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MiniReview

Type III secretion: The bacteria-eukaryotic cell express

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Abstract

Type III secretion (T3S) is an export pathway used by Gram-negative pathogenic bacteria to inject bacterial proteins into the cytosol of eukaryotic host cells. This pathway is characterized by (i) a secretion nanomachine related to the bacterial flagellum, but usually topped by a stiff needle-like structure; (ii) the assembly in the eukaryotic cell membrane of a translocation pore formed by T3S substrates; (iii) a non-cleavable N-terminal secretion signal; (iv) T3S chaperones, assisting the secretion of some substrates; (v) a control mechanism ensuring protein delivery at the right place and time. Here, we review these different aspects focusing in open questions that promise exciting findings in the near future.

Keywords: Bacterial pathogenesis; EPEC; *Salmonella*; *Shigella*; Type III secretion; *Yersinia*

1. Introduction

Type III secretion (T3S) systems are protein export devices essential for the interaction between Gram-negative bacteria and their eukaryotic hosts. These systems are present in many pathogenic bacteria for animals and plants, but also in endosymbionts. Bacteria use T3S to translocate proteins across lipid membranes and to inject some of them, called "effectors", into the cytoplasm of host cells. Inside the eukaryotic cell cytoplasm, the effectors thwart or hijack host cell signaling to the benefit of the bacteria, in the case of pathogens, or both organisms, in the case of symbionts.

With the sequencing of bacterial genomes, the list of species known to harbour T3S systems has become too long to be listed exhaustively (Table 1). Among the animal pathogens, T3S have been most intensively studied in *Yersinia* spp., *Salmonella* spp., *Shigella* spp., *Pseudomonas aeruginosa*, and enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC). The most studied T3S system from a plant pathogen is probably that of *P. syringae*, but this secretion system has also been identified and studied in several other plant pathogens.

The T3S substrates are synthesized in the bacterial cytoplasm and are secreted by a nano-machine, called injectisome, across the bacterial inner membrane, periplasm and the bacterial outer membrane. Some of these secreted proteins, called "translocators" insert in the host cell plasma membrane and mediate the translocation of the effectors across eukaryotic plasma or vacuolar membranes into the host cell cytosol. Secretion of the effectors across the bacterial membranes and translocation through the eukaryotic membrane is thought

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Table 1
T3S systems of bacterial pathogens for mammals

Bacterium	Diseases	T3S system(s) function(s)[a]
Aeromonas spp.	Opportunistic human pathogen causing gastroenteritis and septicemia (*A. hydrophila*)	Asc
	Furunculosis in salmonids (*A. salmonicida*)	Cytotoxicity on fish cells
Bordetella spp.	Respiratory tract infections, whooping cough (*B.pertussis*, *B. parapertussis*) Infection of four-legged animals (*B. bronchiseptica*)	Bsc Establishment of long term infection Downregulation of inflammation
Burkholderia spp.	Melioidosis: pneumonia and skin abcesses (*B. pseudomallei*)	Bp1 (*B. pseudomallei*) ?
	Glanders (*B. mallei*)	Bp2 (*B. pseudomallei*; *B. mallei*; *B. thailandensis*) ?
	Pulmonar infection of cystic fibrosis patients (*B. cepacia* complex)	Bp3 (*B. pseudomallei*; *B. mallei*; *B. thailandensis*) Survival and escape from macrophage vacuoles; Invasion of non-phagocytic cells Bcsc (*B. cepacia* complex) Important for virulence in the murine model
Chlamydiaceae	Sexual transmitted diseases, trachoma (*C. trachomatis*) Pneumonia, atherosclerosis (*C. pneumoniae*)	Sct Cell internalization
Chromobacterium violaceum	Sporadic infections in humans and mammals resulting in fulminant septicaemia that resembles melidiosis	Civ Similar to Inv (SPI-1) and Mxi-Spa Csa Similar to Ssa (SPI-2)
Citrobacter rodentium	Murine colonic hyperplasia, attaching/effacing lesions 1	Esc Adhesion to the intestinal epithelium Cytoskeleton rearrangements
Desulfovibrio vulgaris	Implicated in ulcerative colitis	Dsc ?
Edwardsiella tarda *Edwradsiella ictaluri*	Hemorrhagic septicemia in fishes, diverse infections in humans	Eds Survival and replication in fish phagocytes
Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC)	Diarrhoea, attachment/effacement lesion (EPEC)	Esc
	Diarrhoea, attachment/effacement lesion, haemorrhagic colitis (EHEC)	Colonization Cell attachment Actin remodelling Pedestal formation
Pseudomonas aeruginosa	Pneumonias and chronic bronchopneumonia in patients with cystic fibrosis; ulcerative keratitis	Psc Cytotoxicity Block phagocytosis

