Type III Protein Secretion in Plant Pathogenic Bacteria

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Many gram-negative plant and animal pathogenic bacteria employ a type III secretion system (T3SS) to subvert and colonize their respective host organisms. The T3SS injects effector proteins directly into the cytosol of eukaryotic cells and thus allows the manipulation of host cellular activities to the benefit of the pathogen. In plant pathogenic bacteria, T3SSs are encoded by *hrp* (for hypersensitive response and pathogenicity) genes, which are so named because they are required for bacteria to cause disease in susceptible plants and to elicit the hypersensitive response in resistant plants (Lindgren et al., 1986). The hypersensitive response is a rapid local cell death at the infection site that restricts bacterial multiplication and is triggered by individual effector proteins in plants carrying a corresponding resistance gene (Dangl and Jones, 2001). *hrp* genes were found in almost all major gram-negative bacterial plant pathogens (e.g. *Pseudomonas syringae*, *Xanthomonas* spp., *Ralstonia solanacearum*, and *Erwinia* spp.), illustrating a central role of the T3SS in mediating diverse plant-bacteria interactions (Alfano and Collmer, 2004; He et al., 2004; Büttner and Bonas, 2006). In this Update, we highlight some basic as well as recent experiments that have collectively yielded molecular insights into general principles and unique properties of T3SSs in plant pathogenic bacteria. Environmental conditions that influence the level of *hrp* gene expression during the infection have been reviewed recently (Tang et al., 2006) and will not be discussed here.

T3SSs: FILAMENTOUS SUPRAMOLECULAR STRUCTURES

Delivery of effector proteins from the cytoplasm of gram-negative bacteria to the plant cell interior requires the T3SS to transport proteins across multiple physical barriers: the two bacterial membranes separated by a peptidoglycan layer and the plasma membrane of the plant cell, which is surrounded by a thick cell wall (Fig. 1A). It is widely believed that the T3SS provides a continuous channel for effector proteins to travel from the bacterial cytoplasm directly into the cytoplasm of eukaryotic cells. Central to this belief is the observation that T3SSs in different bacteria invariably assemble filamentous supramolecular structures (He et al., 2004).

Although the first T3SS-associated filamentous structure was discovered in the plant pathogen *P. syringae* (Roine et al., 1997), the most elegant and seminal work in the characterization of T3SS supramolecular structures was carried out in the mammalian pathogen *Salmonella enterica*. Work of Kubori and colleagues (1998) revealed that the T3SS of *S. enterica* consists of two pairs of rings that interact with the cytoplasmic and the outer membrane, respectively, and an extracellular filamentous extension that is 8 nm in diameter and 80 nm in length, resembling a needle, which is also present in other animal pathogenic bacteria (Kubori et al., 1998; Marlovits et al., 2004, 2006; Galán and Wolf-Watz, 2006; Fig. 1B). The needle presumably serves as conduit for secreted proteins to the eukaryotic plasma membrane. Notably, in enteropathogenic *Escherichia coli*, the T3SS needle is connected to an additional filament that consists of the EspA protein and reaches a length of up to 600 nm (Daniell et al., 2001; Sekiya et al., 2001). It was therefore suggested that the EspA filament helps to penetrate the glycocalyx on the surface of the intestinal epithelium. A similar structure is associated with the needle of one T3SS from *Salmonella typhimurium* (Chakravortty et al., 2005).

So far, a complete T3SS supramolecular structure has not been purified from plant pathogenic bacteria. However, as mentioned above, T3SS filaments called Hrp pili have been found and characterized in all major plant pathogens that contain an active T3SS (Roine et al., 1997; Van Gijsegem et al., 2000; Jin et al., 2001; Weber et al., 2005; Table I). The essential contribution of Hrp pili to T3S and the results of in situ immunogold labeling experiments suggest that Hrp pili similarly to T3SS needles provide a protein transport channel for effector proteins to the host-pathogen interface (Jin and He, 2001; Li et al., 2002). Since Hrp pili are much longer (in the micrometer range) than the needle extension from animal pathogenic bacteria, they presumably span the thick plant cell wall, which is a major obstacle in the interkingdom protein transport between plant pathogenic bacteria and their host cells (Fig. 1A).

