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Type III secretion: a bacterial device for close combat with cells of their eukaryotic host

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Salmonella, *Shigella*, *Yersinia*, *Pseudomonas aeruginosa*, enteropathogenic *Escherichia coli* and several plant-pathogenic Gram-negative bacteria use a new type of systems called 'type III secretion' to attack their host. These systems are activated by contact with a eukaryotic cell membrane and they allow bacteria to inject bacterial proteins across the two bacterial membranes and the eukaryotic cell membrane to reach a given compartment and destroy or subvert the target cell. These systems consist of a secretion apparatus made up of about 25 individual proteins and a set of proteins released by this apparatus. Some of these released proteins are 'effectors' that are delivered by extracellular bacteria into the cytosol of the target cell while the others are 'translocators' that help the 'effectors' to cross the membrane of the eukaryotic cell. Most of the 'effectors' act on the cytoskeleton or on intracellular signalling cascades. One of the proteins injected by the enteropathogenic *E. coli* serves as a membrane receptor for the docking of the bacterium itself at the surface of the cell.

Keywords: bacterial pathogenesis; *Salmonella*; *Shigella*; *Yersinia*; enteropathogenic *E. coli*; translocation

1. INTRODUCTION

For millions of years, eukaryotes gradually built up their multicellular complexity and some cells, such as the epithelial cells and the phagocytes, specialized in the exclusion and clearing of intruding micro-organisms. The latter, on the other hand, remained unicellular but developed an impressive pool of genes that they exchanged more or less freely and, by doing so, they acquired an extraordinary adaptative potential, which perpetuates conflicts and equilibria that date from the era when the unicellular state was the rule.

For a rather long period of time, it was assumed that Gram-negative bacteria do not 'secrete' proteins in their environment; they were only supposed to export proteins in their strategical periplasm. However, research of the past two decades revealed that Gram-negative bacteria do indeed transfer proteins across their sophisticated outer membrane. They do this by a variety of systems that are now classified in four major types and several minor ones (Salmond & Reeves 1993). Type I, exemplified by the haemolysin secretion system of *Escherichia coli* is a rather simple system based on only three proteins that belong to the universal multidrug resistance (MDR) type of efflux pumps. Type II is a very complex apparatus that extends the general secretory pathway and transfers fully folded enzymes or toxins from the periplasm to the extracellular medium, across the outer membrane. Type IV, another complex system that transfers pertussis toxin among others, is related to the apparatus of *Agrobacterium*, which transfers DNA to plant cells (Covacci *et al*. 1999). Finally, type III, the object of this review, is a sophisticated apparatus that allows bacteria adhering at the membrane of a eukaryotic host cell or of an intracellular organelle, to inject specialized proteins across this membrane. The injected proteins subvert the functioning of the aggressed cell or destroy its communications, favouring the entry or survival of the invading bacteria. Type III is thus not a secretion apparatus in the strict sense of the term but rather a complex weapon for close combat. It is used by a growing number of animal pathogens but also by a number of plant pathogens. The type of intercellular communication this device allows is not restricted to pathogenesis: it is also used to initiate symbiosis by *Rhizobium* spp. (Viprey *et al*. 1998). The rule seems to be that the communication event follows close contact between the players.

'Type III secretion' contributes to a number of totally different diseases with different symptoms and severities, going from a fatal septicaemia to a mild diarrhoea or from a fulgurant diarrhoea to a chronic infection of the lung. Among the animal pathogens, type III systems have been extensively studied in *Yersinia* spp. (reviewed by Cornelis 1998; Cornelis *et al*. 1998), *Salmonella* spp. (reviewed by Galan 1998), *Shigella* spp. (reviewed by Van Nhieu & Sansonnetti 1999) and pathogenic *E. coli* (enteropathogenic *E. coli*s (EPECs) and enterohaemorrhagic *E. coli*s (EHECs)) (Jarvis *et al*. 1995; Elliott *et al*. 1998; reviewed by Frankel *et al*. 1998; Goosney *et al*. 1999). It has also been described in *Pseudomonas aeruginosa*,