Salmonella spp.	From mild food poisoning to life-threatening systemic infections, depending on the *S. enterica* serovars Example: *S. typhi* causes typhoid fever and *S. typhimurium* causes gastroenteritis	Inv (SPI-1) (*S. enterica*; *S. bongori*) Invasion of non-phagocytic cells; activate inflammation Ssa (SPI-2) (*S. enterica*) Promote intracellular survival
Shigella spp.	Bacillary dysenteria	Mxi-Spa Invasion of non-phagocytic cells; activate inflammation; intracellular survival
Vibrio parahaemoliticus	Gastroenteritis Wound infections	Vcr Cytotoxicity to Hela cells Vpa Enterotoxicity in rabbit ileal loops
Yersinia spp.	Bubonic plague (*Y. pestis*) Gastrointestinal disorders (*Y. pseudotuberculosis* and *Y. enterocolitica*)	Ysc-Yop (*Y. pestis*; *Y. pseudotuberculosis*; *Y. enterocolitica*) Block phagocytosis; inhibition of the pro-inflammatory response Ysa (*Y. enterocolitica*) ? Ypc (*Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*) ?

[a] Some bacteria possess more than one T3S in their genome. The different T3S systems are identified for each bacteria by the name of their injectisome genes. The exception is *Burkholderia*, where the different T3S systems are named Bp1, 2, 3 (for *B. pseudomallei* T3S 1, 2, and 3). The two *Salmonella* T3S systems (Inv and Ssa) are also named in parentheses by the pathogenicity island that encode them: *Salmonella* pathogenicity Island-1 and -2 (SPI-1 and SPI-2). See text for references.

to occur in one step. Secretion of some of these proteins is assisted in bacteria by specialized and strictly cytosolic T3S chaperones. Besides the effectors and translocators, T3S systems also secrete components of the injectisome and regulatory proteins. The whole process is tightly controlled to allow efficient secretion of proteins at the right place and time.

The T3S pathway is also used for the export of flagellar proteins. Injectisomes and flagellae are evolutionary related and share a remarkably similar basal body structure. In this review, we will focus on the injectisome function. We apologize to the authors of many important contributions that could not be cited here owing to space restrictions.

2. The injectisome nanomachine

The electron microscopy (EM) visualization of the first injectisome, from *Salmonella enterica*, was reported in 1998 [1]. Afterwards, EM studies allowed the visualization of the complete injectisomes from *Shigella flexneri* [2] and EPEC [3]. Recently, cryo-EM analyses allowed the visualization at 17 Å resolution of the *S. enterica* injectisome [4]. All these studies indicate that the injectisome nanomachine consists of two distinct parts (Fig. 1): (i) a cylindrical base, similar to the flagellar basal body, composed of two pairs of rings that span the inner and outer bacterial membranes, joined together by a rod; (ii) the base is physically linked to a hollow, elongated, and stiff needle-like structure. In EPEC, a long flexible structure, called the EspA filament, extends the needle (Fig. 1) [3,5]; in plant pathogens, a long and thin structure, called the Hrp pilus extends from the base of the structure [6]. It is generally thought that the injectisome serves as a hollow conduit for travelling of the secreted proteins. Indeed, EM experiments provide evidence for the extrusion of the *P. syringae* effector proteins AvrPto and HrpZ from the tip of the Hrp pilus [6].