Interestingly, the amino acid sequences of the major subunits of Hrp pili are hypervariable in different
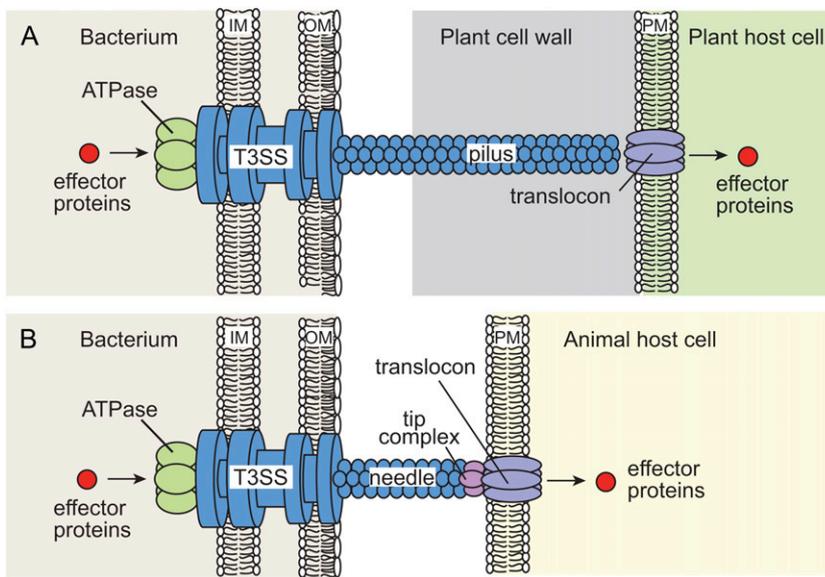
Figure 1. Schematic representation of the T3SS from plant (A) and animal (B) pathogenic bacteria. The secretion apparatus spans both bacterial membranes and is associated with a cytoplasmic ATPase. The T3SS from plant pathogenic bacteria is connected to an extracellular pilus that presumably spans the plant cell wall. The T3SS system from animal pathogenic bacteria is associated with a short extracellular needle, which serves as a transport channel for secreted proteins. The needle is linked via the so-called tip complex to the translocon, which forms a proteinaceous channel in the host plasma membrane and allows transport of effector proteins into the host cell cytosol. Evidence for the presence of a tip complex in plant pathogenic bacteria is still missing. IM, Inner membrane; OM, outer membrane; PM, plasma membrane.

subspecies of bacterial pathogens, although the predicted secondary structures of these proteins are remarkably similar, consisting almost exclusively of α-helices (Lee et al., 2005; Weber and Koebnik, 2005). This observation led to the speculation that extracellular Hrp pili in plant pathogens may be rapidly evolving to avoid recognition by the plant defense surveillance system. Indeed, two studies have provided evidence for strong positive selection (generation of new beneficial alleles) or diversifying selection (generation of multiple different alleles) of Hrp pilus protein sequences in *Xanthomonas* spp. and *P. syringae*, respectively (Guttman et al., 2006; Weber and Koebnik, 2006).

HOW ARE SUBSTRATE PROTEINS RECOGNIZED BY THE T3SS?

T3S substrate proteins possess noncleavable secretion signals in the N-terminal protein regions, but no discernible amino acid or peptide similarities can be found (Michiels and Cornelis, 1991; Arnold et al., 2009; Samudrala et al., 2009). In fact, there has been some debate as to whether it is the amino acid or mRNA sequence that is recognized by the T3SS. The prevailing view is that it is the amphipathic nature and the amino acid composition of the N-terminal region of T3S substrate proteins that serves as a secretion signal (Galán and Wolf-Watz, 2006; Arnold et al., 2009; Samudrala et al., 2009). This view is consistent with the finding that some specific biophysical features are present in the first 50 amino acids of effector proteins from *P. syringae*: (1) solvent-exposed amino acids in the first five amino acids, (2) the lack of Asp or Glu residues in the first 12 amino acids, and (3) the amphipathicity and the enrichment of polar residues

in the first 50 amino acids (Guttman et al., 2002; Petnicki-Ocwieja et al., 2002). Furthermore, recent bioinformatic analyses of effector proteins from plant and animal pathogenic bacteria revealed that the N-terminal 25 amino acids are enriched in Ser and coiled regions but lack Leu (Arnold et al., 2009; Samudrala et al., 2009). Taken together, these features may make the N-terminal regions of T3S substrate proteins structurally flexible and probably unfolded, an important prerequisite for their transport through the narrow inner channel of the T3SS, which is presumably only 2.8 nm in diameter as was shown for the T3SS from animal pathogenic bacteria (Marlovits et al., 2004, 2006; Galán and Wolf-Watz, 2006).

