature of the type III secretion signal identified in *Yersinia* is interesting because similar N-terminal secretion signals were reported in *P. syringae*. Gene fusion analysis of AvrRpt2 revealed that the N-terminal region of the effector protein is important for secretion and the remaining part of AvrRpt2 is important for its biological function [125,126]. Recently, by comparing the N-terminal
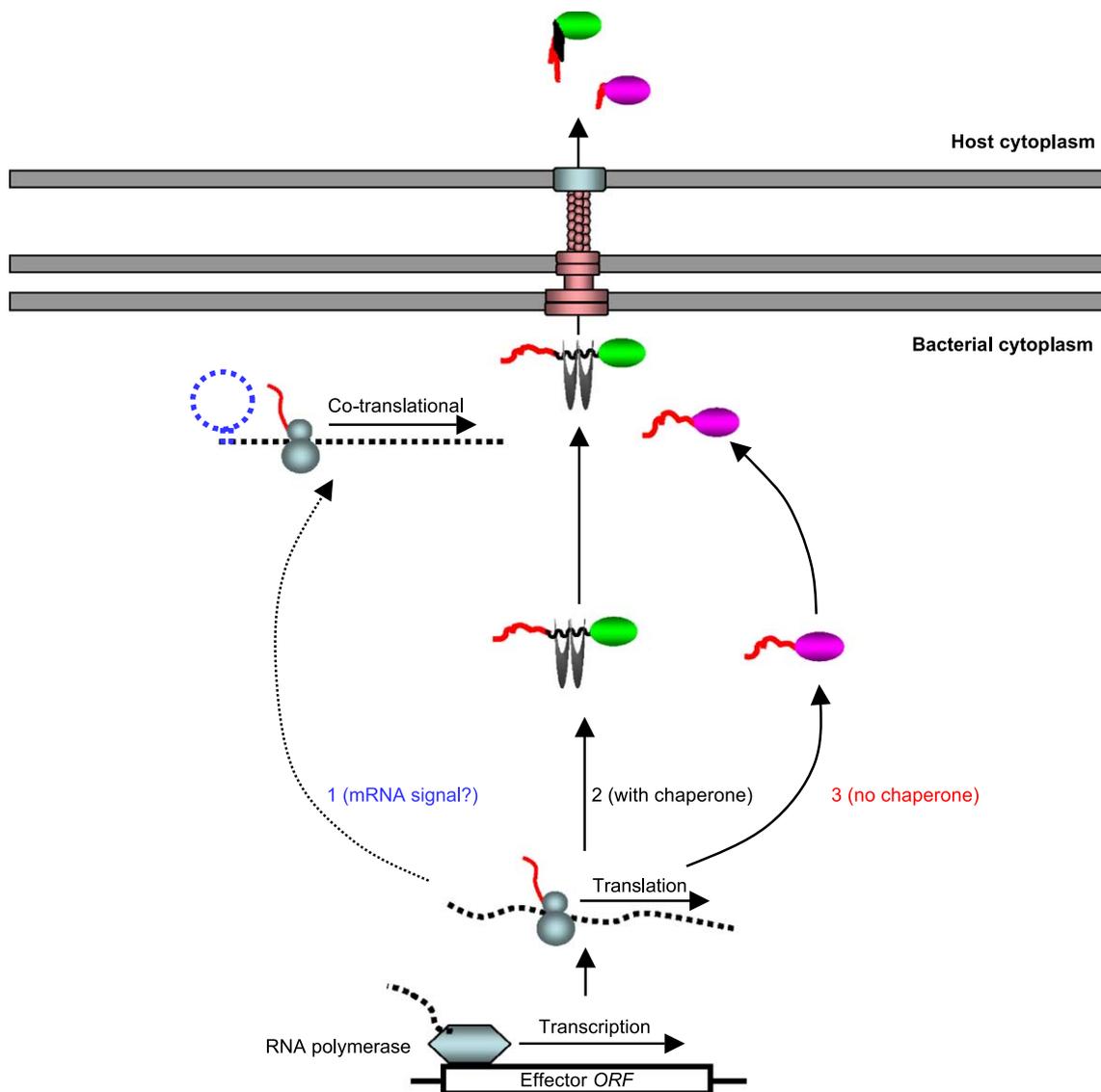
Fig. 4. A diagram of type III secretion signals and functions of chaperones. Three type III secretion signals have been described: 1, 5′ mRNA (a stem loop colored blue); 2, the chaperone–effector binding domain complex (the chaperone homodimer depicted as two horseshoes and the effector-binding domain indicated by a short black wavy line); 3, N-terminal peptide (indicated by a red wavy line). Binding of chaperones to cognate effectors could prevent premature folding, aggregation, and/or complex formation in the bacterial cytoplasm. Some chaperones may also function in feedback gene regulation. See text for further discussion on the evidence for each secretion signal and the various roles of chaperones. The mRNA signal requires secretion to be co-translational, whereas the other two signals do not. Effectors with chaperones may be secreted first because they carry both the chaperone signal (black wavy line) and the N-terminal signal (red wavy line). Effectors assume their final conformations inside the host cell and chaperones stay in the bacterial cytoplasm.

amino acid compositions of 13 known effector proteins from *P. s.* pv. *maculicola*, Guttman et al. [127] reported that the first 50 amino acids of these effector proteins had a high proportion of Ser and a low proportion of Asp residues. Similarly, Petnicki-Ocwieja et al. [128] identified specific biophysical features of the first 50 amino acids of effector proteins in *P. syringae pv. tomato*: (i) the presence of solvent-exposed amino acids in the first five amino acids, (ii) the lack of Asp or Glu residues in the first 12 amino acids, and (iii) the amphipathicity of the first 50 amino acids. Based partly on such N-terminal amino acid properties, Petnicki-Ocwieja et al. [128] identified 38 putative effector genes in the genome of *P. s.* pv. *tomato* DC3000.

Indeed, some of these candidates were confirmed experimentally to be secreted and/or translocated.