The proper assembly of the injectisome requires around 25 proteins, generally encoded by genes clustered in one locus. The precise function and localization of these proteins is only known for a reduced set. The inner pair of rings of the base is made of at least two proteins (called PrgH and PrgK in *S. enterica* [7], and MxiG and MxiJ in *Shigella* [2,8]) (Fig. 1). The outer pair of rings, associated with the outer membrane and the peptidoglycan layer, is made of proteins of the secretin family (InvG in *Salmonella* [7], MxiD in *Shigella* [2,8], and YscC in *Yersinia* [9]) (Fig. 1). Cryo-EM observations and image processing analysis revealed that the base is hollow, and that an "inner rod" channel, made of PrgJ, in *Salmonella*, is mounted upon needle assembly [4]. Besides the proteins that constitute the structural base of the injectisomes, a set of inner membrane and cytoplasmic proteins are highly conserved throughout

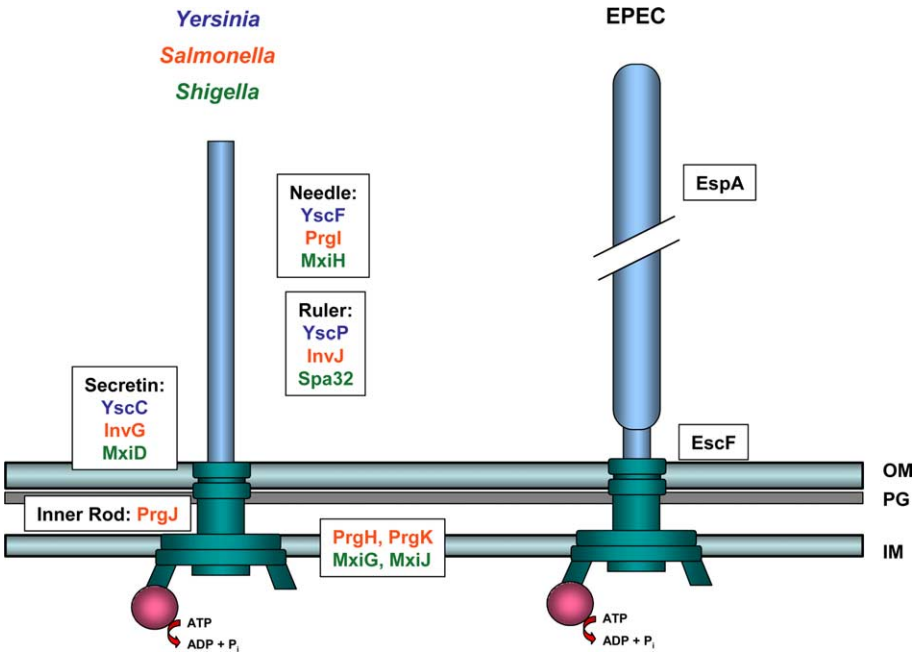

Fig. 1. The T3S injectisome nanomachine. Schematic drawing of T3S injectisomes representative of *Yersinia*, *Salmonella* and *Shigella* (left part), and enteropathogenic *E. coli* (EPEC) (right part). The schemes show the needle (prolonged by the EspA filament, in the case of EPEC) protruding from the bacterial membrane, and the basal body spanning the outer membrane (OM), the peptidoglycan layer (PG) and the inner membrane (IM) of the bacterium. The constituents of the injectisome whose localization is known are indicated. The colour code indicates the protein in relation to the bacteria where they are expressed. The main components of the needle (YscF, PrgI, MxiH, EscF), of the two rings spanning the outer membrane (YscC, InvG, MxiD secretins), the two rings in close proximity to the inner membrane (PrgH, PrgK, MxiG, MxiJ), and an inner rod mounted upon needle assembly (PrgJ) are outlined. There is also evidence that the YscP ruler is associated with the needle [11], and the same might be true for InvJ and Spa32. Also schematically shown (red circle) is the ATPase that is thought to energize T3S.

all T3S systems, including the flagellum. These include the ATPase (YscN in *Yersinia*) that should be the energy source for the apparatus.