For some T3S substrate proteins, however, the presence of an N-terminal secretion signal may not be sufficient for maximal secretion. In these cases, specific T3S chaperone proteins are needed. T3S chaperones are generally small (<170 amino acids), acidic (pI < 5.5), and often contain an amphipathic α-helix near the C terminus (Parsot et al., 2003). Interestingly, the chaperone-encoding genes are often located adjacent to the cognate effector genes, suggesting strong selection for their coexistence in the genome. Although many T3S chaperones are specifically required for the secretion of a cognate effector protein, some seem to be more general and are involved in the secretion of many substrate proteins, as reported for HpaB from *Xanthomonas campestris* pv *vesicatoria* (Parsot et al., 2003; Büttner et al., 2004, 2006; Table I). T3S chaperones presumably target their substrates to conserved components of the T3SS, such as the cytoplasmic ATPase that is associated with the secretion apparatus (see below; Fig. 1). There is also some evidence that T3S chaperones may contribute to the stability of at least some effector proteins inside bacteria. In the plant pathogen *Erwinia amylovora*, for example, the T3S

Table I. *Contribution of secreted and cytoplasmic control proteins from plant pathogenic bacteria to virulence*

Bacterial Species/Predicted Protein Function	Protein[a]	Localization[b]	Contribution to T3S and Virulence	Reference
E. amylovora:				
Pilus protein	HrpA	S	Essential for disease and T3S	Jin et al. (2001)
Translocon proteins	HrpK	S	Homologous to HrpK1 from *P. syringae*, dispensable for virulence	Nissinen et al. (2007)
Control protein	HrpN	S+T	Harpin, contributes to translocation of the effector DspA/E	Bocsanczy et al. (2008)
	HrpJ	S	Required for virulence, contributes to secretion of harpins and translocation of the effector DspA/E	Nissinen et al. (2007); Bocsanczy et al. (2008)
P. syringae pv *tomato*:				
Pilus protein	HrpA	S	Essential for disease and T3S	Roine et al. (1997)
Translocon protein	HrpK1	S+T	Contributes to disease and effector protein translocation	Petnicki-Ocwieja et al. (2005)
Control proteins	HrpZ1	S+T	Harpin, forms ion channels	Lee et al. (2001); Kvitko et al. (2007);
	HrpW1	S+T	Harpin, C-terminal pectate lyase domain	Engelhardt et al. (2009)
	HopAK1	S+T	Harpin, C-terminal pectate lyase domain HrpZ1, HrpW, and HopAK1 contribute to effector protein translocation and disease	
	HrpH	T	HrpH, HopP1, and HopAJ1 are predicted	Oh et al. (2007)
	HopP1	T	lytic transglycosylases and contribute to	
	HopAJ1	NT	effector protein translocation and disease	
	HrpJ	S+T	Required for disease, contributes to T3S	Fu et al. (2006)
	HrpP	T	Predicted T3S4 protein, essential for disease	Morello and Collmer (2009)
R. solanacearum:				
Pilus protein	HrpY	S	Essential for disease and T3S	Van Gijsegem et al. (2000)
Translocon protein	PopF1	S	Essential for disease	Meyer et al. (2006)
	PopF2	S	Dispensable for disease	Meyer et al. (2006)
Control proteins	HpaB	n.a.	Required for disease	Mukaihara et al. (2004)
Xanthomonas spp.:				
Pilus protein	HrpE$_{Xcv}$	S	Essential for disease and T3S	Weber et al. (2005)
Translocon protein	HrpF$_{Xcv}$	S	Essential for disease and effector protein translocation	Büttner et al. (2002)
	HrpF$_{Xoo}$	S	Contributes to disease	Sugio et al. (2005)
Control proteins	HpaA$_{Xcv}$	S+T	Contributes to disease, secreted regulator of HpaB	Lorenz et al. (2008a)
	HpaB$_{Xcv}$	C	Global T3S chaperone, essential for disease	Büttner et al. (2004)
	HpaC$_{Xcv}$	C	T3S4 protein, contributes to disease	Lorenz et al. (2008a)
	HpaH$_{Xcv}$	n.a.	Predicted lytic transglycosylase, contributes to effector protein translocation and disease	Büttner et al. (2007)