4.7. Type III chaperone proteins

Secretion of many type III effectors requires accessory proteins collectively called chaperone proteins. These proteins are small (< 170 aa), acidic (pI < 5.5), and often contain an amphipathic α helix near the C-terminus [129–133]. Somewhat surprisingly, TTSS chaperones are not related to each other in their primary sequences, nor are there sequence similarities between the chaperone-binding domains of different effector proteins. Since the discovery of the first

chaperone SycE (for YopE) in *Yersinia* a decade ago [134], about 30 chaperone proteins have been found in various bacteria (Table 2) and more are predicted from genome-wide analyses [133,135]. The chaperone-encoding genes are almost always located adjacent to the cognate effector genes, suggesting strong selection for their co-existence in the genome. The widespread presence of chaperone proteins in TTSS-containing plant and mammalian pathogenic bacteria suggests an important role in type III secretion.

Chaperones are also found in the flagellar assembly system. As in the virulence-associated TTSS, flagellar system chaperones are dissimilar in the amino acid sequence, but all are acidic polypeptides of approximately the same size (120–140 residues) [136]. However, there are also significant differences between chaperones of virulence-associated TTSSs and those of the flagellar TTSS. For example, unlike virulence-associated TTSS chaperones, which generally bind to the N termini of their cognate

effectors, flagellar chaperones interact with the C-termini of their binding partners [136].

Despite the apparently universal presence of chaperones in TTSSs, the precise mechanisms by which these accessory proteins facilitate the secretion of the cognate effector proteins through the TTSS are poorly understood. Here, we discuss recent structural analyses of several chaperones and three roles (antifolding, secretion signal, and effector stabilization) for which there is some experimental support. However, it is often difficult to separate completely the different roles because they may be related to each other.

4.7.1. High-resolution chaperone structures

Although the TTSS chaperones are not related in primary sequences, recent structural studies revealed remarkably conserved folds among four crystallized chaperones: SycE, SicP, CesT, and SigE [137–140]. These chaperones form a

Table 2
Known and predicted type III chaperones

Bacteria	Chaperone	Size (aa[a])	pI	Substrate(s)	Function(s) in addition to secretion	Reference
Animal pathogen						
E. coli (EPEC)	CesD	151	7.4	EspB, EspD		[251]
	CesF	127	4.2	EspF		[252]
	CesT	156	4.3	Tir	Substrate stabilization[b]	[253]
Pseudomonas aeruginosa	Orf1	116	5.0	ExoS		[254]
	PcrH	168	4.4	PopB, PopD		[255]
	SpuC	137	4.4	ExoU		[256]
Salmonella	FlgN	140	5.3	FlgK, FlgL, FlgM	Substrate stabilization	[154,257]
	FliS	135	4.7	FliC	Anti-polymerization	[136]
	FliT	122	4.9	FliD	Substrate stabilization	[153]
	InvB	135	4.4	SicP/SspA		[258]
	SicA	165	4.6	SipB/SspB, SipC/SspC, InvF	Substrate stabilization, regulation	[234,259]
	SicP	116	3.9	SptP	Substrate stabilization, anti-folding	[139]
	SigE	113	3.9	SigD/SopB	Substrate stabilization	[260]
	SseA	108	10.2	SseB, SseD		[261]
Shigella	IpgA	129	4.5	IcsB	Substrate stabilization	[262]
	IpgC	155	4.4	IpaB, IpaC, MxiE	Substrate stabilization, regulation	[152, 263]
	IpgE	120	4.0	IpgD	Substrate stabilization	[264]
	Spa15	133	4.2	IpaA, IpaB, OspC3	Substrate stabilization, anti-folding	[265]
Yersinia	CesD2	135	5.3	EspD		[266]
	SycB	169	4.5	YspB, YspC	Substrate stabilization	[267]
	SycD/LcrH	168	4.5	YopB, YopD	Substrate stabilization, regulation	[146,225,230]
	SycE/year	130	4.5	YopE	Substrate stabilization, anti-folding	[144,150]
	SycH	143	4.8	YopH, LcrQ/YscM1, YscM2	Regulation	[226,268]
	SycN	123	5.1	YopN, YscB	Substrate stabilization	[269,270]
	SycT	130	4.4	YopT		[271]
Plant pathogen						
Erwinia	DspF/B	124	4.5	DspE/A	Substrate stabilization	[132,142]
Pseudomonas syringae	AvrF/CEL ORF2	129	5.3	AvrE	(putative)[c]	[133]
	AvrPphF (ORF1)	135	6.1	AvrPphF (ORF2)	(putative)	[133]
	CEL ORF8	134	6.8	CEL ORF7	(putative)	[133]
	EEL ORF6	120	6.3	EEL ORF5	(putative)	[133]
	ShcA	126	4.8	HopPsyA		[133]
	ShcM	165	5.3	HopPtoM		[129]

[a] Amino acids.

[b] Substrate can be type III effectors, translocators, or flagellar subunits.

[c] Putative chaperones predicted based on protein properties and gene locations next to *P. syringae* effector genes.

compact and globular homodimer and interact with the chaperone-binding domains in the respective effectors, mainly via four hydrophobic patches. Mutations in these hydrophobic patches affect the chaperone–effector interaction [140]. In the chaperone–effector N-terminus complex, the chaperone-binding regions of effectors are largely unfolded and are wrapped around the chaperone dimer [138,139]. No significant differences between the conformations of SycE in its complexed and uncomplexed states were observed [138].

The structure of the flagellar system chaperone FliS complexed with the C-terminus of flagellin (FliC) has also been elucidated, revealing novel folds that are distinct from the virulence-associated TTSS chaperones [140]. Furthermore, FliS undergoes a substantial conformational change when binding to the C-terminus of FliC, in contrast to the virulence-associated TTSS chaperone SycE. Finally, FliS appears to act as a monomer, instead of a homodimer. Despite these distinct features, the actual interface between FliS and FliC is dominated by hydrophobic interactions, as is also the case in the virulence-associated TTSS chaperone–effector complexes. Furthermore, FliC is wrapped around the outside of FliS in an extended, horseshoe-like conformation with secondary (helical) but no tertiary structure, similar to the interaction between virulence-associated TTSS chaperones and their cognate effectors. Thus, there appears to be a generally conserved mode of recognition between chaperones and effectors in both the virulence-associated TTSS and the flagellar TTSS.