The injectisome needle is a straight hollow tube made by the polymerization of a major subunit (PrgI in *Salmonella* [1], MxiH in *Shigella* [2,8], YscF in *Yersinia* [10], and EscF in EPEC [3]) (Fig. 1). The injectisome needle length is defined, varying between 45 and 80 nm according to the bacterial species. Proteins called YscP, in *Yersinia* [11], Spa32, in *Shigella* [12], and InvJ, in *Salmonella* [7], control the needle length (Fig. 1). The precise needle length seems to have been evolutionary adjusted in relation to the dimensions of other structures at the bacterial surface, like adhesins [13], or lipopolysaccharide [14]. In *Yersinia*, deletions and insertions in YscP lead to shorter and longer needles, respectively. This revealed a linear correlation between the lengths of the needle and YscP, indicating that YscP acts as a molecular ruler. Export of needle subunit proteins are allowed until the needle reaches the length of the extended YscP protein. Then, YscP triggers a substrate specificity switch, which stops YscF secretion and makes the apparatus ready for effector secretion [11]. There is genetic evidence that this substrate specificity switch involves an interaction between YscP and YscU [15], an inner membrane protein. In addition, genetic and bioinformatics analyses indicate that a domain within the C-terminal of YscP mediates the substrate specificity switch, and that this domain is conserved among several other YscP orthologues [16]. There is structural evidence that the injectisome base undergoes conformational changes upon needle assembly [4]. These conformational changes may be a consequence of the substrate specificity switch mechanism, but this still remains to be proven. Thus, there is likely a complex network of protein interactions, mostly still to be unraveled, and subtle conformational changes that ensures a proper needle assembly.

3. The translocators: the injectisome-host cell connection?

Translocation of the effectors across the eukaryotic cell membrane requires a set of proteins called the "translocators" that are also T3S substrates [17–21]. Generally, there are three translocators (YopB, YopD and LcrV in *Yersinia*), two of them (YopB,YopD) with clear hydrophobic domains and the other (LcrV) being an hydrophilic protein. In agreement with their hydrophobic nature, YopB and YopD were shown to insert in the membrane of erythrocytes [22] and to form a pore in erythrocytes [19] and in nucleated cells [23], which presumably mediates effector translocation. The third

translocator, LcrV, is required for pore formation [24], but it does not insert in membranes of infected erythrocytes [22]. These observations suggest a model in which the hydrophilic translocator, acting as an extracellular chaperone, helps the hydrophobic translocators to integrate in the eukaryotic plasma membrane and to form there a pore (Fig. 2). This model is supported by similar observations made in *P. aeruginosa* [25], *Shigella* [26,27] and EPEC [28], but different aspects still need to be challenged. Firstly, the exact composition, stoichiometry and structure of the pore remain to be elucidated. Secondly, the T3S translocation process is thought to be a one-step mechanism, which implies that the pore and the injectisome must be physically connected. However, an interaction between translocator proteins and the needle subunits has never been shown. The only possible exception is in the case of EPEC, where the YopB homologue, EspB, seems to interact with EscF, the needle subunit, via the EspA filament [3,5,29]. If counterparts of YopB/YopD are present in all bacteria carrying T3S systems, the situation regarding LcrV it is more complex. The *P. aeruginosa* PcrV protein is homologous to LcrV, and even complements an LcrV deficiency [20]. However, in *Salmonella* and *Shigella*, there is no LcrV homologous protein but IpaD, in *Shigella*, and SipD, in *Salmonella*, may play an identical role with respect to effector translocation and pore formation [27]. EspA apparently plays a similar role, in the sense that it does not insert in membranes but it is required for pore formation. It is thus very tempting to speculate whether LcrV/PcrV/IpaD/SipD would have a similar role as EspA in linking the pore to the needle. Finally, the translocators must have a secretion priority related to their function. It is noteworthy that LcrV was detected at the bacterial surface before secretion of the effectors is triggered [20].