[a]*Xcv, X. campestris* pv *vesicatoria; Xoo, X. oryzae* pv *oryzae.* [b]Localization: C, cytoplasmic; n.a., not analyzed; NT, not translocated; S, secreted; T, translocated.

chaperone DspF seems to be required for the stability of the effector protein DspA/E, a major virulence factor in this devastating pathogen (Gaudriault et al., 2002). Since the normal folding environment for effector proteins is inside the eukaryotic cell, it was proposed that some T3S chaperones prevent misfolding and thus subsequent degradation of their interaction partners in the bacterial cytoplasm.

TRANSLOCATION OF EFFECTOR PROTEINS INTO THE HOST CELL

How the T3SS penetrates the host plasma membrane is very much an open question. In principle, one could imagine that the T3SS needle/pilus may physically penetrate the membrane and/or cell wall of the eukaryotic cell, as suggested for the *Yersinia* needle (Hoiczyk and Blobel, 2001). Alternatively, the needle/pilus may connect to additional T3SS-associated protein complexes in the eukaryotic cell membrane and/or cell wall to provide a continuous conduit for the delivery of effector proteins into the eukaryotic cell. This hypothesis is supported by the finding that the T3SS secretes several translocator proteins. The function of these proteins is to facilitate the translocation of effector proteins across the eukaryotic cell membrane (i.e. they are not required for secretion of effector proteins across the bacterial envelope). In the mammalian pathogen *Yersinia* spp., three proteins (YopB, YopD, and LcrV) were shown to be involved in the formation of a protein complex (called a translocon) that inserts into the eukaryotic cell membrane. YopB and YopD form a proteinaceous transmembrane chan-

nel that is connected to the needle via a so-called tip complex consisting of LcrV (Håkansson et al., 1996; Neyt and Cornelis, 1999; Mueller et al., 2008). The tip complex presumably facilitates the assembly of the translocon and thus allows the continuous passage of effector proteins into the eukaryotic cell cytosol (Fig. 1B).

YopB, YopD, and LcrV are not conserved among animal and plant pathogenic bacteria, suggesting that the mechanisms underlying effector protein translocation vary among different pathogens. In plant pathogenic bacteria, several putative translocator proteins of the T3SS have been identified, including HrpF from *X. campestris* pv *vesicatoria*, PopF1 and PopF2 from *R. solanacearum*, and HrpK proteins from *P. syringae* and *E. amylovora* (Table I; Fig. 2). It was shown that the secreted HrpF protein from *X. campestris* pv *vesicatoria* is essential for effector protein translocation and induces the formation of ion channels in artificial lipid bilayers, suggesting that it is a component of the predicted translocation channel (Büttner et al., 2002). HrpF is homologous to PopF1 and PopF2 from *R. solanacearum* (Fig. 2), which contribute to bacterial pathogenicity and effector protein translocation but are not required for efficient T3S (Table I). Interest-

ingly, the phenotype of a *popF1 popF2* double mutant can partially be restored upon ectopic expression of *hrpF* from *X. campestris* pv *campestris*, suggesting a functional similarity among putative translocon proteins from *Xanthomonas* spp. and *R. solanacearum* (Meyer et al., 2006).

Notably, HrpF, PopF1, and PopF2 do not share significant protein identity with the predicted translocator HrpK1 from *P. syringae* (Fig. 2). HrpK1 is secreted and translocated by the T3SS and contributes to bacterial pathogenicity and effector protein translocation (Petnicki-Ocwieja et al., 2005; Table I). By contrast, the homologous HrpK protein from *E. amylovora* is dispensable for the bacterial interaction with the plant, suggesting the presence of additional accessory proteins (Table I). What could these other accessory proteins be? Many T3SSs in plant pathogenic bacteria secrete a family of extracellular proteins called harpins (Wei et al., 1992; He et al., 1993; Arlat et al., 1994). Harpins share the general properties of being Gly rich and heat stable and are able to induce a suite of plant defense responses when infiltrated into the plant apoplast at high concentrations (for review, see He et al., 2004). However, the physiological functions of harpins in promoting disease have been enigmatic