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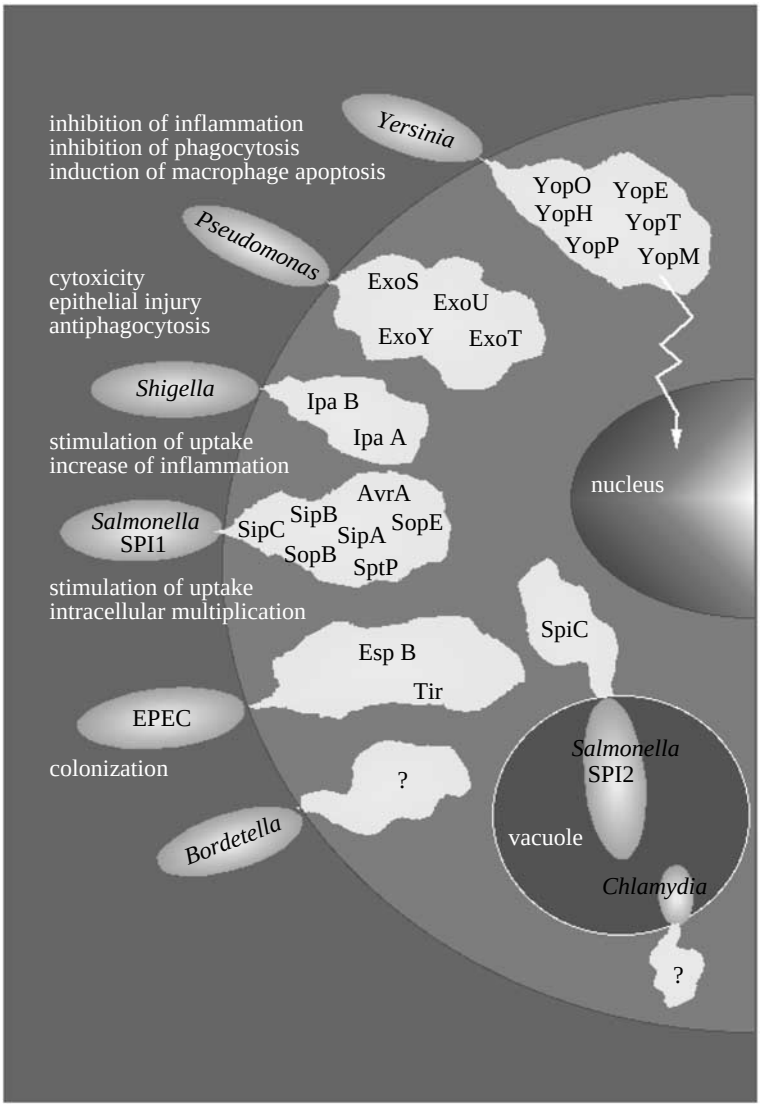

Figure 1. Illustration of the various bacterial pathogens endowed with type III secretion, injecting effectors into the cytosol of a eukaryotic target cell. See table 2 for references.

Chlamydia trachomatis, *Bordetella bronchiseptica* and, recently, in *Bordetella pertussis* (Kerr *et al*. 1999). Surprisingly, *Salmonella typhimurium* and *Yersinia* spp. have not only one type III system but two (Ochman *et al*. 1996; Shea *et al*. 1996; Hensel *et al*. 1997, 1998; Carlson & Pierson, GenBank AF005744), playing their role at different stages of the infection (figure 1). There is also substantial documentation in plant pathogens such as *Erwinia amylovora*, *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas campestris* (Van Gijseghem *et al*. 1995; reviewed by Galan & Collmer 1999).

A secretion system very close to the various type III systems is also dedicated to the export of the components of the flagellum. It appeared recently that a *Yersinia* phospholipase (YplA) involved in virulence and not in motility could be exported by the *Yersinia* flagellar export apparatus (Young *et al*. 1999).

Space restrictions necessitate limitation of this review to aspects that currently seem most worthy of highlighting. The references cited are mostly the more recent or lesser-known studies, and readers are referred to other recent reviews for more detailed information and references. I apologize to the authors of many important relevant studies that could not be cited here.

2. A DEVICE TO INJECT BACTERIAL PROTEINS ACROSS EUKARYOTIC CELL MEMBRANES

(a) *From the* Yersinia *Ysc secretion apparatus* . . . *to the* Salmonella *and* Shigella *'needle'*

The first observation of 'type III secretion' was made with *Yersinia* around 1990. It was the first major outcome of long and tenacious research by a few groups trying to understand the mysterious phenomenon of Ca^{2+} dependency discovered with the plague bacillus in 1961 by Higushi & Smith (1961): when incubated at 37 °C in the absence of Ca^{2+} ions, these bacteria can no longer grow, instead they release large amounts of proteins called Yops in the culture supernatant (Michiels *et al*. 1990). This phenomenon is generally referred to as Yop 'secretion' but it is not a physiological secretion: it is rather a massive leakage resulting from the artificial opening of a tightly controlled delivery apparatus. In spite of the fact that it is presumably artefactual, this observation turned out to be of paramount importance because it allowed one to carry out a genetic analysis, which led to the identification of 29 genes involved in this process of Yop release and called '*ysc*' for Yop secretion. Only a minority of the Ysc proteins have been characterized so far but the Ysc system remains the

archetype of the type III secretons. The *ysc* gene nomenclature has been transposed in EPECs (*esc* genes), as well as in plant pathogens for the type III genes that are conserved (*hrc* genes for hypersensitive response (HR)-conserved): the *hrc* and *esc* genes thus carry the same letter code as their *ysc* homologues. For the sake of clarity and consistency, we will first describe the various Ysc proteins (see Cornelis *et al*. (1998) for details) and mention afterwards the elements that are different or better-known in the other systems.