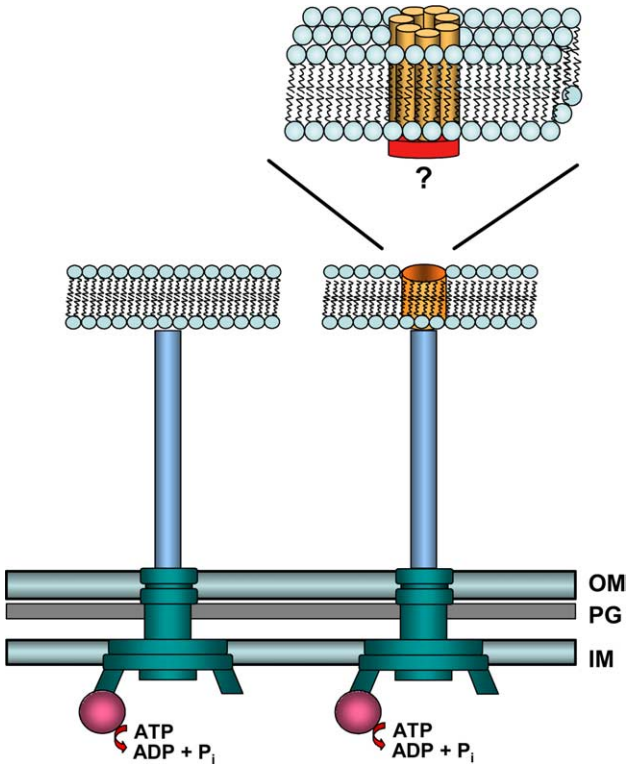

Fig. 2. The T3S traslocation pore. A model for formation of the T3S translocation pore is schematically shown. T3S is triggered upon contact with the eukaryotic cell lipid membrane. The translocators (two hydrophobic proteins and one hydrophilic protein) should have secretion priority, but this has never been experimentally demonstrated. Then, the two hydrophobic translocators (in orange) insert and form a pore in the host cell lipid membrane in a process assisted by the hydrophilic translocator (in red). The structure and stoichiometry of the pore are unknown. The hetero-heptameric pore structure shown is purely hypothetical. It is also unknown if the hydrophilic translocator binds to the assembled pore and/or needle.

4. The T3S signal sequence: a peptide or not a peptide?

At the time of the first observations of T3S, it appeared that substrate recognition occurs by a non-cleavable N-terminal signal [30]. Later, the minimal N-terminal region required for secretion was shown to be around 15 residues for different *Yersinia* T3S substrates [31,32]. However, the exact molecular determinants that allow the injectisome to recognize the T3S substrates among all the other bacterial proteins are still a matter of debate. The observation that led to this controversy was the finding that no mutations could be identified that specifically abolished secretion of the *Yersinia* proteins YopE and YopN, when their N-terminus is fused to a reporter protein [31]. These results suggested that the secretion signal is encoded in the mRNA rather than in the peptide sequence [31]. Today, two orders of evidence indicate that this mRNA signal, if it exists, is not the only T3S recognition motif. First, frameshift mutations that completely alter the amino acid sequence of residues 2-11 of YopE, but leave the mRNA sequence essentially intact, abolished secretion of full-length YopE [33]. In contrast, synonymous mutations in the first 11 codons of yopE, which alter the yopE mRNA sequence but not the YopE amino acid sequence, do not affect YopE secretion [33]. Secondly, and most importantly, the signal mRNA hypothesis predicts that T3S would be co-translational, and it has been demonstrated in *Shigella* that some pre-synthesized and stored effectors are secreted upon addition of an artificial inducer of secretion [34]. In contrast, reports favoring a role of an mRNA signal on T3S have been published in recent years. For example, it was reported that synonymous mutations in yopE prevent T3S secretion of hybrid proteins [35].

Both hypotheses are hard to reconcile, but so far a defined model that could explain the recognition mechanism by which T3S substrates are selectively engaged by the injectisome is lacking. Studies on secretion by

the bacteria flagellum indicate that the secretion signal might be a disordered N-terminus, but it is difficult to imagine how such an unstructured element would be recognized by the injectisome.

5. The multiple roles of T3S chaperones

Secretion of some, but not all, T3S substrates requires the assistance in the bacterial cytoplasm of a particular type of chaperones, which are a hallmark of T3S. They are low molecular mass acidic proteins (ca. 15 kDa) that specifically interact with only one or a few T3S substrates and are required for proper secretion of their cognate substrate.