translocator proteins		HrpF$_{Xcv}$	PopF1$_{Rsol}$	PopF2$_{Rsol}$	HrpK1$_{Psyr}$	HrpK$_{Eam}$	YopB$_{Yent}$
	HrpF$_{Xcv}$	100	48	43	n.s.	n.s.	32[a]
	PopF1$_{Rsol}$	48	100	81	n.s.	n.s.	n.s.
	PopF2$_{Rsol}$	43	81	100	n.s.	24[b]	n.s.
	HrpK1$_{Psyr}$	n.s.	n.s.	n.s.	100	41	26[c]
	HrpK$_{Eam}$	n.s.	n.s.	24[b]	41	100	n.s.

T3S4 proteins		HpaC$_{Xcv}$	HpaP$_{Rsol}$	HrpP$_{Psyr}$	YscP$_{Yent}$
	HpaC$_{Xcv}$	100	27[d]	n.s.	n.s.
	HpaP$_{Rsol}$	27[d]	100	n.s.	n.s.
	HrpP$_{Psyr}$	n.s.	n.s.	100	n.s.

Figure 2. Protein identities among selected putative translocators and T3S4 proteins, respectively, from plant pathogenic bacteria and the animal pathogen *Yersinia enterocolitica*. Full-length protein sequences were compared using the BLASTP program (http://blast.ncbi.nlm.nih.gov). Numbers refer to the percentage of protein identity. Boxes with protein identities of 75% to 100% are shaded in dark gray, and boxes with protein identities of 25% to 50% are in light gray. The following protein sequences were used: HrpF (*X. campestris* pv *vesicatoria* strain 85-10, AAB86527), PopF1 (*R. solanacearum* GMI1000, CAD18706), PopF2 (*R. solanacearum* GMI1000, CAD18051), HrpK1 (*P. syringae* pv *tomato* DC3000, AAO54927), HrpK (*E. amylovora*, AAX39435), YopB (*Y. enterocolitica*, AAK69211), HpaC (*X. campestris* pv *vesicatoria* strain 85-10, CAJ22055), HpaP (*R. solanacearum* GMI1000, CAB58249), HrpP (*P. syringae* pv *tomato* DC3000, AAG33881), and YscP (*Y. enterocolitica*, AAK69225). n.s., Not significant (protein identity among full-length proteins was defined as not significant when regions with identical residues were smaller than 100 amino acids); *Eam*, *E. amylovora*; *Psyr*, *P. syringae* pv *tomato* DC3000; *Rsol*, *R. solanacearum* GMI1000; *Xcv*, *X. campestris* pv *vesicatoria* strain 85-10; *Xoo*, *X. oryzae* pv *oryzae* PXO99A; *Yent*, *Y. enterocolitica*. [a], The region with 32% protein identity is restricted to 137 amino acids; [b], the region with 24% protein identity is restricted to 205 amino acids; [c], the region with 26% protein identity is restricted to 105 amino acids; [d], the region with 27% protein identity is restricted to 147 amino acids.

ever since their discovery. Recent experimental evidence reported for *P. syringae* and *E. amylovora* suggests an exciting possibility that harpins are important for the translocation of effector proteins into the plant cell. For example, deletion of all four harpin-encoding genes (*hrpZ1*, *hrpW1*, *hopAK1*, and *hopP1*; Table I) in *P. syringae* pv *tomato* strain DC3000 leads to reduced effector protein translocation (Kvitko et al., 2007). This deleterious effect is severely enhanced upon additional deletion of the putative translocator gene *hrpK1* (Kvitko et al., 2007). Importantly, effector secretion in culture is not affected in this polymutant deleted in harpin genes and *hrpK1*, suggesting additive effects of harpins and HrpK1 in effector translocation. Notably, HrpN, a major harpin secreted by *E. amylovora*, was recently shown to be essential for translocation of the DspA/E effector protein into the plant cell (Bocsanczy et al., 2008; Table I).