4.7.2. Chaperones as antifolding factors

The observation of the largely unfolded conformation of the chaperone-binding domain of the *Salmonella* effector SptP led Stebbins and Galan [139] to speculate that chaperones may function to maintain effectors in an unfolded, secretion-competent conformation (Fig. 4). This speculation is perhaps easy to accept because the central channel of the needle/EspA filament/flagellar filament is only 2–2.5 nm in diameter [62,95], too small for passage of completely folded effectors, some of which can be as large as 200 kDa [142,143]. Furthermore, two recent studies show that when YopE is fused to a tightly folded protein, dihydrofolate reductase (DHFR), the fusion protein cannot be secreted by *Yersinia* [144,145]. Interestingly, however, when YopE is fused to a misfolded DHFR mutant, the fusion protein is secreted efficiently [144]. Thus, the *Yersinia* TTSS can apparently accommodate heterologous proteins that are not tightly folded. Taken together, these results strongly suggest that unfolded or partially folded polypeptides are secreted through the TTSS and that chaperone-mediated unfolding of the N-terminus may play an important role in at least initiating the unfolding process.

Results from recent studies of chaperones SycE, SigE, and CesT, however, argue against the role of chaperones in global antifolding. Binding of SycE, SigE, and CesT to the cognate effectors YopE, SigD, and Tir, respectively, did not lead to global unfolding of these effectors [138,140]. The effectors in the chaperone-bound complexes still have normal biological activity in vitro. This, of course, does not preclude the possibility that in vivo, chaperones unfold the N-terminus of effector proteins and that additional accessory proteins at the base of the TTSS (e.g., HrcN ATPase) unfold the remainder of the protein to complete the secretion process. Furthermore, some chaperones, such as *Yersinia* SycD and *P. syringae* ShcM, bind their cognate effectors in multiple regions [129,146]. It would be interesting to determine and compare the structural bases of the SycD–YopB and ShcM–HopPtoM complexes with that of the SycE–YopE complex. It could be that SycD and ShcM have more extensive interactions with their effectors.

4.7.3. Chaperones as secretion signals

Birtalan et al. [138] propose that TTSS chaperones may function as part of a structure-based type III secretion signal (Fig. 5). They argue that the surprising conservation of the chaperone–effector binding complexes in the otherwise dissimilar effector–chaperone partners YopE–SycE and SptP–SicP suggests strong selective evolutionary pressure to maintain this particular stereochemical conformation. This proposal appears to be particularly attractive for the YopE–SycE interaction in *Yersinia*. YopE has two secretion signals, one in the extreme N-terminus (the first 15 codons) and the other immediately downstream (from 15 to 100 codons); the latter is recognized in a SycE-dependent manner [147]. Why do some type III effectors carry two secretion signals and others only one? The first clue came from the study of Boyd et al. [148], which showed that SycE and the SycE-binding domain in YopE are necessary for secretion only in the wild-type bacteria. In a *Yersinia* mutant that is deleted for all known effector genes, SycE and the SycE-binding domain are dispensable. This finding strongly suggests that SycE and the SycE-binding domain in YopE are involved in prioritizing YopE secretion in the presence of other effectors in the wild-type bacterium. Why would bacteria evolve to prioritize secretion of different effectors that enter the same host cell? A prevailing theory is that those *Yersinia* effectors that have two secretion signals (e.g., the host cytoskeleton regulators YopE, YopH, and YopT) are probably needed immediately upon host contact to rapidly disable the extremely fast host phagocytic response. These effectors are delivered from a presynthesized pool before host contact [123,138,148]. The other effectors that bear only one secretion signal may not be needed for this rapid virulence activity and can therefore be delivered more slowly via the N-terminal signal in a co-translational or posttranslational manner.