There are examples of proteins requiring T3S chaperones among the different types of T3S substrates. Chaperones of effectors have been most studied ones. The archetype of the T3S chaperones is SycE from *Yersinia* [36]. SycE is a dimer which binds to the N-terminal region of YopE. This seems to be the case for most if not all the effector chaperones. In the absence of SycE, YopE is unstable and rapidly degraded [36]. The reason why SycE is necessary to stabilize YopE became apparent only recently. SycE is necessary to mask an aggregation-prone region between amino acids 50–77 of YopE, since the presence of this domain creates the requirement of SycE for YopE secretion and stabilization [37]. This domain is not required for the catalytic activity of YopE but is rather involved in the proper targeting of YopE to host cell membranes [38]. Although YopE deleted of amino acids 50–77 still exerts its long-term toxic effect on eukaryotic cells [37,38], it is likely that such an activity is not efficient in the context of the infection. Thus, SycE protects a region of YopE that is required for its function but that hampers its secretion. In addition, SycE was also shown in some conditions to be required for YopE recognition by the injectisome [33,39]. A strong argument in favor of a targeting role for T3S chaperones comes from the observation that the *Salmonella* effectors SptP and SopE, in the absence of their chaperone binding sites, are secreted through the flagellar T3S rather than through the injectisome [40]. A targeting role could also apply to Spa15, an effector chaperone from *Shigella*. In the absence of this chaperone, post-translational secretion is abolished but co-translational secretion is unaffected [34]. In this case, the chaperone could not only pilot the T3S substrate to the T3S apparatus but it could also maintain it in a secretion competent state. However, it is difficult to conclude that the role of effector chaperones is to prevent folding of their partner. Indeed, SycE bound to YopE does not abrogate the catalytic activity of YopE [41] and hence, presumably does not prevent its folding. Similar observations were made for the complexes between EPEC effector Tir and its chaperone, and the *Salmonella* effector SopB and its chaperone SigE [42]. However, this does not exclude that in some cases chaperones may facilitate export by preventing folding. It was indeed shown that SycE allows secretion of YopE-DHFR hybrid proteins which can otherwise not be secreted presumably because they fold irreversibly [43].

The two hydrophobic translocators share one common chaperone. In the *Yersinia* Ysc-Yop injectisome, YopB and YopD share the SycD chaperone [44]. In the *Shigella* injectisome, the hydrophobic translocators IpaB and IpaC share the IpgC chaperone [45]. IpgC stabilizes IpaB and IpaC and prevents their premature association in the cytoplasm of *Shigella* cells [45]. SycD masks the hydrophobic domains in YopB, neutralizes their potential toxicity, and may prevent premature association of YopB/YopD with LcrV [44]. Some proteins that belong to the distal part of the injectisome also have a cytosolic chaperone but their role is even less documented than for the others. The chaperones were also shown to be involved T3S control (see below).

Most T3S substrates exert their action at specific sites in the host eukaryotic cell. Their design must have been shaped by two evolutionary pressures: their precise function/activity at certain localization and time, and their need to be secreted/translocated through two or three lipid membranes. The diverse functions of T3S chaperones could reflect an evolutionary conflict between these two pressures, which might have also created the need for the chaperone in some cases, but not in others.

6. Regulation: what makes T3S work when it must work

T3S is regulated at different levels. Transcription of the genes encoding the secretion apparatus is regulated by transcriptional regulators, in response to different environmental stimuli. This is achieved by the action of classical regulatory proteins such as AraC-like transcriptional activators, histone-like proteins and two-component regulatory systems. This level of control is thought to ensure expression and assembly of the injectisome and a certain level of expression of T3S substrates. The activity of the injectisome is itself tightly regulated, and secretion only occurs when a specific signal triggers it, which in turn boosts expression of T3S genes. Based on solid evidence from *Yersinia* and *Shigella* [17,46,47], the signal that triggers T3S is commonly viewed as contact with a host cell (Fig. 3). The mechanisms by which the activity of the injectisome remains blocked in the absence of triggering are not totally understood. In *Yersinia*, at least five proteins, YopN and its chaperones SycN and YscB [48,49], TyeA [50] and LcrG [51] are required for this control and for proper effector