It is currently unclear how harpins facilitate effector translocation. Several harpins contain intriguing motifs that suggest potential interactions with plant cell wall components. For example, the *P. syringae* harpins HrpW1 and HopAK1 contain a C-terminal pectate lyase-like domain (Kvitko et al., 2007). This domain in HrpW1 binds to calcium pectate, a major plant cell wall component (Charkowski et al., 1998). Even HrpZ1, which lacks an obvious plant cell wall-interacting domain, also seems to bind to the plant cell wall (Hoyos et al., 1996). These observations suggest that harpins may be involved in modifying (loosening?) the plant cell wall to facilitate the initial penetration of the T3SS pilus. Although very attractive, this possibility is not consistent with results from further genetic analyses of *hrpK1* and harpin genes in *P. syringae*. Most strikingly, the polymutant lacking both *hrpK1* and the four harpin genes could be complemented by either *hrpK1* or individual harpin genes (Kvitko et al., 2007). These observations suggest that HrpK1 and harpins are functionally redundant and act at the same step of effector translocation. Notably, harpins are found to be associated with synthetic lipid membranes and to form pores (Lee et al., 2001; Racapé et al., 2005; Engelhardt et al., 2009). Further studies are needed to determine whether harpins and HrpK proteins form functional complexes in the plant plasma membrane and/or the plant cell wall in vivo. If such complexes are found and shown to be important for effector translocation, the next step would be to study a possible physical connection with the T3SS pilus in planta.

HOW DO BACTERIAL PATHOGENS COORDINATE T3SS ASSEMBLY AND EFFECTOR TRANSLOCATION?

T3S is presumably a hierarchical process. As the T3SS appears to be dedicated to delivering effector proteins, which function inside the eukaryotic cell, it would make sense if secretion and translocation of effector proteins occur after the T3SS is fully assembled to prevent excessive leakage of effector proteins into the extracellular milieu. If this is indeed the case, how does the T3SS prevent secretion of effector proteins before the extracellular parts (pilus and translocon) of the T3SS are assembled? Studies of the T3SS in animal pathogens suggest a fascinating substrate specificity switch process from secretion of the needle structural proteins to secretion of translocators and effector proteins (Cornelis et al., 2006; Ferris and Minamino, 2006). As a consequence, the needle extension in mammalian pathogens has a relatively well-defined length (e.g. 80 nm in *Salmonella*; Cornelis et al., 2006; Galán and Wolf-Watz, 2006). The substrate specificity switch is presumably mediated by T3S substrate specificity switch (T3S4) proteins that are secreted by the T3SS and interact with the C-terminal cytoplasmic domain of a member of the conserved YscU/FlhB protein family. YscU and homologous proteins are inner membrane components of the T3SS and possess a cytoplasmic domain that was proposed to be involved in T3S substrate recognition (Cornelis et al., 2006; Ferris and Minamino, 2006). In animal pathogenic bacteria, T3S4 proteins presumably induce a conformational change in the cytoplasmic domain of YscU or homologous proteins and thus alter the substrate specificity of the T3SS. According to a model proposed for the T3S4 protein YscP from *Yersinia* spp., the N terminus of YscP is attached to the growing needle, whereas the C terminus interacts with YscU and triggers the substrate specificity switch once the T3S4 protein is stretched (Journet et al., 2003; Cornelis et al., 2006; Ferris and Minamino, 2006). This hypothesis implies that T3S4 proteins act as molecular rulers that determine needle length. Notably, however, this molecular ruler model was challenged by the finding that T3S4 proteins control the formation of an inner rod structure inside the base of the T3SS (i.e. beneath the needle extension), suggesting that it is the formation of the inner rod that induces the switch in T3S substrate specificity (Marlovits et al., 2004, 2006; Wood et al., 2008).