4.7.4. Chaperones as stability factors

Some chaperones are required for the stability of effectors in bacteria. Examples can be found in both mammalian and plant pathogens. For example, YopE is unstable and rapidly degrades in the absence of its chaperone SycE
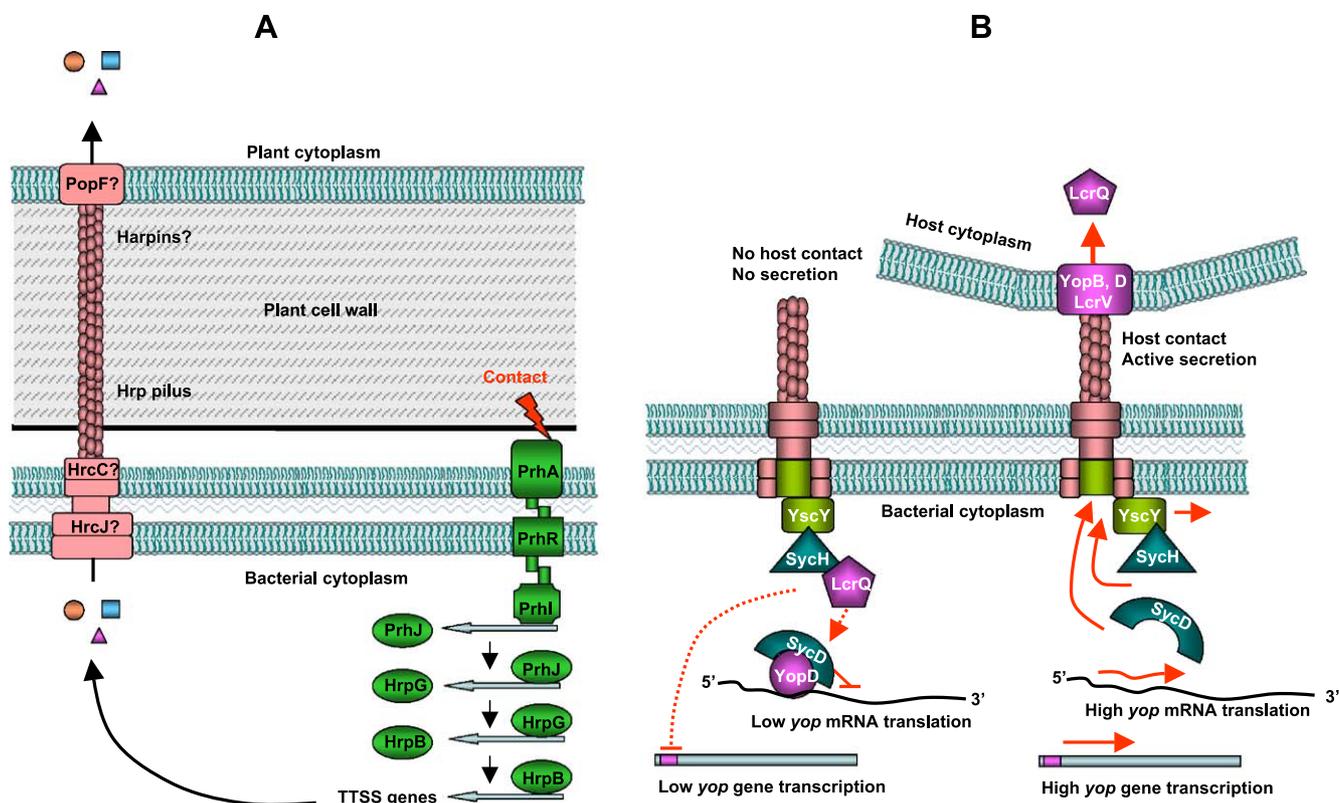
Fig. 5. (A) A signal transduction pathway for host contact-dependent activation of TTSS gene expression in *R. solanacearum*. The plant cell contact signal is sensed by the PrhA–PrhR–PrhI three-component signal transduction system, which results in expression of the cytoplasmic regulator PrhJ. Based on studies of other analogous systems, PrhA, PrhR, and PrhI likely interact directly to transduce the external signal. PrhJ activates the expression of HrpG, which then activates the *hrpB* regulatory gene. HrpB activates all other TTSS transcriptional units. Other environmental signals (e.g., metabolite signals; not shown) also influence this pathway (e.g., at HrpG and HrpB steps). (B) Negative feedback regulation via effector–chaperone complexes in *Yersinia*. The LcrQ–SycH complex, together with YscY, may impose a secretion hierarchy at the base of the TTSS. Before host contact, the *Yersinia* TTSS is closed, resulting in intracellular accumulation of the LcrQ–SycH(–YscY) and YopD–SycD complexes. Accumulation of these complexes is correlated with repression of transcription of *yop* genes, destabilized *yop* mRNA, and/or Yop protein translation. Host contact triggers secretion, allowing LcrQ and YopD to be secreted. Secretion of LcrQ and YopD would result in a higher concentration of free chaperones SycH and SycD. The presence of free SycH and SycD and/or the absence of LcrQ and YopD relieve transcriptional and posttranscriptional repression of Yop production.

[149,150]. In a *sycE* mutant, YopE protein lacking the SycE-binding region is more stable than wild-type YopE protein, suggesting that the SycE-binding region is involved in YopE instability [151]. In this case, however, it is difficult to separate the SycE-mediated YopE stability from SycE function in YopE secretion, because the aggregation-prone nature of the SycE-binding domain is likely a side effect of having to bind SycE as a highly extended peptide [138].

Shigella IpaB and IpaC translocators, which form a complex in the eukaryotic cell membrane, are independently associated with chaperone IpgC in the cytoplasm and both are degraded in the *ipgC* mutant strain [152]. The instability is presumably caused by premature interaction between IpaB and IpaC in the bacterial cytoplasm of the *ipgC* mutant, instead of the normal interaction within the host membrane. Similarly, chaperone SicA (an IpgC homolog; Table 1) is required for stability of translocators SipB and SipC in the cytoplasm of *Salmonella*. In *Yersinia*, SycD shares sequence similarity with IpgC (Table 1) and is required for the

stability of the YopB–YopD complex, presumably preventing premature interaction of the YopB–YopD complex with LcrV in the bacterial cytoplasm [146]. In the plant pathogen *E. amylovora*, DspF is required for the stability of DspE [132].

It is likely that the requirement for chaperone-mediated stability of effectors reflects the fact that the effectors need to be partially unfolded to get through the type III secretion machinery. It is also possible that the normal folding environment for effector proteins is inside a eukaryotic cell/membrane. The effector proteins may be misfolded in the bacterial cytoplasm in the absence of proper chaperones, leading to degradation. Similarly, chaperones are required for stability of many flagellar structural subunits in the bacterial cytoplasm. Flagellar chaperones bind to the C-terminal region of flagellar subunits. The C-termini of these subunits are normally involved in polymerization by which various parts of the flagellum form in the extracellular space [63,136,141,153]. Degradation of flagellar subunits has been observed in chaperone mutant strains [154].

4.8. Type III translocation across the host membrane/cell wall

How do effector proteins cross the host cell wall and/or membrane before entering the host cytosol? The simplest hypothesis is that the TTSS needle and pilus physically penetrate all host surface barriers, as suggested for the *Yersinia* needle [54]. Alternatively, the needle and pilus connect themselves to additional TTSS-associated protein complexes in the host cell membrane and/or cell wall to provide a continuous conduit for effector proteins. Current experimental evidence, discussed below, supports the latter hypothesis.

4.8.1. Mammalian pathogens

In mammalian pathogenic bacteria, a class of type III-secreted proteins called translocators (Tables 1 and 3) are required for the translocation of other effector proteins into the host cells.