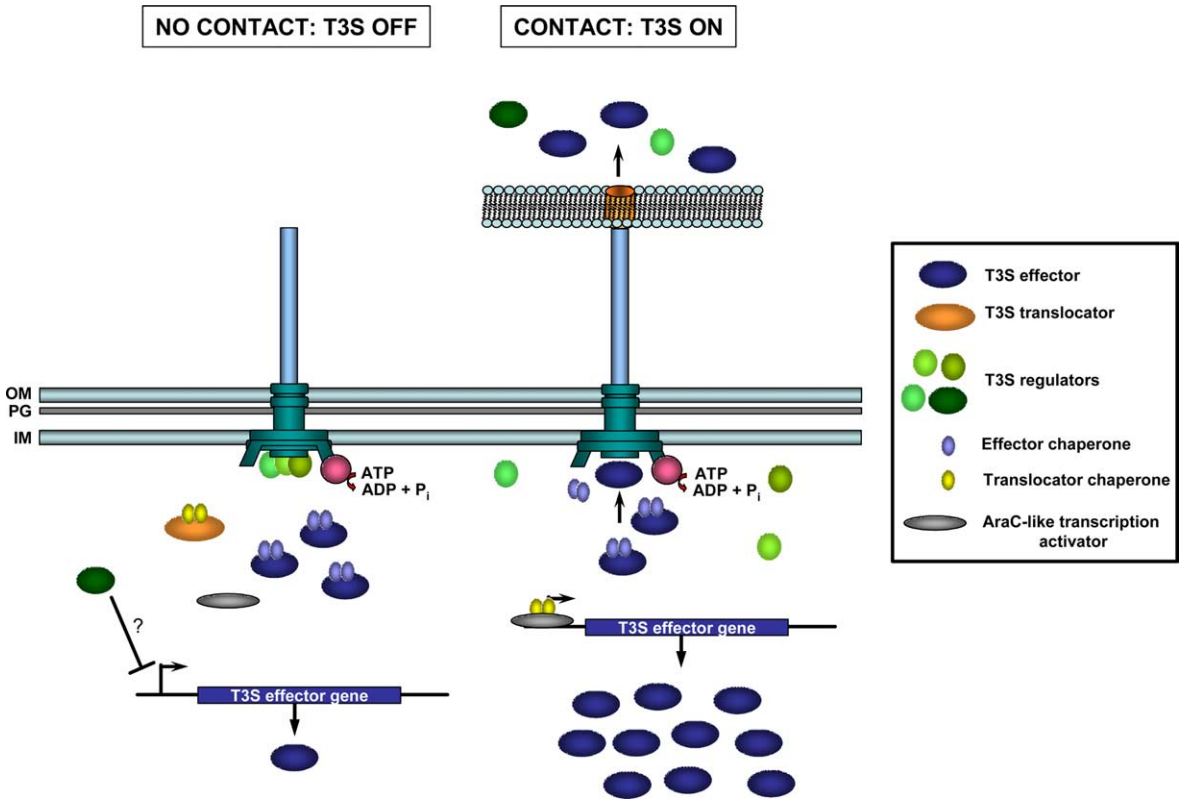

Fig. 3. Regulation of T3S. A general model on the mechanisms that were described to control T3S is shown. The model summarizes information from different T3S systems; it does not mean that all mechanisms are operative in a single bacterium. In T3S systems, a first response to specific host environmental conditions ensures injectisome assembly and a certain expression level of T3S effectors. However, secretion only occurs when a specific signal triggers T3S. In the absence of contact, secretion is prevented by a multiprotein complex of T3S regulators that somehow blocks the access of T3S substrates to the injectisome. In non-secreting conditions, transcription from T3S substrates promoters is limited. This has been shown to be due to the indirect and uncharacterized action of a negative regulator of T3S transcription and to the inability of an AraC-like transcription activator to have high-affinity access to the T3S promoters. Upon contact with the eukaryotic host cell plasma membrane, the complex blocking the access to the injectisome is disassembled and secretion starts. This leads not only to the secretion of T3S effectors but also of some T3S regulators. In addition, the T3S chaperones are now free in the cytoplasm. The consequences are that the negative regulator of T3S transcription cannot exert its action any more (because is secreted) and the AraC-like transcription activator forms a high-affinity complex for T3S promoters with the now free T3S translocator chaperones, boosting transcription and expression of T3S effector genes. The function of T3S chaperones is far more complex (see text for a discussion) than what is depicted in this model on T3S control. The injectisome and translocation pore are represented as in Figs. 1 and 2. OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

translocation. YopN, SycN, YscN and TyeA form a complex, likely in the bacterial cytoplasm, which should block T3S in non-triggering conditions [52]. LcrG is a cytosolic protein that interacts with LcrV [53], and its regulatory role is likely to be related to its action on LcrV. However, since all proteins are required to achieve a tight control of secretion, they all must contribute to one complex trigger mechanism (Fig. 3).