It is currently unknown whether plant and animal pathogenic bacteria employ similar mechanisms to control substrate specificity and length of extracellular appendages of the T3SS. In plant pathogenic bacteria, it is not yet clear whether or not T3SS pili have a defined length in vivo. In vitro sample preparation often shears long Hrp pili into shorter fragments, making it impossible to accurately estimate the full length of Hrp pili. Nevertheless, putative T3S4 proteins have also been identified in plant pathogenic bacteria. In *X. campestris* pv *vesicatoria*, the predicted T3S4 protein HpaC was shown to switch the substrate specificity of the T3SS from secretion of the putative inner rod protein HrpB2 to secretion of translocators and effector proteins. HpaC interacts with and presumably induces a conformational change in the C-terminal domain of HrcU, which is a member of the YscU/FlhB protein family (Lorenz et al., 2008b; Fig. 3).
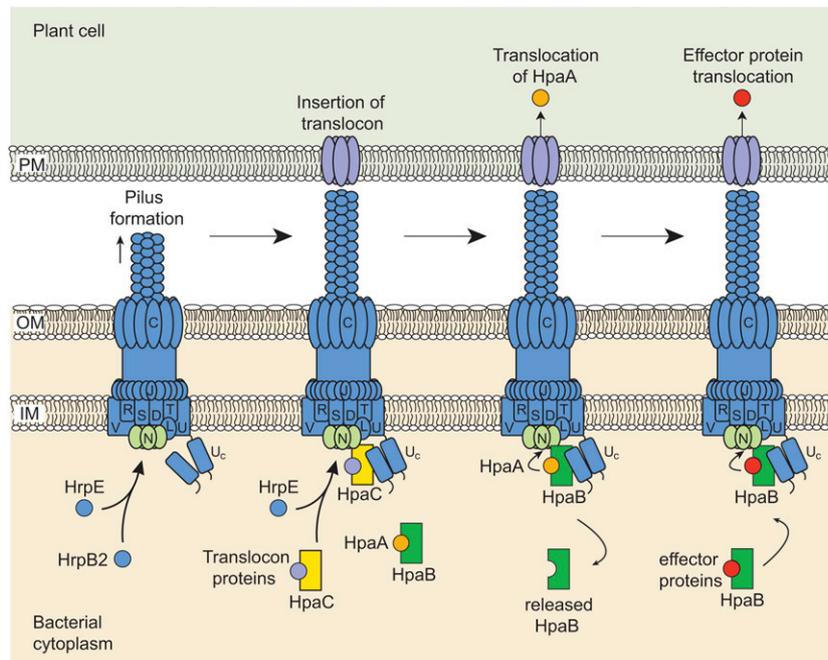
Figure 3. Model of T3S in the plant pathogenic bacterium *X. campestris* pv *vesicatoria*. The secretion apparatus consists of approximately 20 components, nine of which, labeled with single letters here, are designated Hrc (Hrp conserved) because they are conserved among plant and animal pathogenic bacteria. The pilus protein HrpE and the putative inner rod protein HrpB2 are the first substrates that travel the T3SS. A yet unidentified signal activates a switch in the T3S substrate specificity that depends on the cytoplasmic T3S4 protein HpaC and an inner membrane component of the T3SS, HrcU. HrcU consists of four transmembrane helices and a C-terminal cytoplasmic domain (U_C), which is proteolytically cleaved. HpaC presumably induces a conformational change in the C-terminal cytoplasmic domain of HrcU and activates secretion of translocon and effector proteins. Targeting of effector proteins to the secretion apparatus depends on the global T3S chaperone HpaB, which binds to multiple effector proteins. The activity of HpaB is normally inhibited by HpaA, which binds to HpaB in the bacterial cytoplasm. Secretion and translocation of HpaA after assembly of the T3SS liberates HpaB and is thus a prerequisite for the efficient translocation of effector proteins (Lorenz et al., 2008a). Secretion of all known T3S substrates depends on the ATPase HrcN, which was shown to disassemble HpaB-effector complexes. IM, Inner membrane; OM, outer membrane; PM, plasma membrane.

In contrast to T3S4 proteins from animal pathogenic bacteria, however, HpaC is not secreted by the T3SS (Table I). Furthermore, HpaC does not control secretion of the pilus protein HrpE, suggesting that HpaC is not involved in length determination of the pilus (Lorenz et al., 2008b). T3S4 proteins are not highly conserved among different plant and animal pathogenic bacteria, and no significant sequence identity is detected between HpaC from *X. campestris* pv *vesicatoria* and the predicted T3S4 protein HrpP from *P. syringae* (Fig. 2). HrpP is secreted and translocated by the T3SS from *P. syringae* and is required for efficient secretion of all T3S substrates that were tested, including the pilus protein HrpA and the predicted inner rod component HrpB (Morello and Collmer, 2009). Thus, HrpP presumably does not act similarly to known T3S4 proteins from animal pathogenic bacteria that switch the substrate specificity of the T3SS from early (inner rod proteins and needle proteins) to late (translocators and effector proteins) T3S substrates.