In *Yersinia*, the translocator proteins are YopD, YopB, and LcrV [2,3,155,156]. Lipid membrane incubated with these three proteins contains ion-conducting channels [157]. LcrV alone has channel-forming activity [158] and YopB alone has membrane-disrupting activity [159]. Interestingly, antibody raised against LcrV can prevent effector translocation [156]. YopB and YopD contain hydrophobic as well as hypothetical coiled-coil sequences that are often involved in protein–protein interaction. YopB, YopD and LcrV over-

expressed in the *E. coli* cytosol all interact with each other [146,160]. However, the YopB/YopD/LcrV-mediated channel has not been visualized in the host membrane using electron microscopy. Whether or not this channel is actually involved in translocation remains to be experimentally proven.

In addition to their role in channel formation, translocators can have other functions. For example, YopD and LcrV exert regulatory functions on the TTSS, and YopD was shown to be translocated across the plasma membrane [161,162]. Besides YopB and YopD, YopK also modulates the translocation step in *Yersinia* [163].

PopB, PopD, and PcrV, which are *P. aeruginosa* homologues of the respective *Yersinia* counterparts, are also required for pore formation in the host cell membrane [164–166]. An antibody raised against PcrV also blocks protein translocation [167]. However, in contrast to the situation in *Yersinia*, PcrV expressed in *E. coli* does not interact with PopB and PopD in vitro and does not form pores by itself [165,168]. Instead, PopB and PopD form ring-like structures of 80 Å in outer diameter and with a 40-Å-wide centralized hole in artificial membranes independent of PcrV [168].

In pathogenic *E. coli*, EspB [169] and EspD [170,171], both of which share some sequence similarity with *Yersinia* YopB [172], insert into the host membrane and are required for pore formation [173]. A TTSS-dependent pore-like structure containing EspB and EspD has been visualized by atomic force microscopy [173]. The pore appears to be composed of six to eight subunits constituting a doughnut-shaped structure with a relatively large (>50 nm) outer diameter and a central cavity of ~ 8 nm. The opening of the pore displays a funnel-like morphology with the internal canal narrowing toward the cytoplasm. The funnel-like opening led to speculation that the EspA filament could be locked into this opening. Indeed, the interaction between EspA and EspB was detected and, furthermore, during infection EspA and EspB are localized in similar regions. These results raise the possibility that the EspA filament and the translocation pore may physically connect during delivery in vivo [174].

In *Shigella/Salmonella*, secreted IpaB/SipB (YopB homologues) and IpaC/SipC are required for effector translocation [175,176]. During infection, IpaB and IpaC insert into host cell membranes and are required for TTSS-dependent pore formation in the host membrane [177,178]. Purified IpaB or IpaC can individually integrate into model, erythrocyte, and cultured mammalian cell membranes, but without disrupting bilayer integrity [179,180], suggesting that formation of pores in *Salmonella* and *Shigella* requires the presence of both IpaB and IpaC proteins. Similarly, the SipB protein integrated into both mammalian cell membranes and phospholipid vesicles, again without disturbing bilayer integrity [181–183]. Biochemical analysis suggests that IpaB assembles into trimers via an N-terminal domain predicted to form a trimeric coiled-coil, and is predominant-

Table 3
Proteins involved in translocation across the host cell wall/membrane[a]

Bacterium	Protein	Structural features[b]	Necessary, and/or sufficient for pore formation in membranes	Interaction partners
Yersinia	YopB	2 TM, 1 CC	Yes, no	YopD, LcrV
	YopD	1 CC	Yes, –[c]	YopB, LcrV
	LcrV		Yes, yes	YopD, YopB
Shigella	IpaB	2 TM, 1 CC	Yes, –	IpaC, SipC
	IpaC	2 TM	Yes, –	IpaB, SipB
	IpaD			
Salmonella	SipB	2 TM, 1 CC	Yes, –	SipC, IpaC
	SipC	1 TM	Yes, –	SipB, IpaB
	SipD			
E. coli	EspD	2 TM	Yes, –	EspB
	EspB	1 TM	Yes, –	EspD
P. aeruginosa	PopB	2 TM, 1 CC	Yes, yes	
	PopD	1 TM	Yes, yes	
	PcrV		Yes, no	
Xanthomonas	HrpF	1 TM	–, yes	
Ralstonia	PopF2	3 TM	–, –	
P. syringae	HrpK	1 TM	–, –	
	HrpZ	1–2 TM	–, yes	
Sinorhizobium	NolX	2 TM	–, –	

[a] Modified from Table 1 of Ref. [250], which contains additional references on the properties of these translocators.
[b] TM, transmembrane domain; CC, predicted coiled coil.
[c] –, not determined.

ly alpha-helical. Upon lipid interaction, two transmembrane domains (residues 313–333 and 399–419) penetrate the bilayer, allowing the intervening hydrophilic region (334–398) to cross the membrane [180]. Analogous to IpaB, the two transmembrane domains (residues 320–353 and 409–427) of SipB insert into the bilayer, allowing the hydrophilic region between the hydrophobic domains (354–408) to cross the bilayer. In addition to the two hydrophobic domains, SipB membrane integration requires an additional helical C-terminal region (428–593). A C-terminal recombinant polypeptide (residues 428–593) inhibits SipB insertion and bacterial entry into cultured cells [184]. Similarly, a peptide derived from IpaB is a potent inhibitor of *Shigella* entry into host cells [180]. SipB/IpaB and SipC/IpaC also have been shown to interact with each other [185–188], consistent with complex formation in vivo.

4.8.2. Plant pathogens

The TTSS translocation complex in plant pathogenic bacteria has been much less studied. Emerging evidence points to a mechanism probably similar to that in mammalian pathogenic bacteria, but plant pathogenic bacteria encounter a unique cell wall barrier (>100 nM) during type III secretion. Therefore, these pathogens may also produce unique translocators that presumably assist the Hrp pilus in overcoming the cell wall barrier.