Recent reports suggest that the bacteria could sense cholesterol in eukaryotic cell lipid membranes to trigger T3S. In *Shigella*, contact haemolysis due to the insertion of the T3S pore depends on the presence of cholesterol on the host cell surface, which was related to the requirement of cholesterol for the bacteria to trigger T3S [54]. However, it was recently shown that IpaB and SipB are cholesterol binding proteins and that T3S effector delivery by *Shigella*, *Salmonella*, and EPEC does not occur into cholesterol-depleted cells [55]. These observations suggest a model in which the T3S triggering signal is not dependent on host cell contact, but more specifically requires the assembly of the translocation pore in the host cell lipid membrane. The host cell membrane would provide the lipid environment required for the formation of the pore complex, which would then be the last injectisome assembly step. Indeed, if pore assembly is physically linked to the injectisome, then it can be envisioned that pore formation would generate conformational changes triggering T3S of effector proteins. This is in agreement with observations indicating that the needle tip is the T3S sensor [8,13].

Another question is how the activity of the injectisome boosts the expression of T3S proteins. This has been shown to happen by at least two mechanisms. One is the T3S of a regulatory protein (Fig. 3). In *Yersinia*, the feed-back control mechanism that keeps

expression of T3S genes at low levels when the injectisome is not operating is relieved by the export of the LcrQ/YscM protein [47]. However, LcrQ/YscM does act directly to repress transcription and it does not block the activity of the activator of *Yersinia* T3S genes [56]. The second mechanism exploits the free state of T3S chaperones when the injectisome is active (Fig. 3). This is the case of SicA, the chaperone of the *Salmonella* SPI-1 translocators [57], IpgC, the chaperone of the *Shigella* translocators [58], and of SycD, the chaperone of the YopB/YopD [59]. Finally, secretion of T3S substrates was proposed to be hierarchical. There is certainly a hierarchy of secretion related to the nature of the T3S substrates. However, a hierarchy in secretion of T3S effectors is yet to be clearly demonstrated. It has been proposed that the T3S chaperones could set such a hierarchy [37,41]. The hypothesis is that proteins having a chaperone would have a secretion-advantage over those not having one, but the experimental data supporting this model is minimal.

7. Conclusions

Remarkable progress has been achieved in our understanding of T3S since the experimental discovery of this sophisticated export pathway in 1994 [17,18]. However, many important questions remain unanswered and others arise from the models that are now proposed. We know what the injectisome looks like and high definition cryo-EM pictures have shown in detail its structure before and after needle assembly. We also know that this needle has a precise length and why. However, the exact molecular mechanisms by which the injectisome is assembled, including needle length control and substrate specificity switch, have only begun to be unravelled. Our knowledge of the function of the about 25 genes encoding the injectisome is optimistically restricted to around half of them. A small set of T3S substrates is dedicated to effector translocation probably through a pore. But, what is the exact composition and structure of this pore? How is the pore connected to injectisome? How is the T3S of the translocators regulated in time? Regarding the secretion signal there is still a dispute on whether this signal is an mRNA or a peptide sequence. The chaperones are a trademark of T3S and have been intensively studied since its discovery, and we now know the crystal structures of some of them, but the basic question remains unanswered: why some T3S substrates require a chaperone whereas others do not? We know that T3S requires a strict regulatory mechanism to allow effector delivery only at a certain time and at a certain place. The physiological signal that triggers T3S is likely to be host cell contact, but this has been only shown for *Shigella* and *Yersinia*. Generally, our knowledge of the mechanisms that control T3S is limited. Finally, both the setting of a secretion hierarchy and the role of cholesterol in T3S triggering or pore formation are recent trendy topics but further studies are required to understand their possible role in T3S.

In conclusion, the following years in T3S research promise to be as exciting as the past 15 years. The incoming and forthcoming studies are expected not only to result in a better understanding of the system itself, but also to lead to the discovery of new concepts in molecular biology, microbiology and biochemistry, and to findings that may contribute to the design of new drugs to combat many important bacterial pathogens.

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