In addition to T3S4 proteins, T3S in plant pathogenic bacteria is controlled by other accessory proteins that

act in the bacterial cytoplasm or are secreted by the T3SS (Table I). For instance, it was shown that the secreted HrpJ proteins from *E. amylovora* and *P. syringae* are required for efficient T3S (Fu et al., 2006; Bocsanczy et al., 2008). Both proteins contribute to the secretion of harpin proteins and thus may indirectly affect effector protein translocation (Fu et al., 2006; Nissinen et al., 2007; Bocsanczy et al., 2008; see above). In *X. campestris* pv *vesicatoria*, translocation of effector proteins is differentially regulated by the global T3S chaperone HpaB, which specifically promotes the translocation of a certain class of effector proteins (Büttner et al., 2006). The activity of HpaB is presumably controlled by the secreted regulator HpaA that binds to HpaB in the bacterial cytoplasm and allows secretion of extracellular components of the T3SS. After assembly of the T3SS, secretion of HpaA liberates HpaB and thus activates secretion and translocation of effector proteins (Lorenz et al., 2008a). In addition to HpaB, translocation of a certain set of effectors from *X. campestris* pv *vesicatoria* requires HpaH, which is a predicted lytic transglycosylase

that might facilitate assembly of the T3SS (Büttner et al., 2007). Interestingly, experimental evidence for a role of lytic transglycosylases in the regulation of effector protein translocation was also reported for *P. syringae* (Oh et al., 2007; Table I). However, the precise role of lytic transglycosylases from plant pathogenic bacteria during T3S is not yet understood.

ENERGY SOURCE FOR POWERING T3S

The final aspect of T3SSs from plant pathogenic bacteria we would like to highlight concerns the energy source for T3S. All characterized T3SSs contain a cytoplasmic/inner membrane ATPase (HrcN in plant pathogens; Fig. 1) 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 (Abrahams et al., 1994). However, the α-subunit equivalent is not found in T3SSs. Using hydrodynamic, cross-linking, and ultrastructural analyses, Pozidis et al. (2003) found that the *P. syringae* HrcN ATPase 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 microscopy, the dodecamer appears as an organized round particle with an o.d. of 13 nm (Pozidis et al., 2003). The dodecameric HrcN ATPase seems to form double hexameric stacks, as was found for other dodecameric traffic ATPases (Müller et al., 2006). Analysis of the HrcN ATPase from *X. campestris* pv *vesicatoria* revealed multiple protein-protein interactions between HrcN and cytoplasmic and inner membrane components of the T3SS, including the T3S4 protein HpaC and the global T3S chaperone HpaB (Lorenz and Büttner, 2009; Fig. 3). It is likely that the ATPase is required to release and unfold chaperone-bound effectors (Akeda and Galán, 2005; Lorenz and Büttner, 2009; Fig. 3). Furthermore, the HrcN ATPase presumably also serves as part of a docking site for accepting T3S substrates without accompanying T3SS chaperones (Lorenz and Büttner, 2009).

Another potential energy source for T3S is the proton motive force, as reported for the assembly of bacterial flagella (Minamino and Namba, 2008; Paul et al., 2008). However, there is so far no experimental evidence that T3S in plant pathogens can occur in the absence of a functional ATPase (Lorenz and Büttner, 2009). It therefore remains to be determined experimentally whether proton motive force is also an energy source for powering virulence-associated T3SSs in plant pathogens.

CONCLUDING REMARKS

With this update, we hope to give readers an impression of the substantial progress made in the understanding of the T3SS in plant pathogenic bacteria following the initial discovery of the enigmatic *hrp* genes in the 1980s. Many questions remain to be answered: Can we visualize the real-time assembly and action of the T3SS in translocating effectors in planta? How does a bacterium make sure that all necessary effector proteins, which can be as many as several dozens, are injected into a host cell and in a timely manner? Does the assembly of the T3SS activate plant defense responses? If so, what is the nature of such defenses? Can we find chemicals and genetic engineering methods that could effectively and safely inhibit the T3SS during infection? Can we purify the complete T3SS from plant pathogens? Answering these fundamental questions should further advance our basic understanding of the T3S mechanism in plant-bacteria interactions and provide possible solutions to bacterial disease control.

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