In all plant pathogenic bacteria examined, there are TTSS proteins that share weak similarity to the YopB family of translocator proteins (Tables 1 and 3). These include HrpF in *Xanthomonas* (AAB86527), HrpK in *P. syringae* (AAF71497), PopF1/PopF2 in *Ralstonia* (NP_523114, NP_522461), and NolX in *Sinorhizobium* (NP_444155). NolX of *Sinorhizobium* and HrpF of *Xanthomonas* are secreted proteins [17,189]. HrpF is required for translocation of effector proteins into the plant cell [190]. Furthermore, HrpF binds to lipid membrane and forms ion-conducting pores in a planar lipid bilayer system [189]; thus it is one of the *Xanthomonas* translocators. It remains to be determined whether HrpK of *P. syringae*, NolX of *Sinorhizobium*, and PopF1/PopF2 of *Ralstonia* are also involved in protein translocation and pore formation in the host membrane. A recent immunogold localization study, however, suggests that NolX is predominantly localized extracellularly within the infection thread, a unique bacterial-induced tubular structure enveloped by host cell walls, allowing infecting bacteria to reach deep into host tissues as an extracellular bacterium [191].

Plant pathogenic bacteria also secrete a unique family of type III effectors that are not found in mammalian pathogens: the harpin family of effectors. The first member of the harpin family was identified in *E. amylovora* [192]. Proteins of the harpin family are secreted abundantly in culture, are glycine-rich and heat-stable, and induce the HR cell death and associated defense responses in some nonhost plants [7–9,193–200]. Although TTSS-dependent secretion and the ability of purified harpins to activate host defense

response in nonhost are well documented [7–9,199], their precise function during bacterial infection in the native host plant remains elusive. HrpZ and HrpW both are known to be associated with the plant cell wall [201,202]. The association of HrpW with host cell walls is particularly compelling because this protein has a pectate lyase domain. This domain binds to calcium pectate, a major plant cell wall component. However, a recent study shows that HrpZ of *P. syringae* is also associated with synthetic lipid membrane and, remarkably, forms a pore [203]. The HrpZ-mediated ion-conducting pore was permeable for cations but did not mediate fluxes of Cl$^-$. This result raises the possibility that HrpZ may be involved in nutrient release and/or delivery of virulence factors during *P. syringae* colonization of host plants.

The fact that all plant pathogens, but no known mammalian pathogens, secrete proteins of the harpin family suggests that these proteins could be involved in assisting the penetration of the plant pathogen TTSS pilus through the plant cell wall. However, there is no definitive genetic evidence that HrpZ or any other harpin is involved in protein translocation by functioning either as a cell wall-binding protein or a pore-forming translocator. HrpZ is apparently necessary for the function of the *P. syringae* TTSS when a minimal TTSS gene cluster is expressed heterologously in the saprophytic bacterium *P. fluorescens* [10,204]. However, *hrpZ* mutants of *P. syringae* appear normal in virulence [201,204]. These contradictory results may be attributable to the presence of multiple HrpZ-like proteins in *P. syringae*. *P. syringae* and *E. amylovora*, for example, produce a second harpin called HrpW [201,205,206]. However, the *hrpZhrpW* double mutant of *P. syringae* is not affected in virulence [201]. Genome-wide inventory of type III-secreted proteins has revealed additional proteins similar to HrpW in *P. syringae* and *Ralstonia* [127,128,207,208]. Clearly, further mutagenesis experiments will be needed to determine precisely the role of this family of proteins in type III protein translocation and/or other virulence-related functions.

5. Regulation

The assembly and function of the TTSS involve the production of a large number of gene products, which could be very energy-consuming for bacteria. It is therefore understandable that bacteria often do not fully express the TTSS until they enter host tissues. Once bacteria are inside host tissues, expression of these genes is tightly regulated at both transcriptional and posttranscriptional levels. It is now clear from numerous in vitro gene expression studies that conditions activating the expression of TTSS-associated genes are often tailored to specific host–bacteria interactions and generally reflect the host environment that a given bacterium encounters during infection. These conditions may vary greatly among different TTSS-containing bacteria.

As a rule, optimal expression of TTSS-associated genes in mammalian pathogens occurs at 37 °C (the mammalian body temperature), whereas in plant pathogenic bacteria expression of TTSS genes occurs at much lower ambient temperatures (e.g., 20 °C). A variety of other environmental conditions (e.g., osmolarity; concentration of oxygen, calcium, iron, or nucleotides; pH; growth phase; cell density; nutrients; or host signals) regulate the expression of TTSSs in different ways in different bacteria. In response to the host environment, bacterial species have evolved a number of common and unique gene regulators, many of which are located within TTSS-associated PAIs. The most common regulators are members of two-component regulatory systems, AraC family transcription activators, quorum-sensing systems, RNA-binding proteins, and alternative sigma factors (see recent reviews: Refs. [43,209,210]). A recent study also shows the involvement of the Lon protease in the regulation of the *P. syringae* TTSS [211]. Here, we highlight only a few TTSS regulation examples that are either common in several bacteria or particularly intriguing.

5.1. Sensing different niches in the same host: Salmonella SPI-1 and SPI-2 TTSSs

As mentioned above, *Salmonella* contains two TTSSs. The SPI-1 TTSS is involved in bacterial invasion into host cells when bacteria are located extracellularly in the lumen of the small intestine. The SPI-1 TTSS is expressed optimally in growth conditions that reflect those in the lumen of the small intestine, including low oxygen, high osmolarity, slight alkalinity (pH 8), and nutrient-rich media [212]. The SPI-2 TTSS, on the other hand, is required for intracellular replication in both macrophages and epithelial cells. The expression of this TTSS is optimal in acidic, low osmolarity, low Ca^{2+} conditions, and minimal media, which reflect the environmental conditions inside *Salmonella*-containing vacuoles [213–215]. Thus, each of the two TTSSs is expressed optimally only when needed in a specific niche, clearly illustrating the temporal control of type III secretion imposed by differential expression of two TTSSs in the same host.

5.2. Sensing host contact: the R. solanacearum TTSS

Host contact is required for full activation of the expression of TTSS-associated genes in both mammalian (e.g., *Yersinia* spp., *Shigella* spp., *P. aeruginosa*) and plant pathogens (e.g., *R. solanacearum*) during infection. This requirement makes sense because contact with the host signals to bacteria that they are in the right place for maximal type III delivery of effector proteins. But how do bacteria sense physical contact? In a study unrelated to type III secretion, Zhang and Normark [216] showed that in uropathogenic *E. coli*, P pilus-mediated bacterial contact with host cells is required for transcriptional activation of a regulatory protein that is essential for the bacterial iron-starvation response.

The mechanism by which pilus-mediated contact transmits a signal to activate gene expression is not understood. However, it raises the possibility that other surface filaments, e.g., TTSS needles and pili (assembled from a basal level of TTSS expression?), could be involved in sensing host contact. As mentioned above, the *Shigella* needle and EPEC EspA filament have the same helical architecture as the flagellar filament. The flagellar filament undergoes switching between its two alternative helical forms (R form vs. L form) in response to the environmental cues, resulting in changes in the rotation direction of the flagellum and bacterial movement. The drastic architectural changes in the switching of the flagellar filament forms are linked to small changes in the conformation of each flagellar subunit. It is thus tempting to speculate that host contact of TTSS needles and pili triggers the switch of their helical state, producing an instant signal to the base of the TTSS in the inner membrane/cytoplasm to change the substrate selectivity and/or gene expression.

Although the possible involvement of TTSS needles and pili in host-contact sensing remains pure speculation, a nice series of studies has documented a signal transduction pathway that is initiated by host contact and concludes with full activation of the TTSS-associated genes in *R. solanacearum*. This signal transduction pathway consists of at least six genes: *prhA*, *prhI*, *prhR*, *prhJ*, *hrpG*, *hrpB* (Fig. 5A). PrhA shows homology to outer membrane siderophore receptors [217] and may function as a receptor for a plant-derived signal. PrhI belongs to the extracytoplasmic function sigma factors, and PrhR is predicted to be a transmembrane protein. PrhA, PrhJ, and PrhR constitute a "three-component signal transduction system" analogous to those involved in the transcriptional regulation of *E. coli* and *P. putida* iron transport genes [218–220]. PrhJ, HrpG, and HrpB are members of the LuxR/UhpA family, the OmpR family, and the AraC family of regulators, respectively [221,222]. Genetic and expression analyses reveal a regulatory cascade in which plant cell contact signals sensed by the outer membrane protein PrhA are transduced across the bacterial periplasm and the inner membrane, via PrhR and PrhI, to the cytoplasmic regulator PrhJ, which controls the expression of HrpG. HrpG then activates the *hrpB* regulatory gene, and, subsequently, all other TTSS transcriptional units. It remains to be determined whether this signal transduction pathway is a common mechanism for host contact activation of the TTSS in other bacteria.

5.3. Sensing via effector–chaperone complexes

Type III secretion chaperones have traditionally been thought to prevent folding, stabilize effectors, provide a secretion signal, and/or prioritize effector secretion through the TTSS, as discussed above. However, recent studies show that some chaperone proteins are involved in feedback gene regulation, providing temporal and spatial optimization of type III secretion in vivo. So far, the regulatory role

appears to be limited mainly to those homologous chaperones that are involved in the secretion of translocators: SycD/LcrH in *Yersinia* spp., SicA in *Salmonella* spp., and IpgC in *Shigella* spp. (Table 1). The purpose of this type of regulation is probably to establish a secretion and expression hierarchy so that maximal expression and secretion of effectors occur only when the translocator proteins are first secreted to form a pore in the host membrane for subsequent maximal delivery.

The first evidence for negative feedback regulation was found in *Yersinia*. Type III secretion in *Yersinia* spp. is activated in vivo upon host contact and in vitro in low Ca^{2+} medium [37]. Under conditions in which type III secretion does not occur (that is, in the presence of Ca^{2+} or in a type III secretion mutant), expression of *yop* genes is significantly reduced [113,117,223]. Host contact and low Ca^{2+} medium triggers secretion of LcrQ, which was initially thought to be a negative regulatory protein [109]. This phenomenon is reminiscent of secretion-coupled regulation of flagellar assembly genes, where secretion of the negative regulator FlgM, an anti-σ^{28} (FliA) protein, results in the expression of late flagellar biosynthesis genes [224]. A recent study, however, suggests that the negative regulatory effect of LcrQ on *yop* effector expression may be indirect. LcrQ appears to be more involved in substrate specificity/switching, presumably at the base of the TTSS and possibly by interacting with another chaperone protein, YscY (Ref. [225]; Fig. 5B). LcrQ secretion requires its cognate chaperone SycH [226]. Overexpression of LcrQ blocks secretion of some Yop proteins (e.g., YopE and YopM and presumably also a negative regulator) but not others (e.g., YopH) [227]. It was suggested that an LcrQ–SycH complex might impose a secretion hierarchy at the base of the TTSS, allowing LcrQ to be secreted first upon host contact [227]. Secretion of LcrQ would result in a higher concentration of free chaperone SycH. Either the presence of free SycH or the absence of LcrQ somehow signals the TTSS to allow secretion of other proteins (including a negative regulator?) and therefore amplification of Yop production (Fig. 5B).

The identity of the negative regulator(s) in the LcrQ–SycH-mediated regulatory pathway remains uncertain. In addition, although the LcrQ-mediated negative regulation could be achieved by transcriptional repression, posttranscriptional regulation may also be involved. A strong candidate for the latter mechanism is the YopD–SycD complex (Fig. 5B). YopD (a translocator protein) and SycD (a YopD chaperone) are required for LcrQ function [228,229]. The YopD–SycD complex has been shown to bind to the 5′untranslated regions (UTRs) of *yopQ* and *yopE* mRNA and LcrQ exerts its negative effect at the 5′UTR of *yop* genes [230,231]. Furthermore, mutations in the nucleotide sequence 5′-AUAAA-3′, which is found in many 5′ *yop* UTRs, either reduced or abolished LcrQ-mediated regulation, suggesting that the conserved sequence element in the 5′ region of *yop* mRNA is the target for posttranscriptional

regulation by two effector–chaperone complexes (YopD–SycD and LcrQ–SycH), apparently via translation inhibition/mRNA degradation. SycD-assisted secretion of YopD would relieve this posttranscriptional repression and allow for maximal *yop* gene expression.

Chaperone-mediated feedback gene regulation is also present in *Salmonella* and *Shigella*, even though the mechanism appears to be different. In *Salmonella* spp., the SicA chaperone [232] has been shown to function as a coactivator for InvF, an AraC family of transcriptional activators required for the expression of a subset of TTSS-associated genes in the *Salmonella* SPI-1 [233,234]. InvF binds to TTSS gene promoters in vitro, but this binding is apparently not sufficient for its function in vivo. SicA binds to InvF and functions as a coactivator in vivo [234]. The biological significance of SicA-mediated coupling of secretion and gene expression activation, again, likely lies in a secretion hierarchy. Because SicA is a chaperone for the translocators SipB and SipC, secretion of SipB and SipC (analogous to the secretion of YopD in *Yersinia*) results in a higher level of free SicA in the bacterial cytoplasm, which would signal to bacteria that a functional translocator has been assembled and maximal expression and delivery of effector proteins should occur. A similar feedback regulation mechanism has been found in *Shigella*, which involves MxiE (a homolog of InvF; Table 1) and IpgC (a homologue of SicA; Table 1) [235].

5.4. Sensing host defense and hormones

To survive bacterial infection, host species have evolved a variety of defense mechanisms. This has been well documented in numerous plant–pathogen interactions [236,237]. *Nramp1* (*N*atural *r*esistance-*a*ssociated *m*acrophage *p*rotein-*1*) is a mammalian resistance-associated gene that provides protection against several intracellular pathogens, including *S. enterica* serovar *typhimurium* [238,239]. Nramp1 appears to function as a divalent cation (e.g., iron) transport system across the membrane of the parasitic vacuole [240–242]. The defense function of Nramp1 most likely lies in its ability to deplete the SCV of iron and potentially other essential divalent cations during infection, thereby mediating its defense function against intracellular pathogens by starving the bacterium of essential nutrients. It was recently found that Nramp1 exerts a strong positive influence on the activation of the intracellular expression of the SPI-2 TTSS in the macrophages [243]. *S. enterica* serovar *typhimurium* has apparently evolved a means to counteract Nramp1-mediated defense by increasing the expression of an essential virulence system, the SPI-2 TTSS. Expression of SPI-2 TTSS enables bacterial replication at early time points, but eventually Nramp1 enables the host to control and ultimately survive infection with *S. enterica* serovar *typhimurium*. The SPI-2-mediated ability to replicate in the presence of Nramp1, although not to the extent of killing the host (as in hypersusceptible Nramp1$^{-/-}$ hosts),

may reflect the dynamic battle between host and pathogen leading to restricted infection. Restricted infection, instead of rapidly killing the host, is a common phenomenon in plant and mammalian bacterial pathogenesis, and is apparently a very effective means for bacterial survival and spread to other hosts.

Recent studies suggest that bacteria also sense host hormones. For example, the expression of the EHEC TTSS is controlled by *luxS*-mediated quorum sensing, in which gene expression occurs only when bacteria reach a certain population density [244]. The *luxS* gene product makes autoinducer 2 (AI-2), a furanosyl borate diester [245,246], and IA-3, the structure of which has not been determined [247]. AI-3, but not AI-2, is involved in quorum-sensing regulation of the EHEC TTSS. Surprisingly, Sperandio et al. [247] found that an EHEC *luxS* mutant, unable to produce the bacterial autoinducers, still responds to a host cell signal to activate expression of the TTSS. Sperandio et al. identified this signal as the hormone epinephrine and, furthermore, determined that adrenergic antagonists can block EHEC response to epinephrine and inhibit both type III secretion of EHEC in vitro and lesion formation by EHEC in HeLa cells [247]. These findings suggest that the AI-3 and epinephrine probably act through the same receptor/pathway in bacteria to activate TTSS expression.

There is a considerable amount of epinephrine-related compounds in the human gastrointestinal tract [248]. Thus, EHEC has evolved the ability to exploit a host signal present in the infection site and integrate it into an endogenous quorum-sensing mechanism to activate its TTSS. This is perhaps especially important for EHEC O157:H7, given its ability to initiate a successful infection at an unusually low infectious dose (50 bacteria) in one outbreak [249]. The low infectious dose suggests that quorum-sensing signaling might not occur in vivo, at least during the initial stages of infection, if only a few EHEC bacteria are present at the infection site. However, EHEC may take advantage of the existence of host epinephrine-related compounds as well as LuxS-dependent autoinducers (e.g., AI-3), which are likely to be ubiquitously produced by normal flora *E. coli* resident in the human gastrointestinal tract, to activate the TTSS, enabling the low infectious dose of this pathogen.

6. Concluding remarks

In summary, tremendous progress has been made in the understanding of the virulence-associated TTSS following the initial discovery and subsequent largely descriptive characterization of TTSS genes in the 1980s and early 1990s. Now we have begun to understand what the type III secretion structure looks like, how it is assembled and regulated, the molecular signals marking secreted effectors, the putative players involved in the crossing of the host cell wall and membrane, and the major host pathways targeted by type III effectors. Although it has been a long road for those pioneers who discovered the TTSS genes, this is a very exciting time for both pioneers and those who were attracted later to this fascinating area. In the next few years, we will undoubtedly continue to witness fast-paced discoveries in all aspects of the TTSS, including biophysical characterization of both the TTSS structure and individual components of the TTSS, understanding of the fine-tuning mechanisms to optimize TTSS expression and secretion efficiency in vivo, new-technology-driven visualization of the TTSS structure and function during infection, and molecular mechanisms by which the enigmatic type III effectors (especially those from plant pathogens) manipulate the host targets. All these discoveries will ultimately lead us to a more integrated and sophisticated view of the TTSS, which is, without any doubt, a wonderful organelle invented by bacteria that has enabled them to successfully occupy the nutrient-rich, but otherwise extremely hostile niches within eukaryotic organisms.

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