YscC is one of the best known Ysc proteins. It belongs to the family of secretins, a group of outer-membrane proteins involved in the transport of various macromolecules and filamentous phages across the outer membrane (Genin & Boucher 1994). As the other secretins, it exists as a very stable multimeric complex of about 600 kDa that forms a ring-shaped structure with an external diameter of about 200 Å and an apparent central pore of about 50 Å (Koster *et al*. 1997). As a matter of comparison, the PIV secretin of phage f1 has an internal diameter of about 80 Å, allowing the passage of the filamentous capsid with a diameter of 65 Å (Russel 1994). Lipoprotein YscW is ancillary to YscC in the sense that it is required for the proper insertion of YscC in the outer membrane (Koster *et al*. 1997). The Ysc apparatus also contains another lipoprotein called YscJ. Four proteins (YscD, -R, -U and -V, formerly called LcrD) have been shown, and two other (YscS and -T) proteins have been predicted to span the inner membrane. YscN is a 47.8 kDa protein with ATP-binding motifs (Walker boxes A and B) resembling the β-catalytic subunit of F_oF_1 proton translocase and related ATPases (Woestyn *et al*. 1994). It probably energizes the secretion process. Surprisingly, the two proteins YscO and YscP which are also necessary for Yop secretion are themselves released on Ca^{2+}-chelation, suggesting that they belong to the external part of the apparatus (Payne & Straley 1998).

Eight Ysc proteins (YscJ, -N, -O, -Q, -R, -S, -T, -U and -V) have counterparts in almost every type III secreton, including the one serving the flagellum. In the flagellum, the corresponding proteins belong to the most internal part of the basal body, i.e. the MS ring, the C ring and the ATPase. The YscC secretin also has counterparts in almost any type III system but not in the flagellum. The similarity between the inner parts of the type III secreton and the basal body of the flagellum prompted the groups of I. Aizawa and J. Galan to apply the well-established extraction and purification procedures of the basal body to the *Salmonella* type III secreton. This allowed them to visualize under the electron microscope a supramolecular structure that strikingly resembles a needle (Kubori *et al*. 1998). This needle complex is a hollow structure about 1200 Å long composed of two clearly identifiable domains: a needle-like portion projecting outwards from the surface of the bacterial cell and a cylindrical base that anchors the structure to the inner and outer membranes. The base closely resembles the flagellar basal body, further supporting the evolutionary relationship between flagella and type III secretion systems. An immunoblot analysis of the purified needle complex revealed that it is composed of at least three proteins: the secretin homologous to YscC and two lipoproteins, one of which resembles YscJ. More recently, a similar needle-like structure could be seen on the surface of plasmolysed *Shigella* (Blocker *et al*. 1999).

Little is known about the actual mechanism of export. The structure of the needle-like complex suggests that it serves as a hollow conduit through which the 'exported' proteins travel to cross the two membranes and the peptidoglycan barrier. The type III secreton would thus be operating in one step, taking its energy from the hydrolysis of ATP. Whether proteins travel folded or unfolded has not been demonstrated yet but, given the size of the channel, it is likely that they travel at least partially unfolded.

(b) *Other structural components of the type III systems*

Apart from the needle, described in *Salmonella* and in *Shigella*, other structural components have been found to be associated with type III machineries. *P. syringae* pv. tomato produces a filamentous surface appendage 6–8 nm in diameter, called the Hrp pilus, that is dependent on at least two type III system genes, *hrpS* and *hrcC*, encoding the secretin (Roine *et al*. 1997). The major structural protein of this Hrp pilus is encoded by *hrpA*, another essential gene for the type-III-mediated hypersensitive response and pathogenicity.

A filamentous organelle is also associated to the type III system of EPECs (Knutton *et al*. 1998). It has a diameter of about about 7–8 nm and a length of up to 2 μm. It contains EspA (Knutton *et al*. 1998), one of the proteins secreted by the Esc type III secreton of EPECs (Kaper 1998). It seems likely that these EspA filaments would play a role in the translocation process and the authors speculate that they may act as 'molecular go-betweens' transporting proteins from the bacterium to the host cell. However, this has not been demonstrated yet.

Finally, Ginocchio *et al*. (1994) have reported that contact with cultured epithelial cells results in the formation of filamentous appendages on the surface of *S. typhimurium*, but the significance of this observation and its relation to the more recently discovered 'needle' are not clear.

(c) *Translocation of effectors across eukaryotic cell membranes*

Purified secreted Yops have no cytotoxic effect on cultured cells, although live extracellular *Yersinia* have such an activity. Cytotoxicity was nevertheless found to depend on the capacity of the bacterium to secrete YopE and YopD. However, YopE alone was found to be cytotoxic when microinjected into the cells. This observation led to the hypothesis that YopE is a cytotoxin that needs to be injected into the eukaryotic cell's cytosol by a mechanism involving YopD, in order to exert its effect (Rosqvist *et al*. 1991). In 1994, this hypothesis was demonstrated by two different approaches. The group of Hans Wolf-Watz used immunofluorescence and confocal laser scanning microscopy examinations (Rosqvist *et al*. 1994) while the group of Guy Cornelis introduced a reporter enzyme strategy based on the calmodulin-activated adenylate cyclase (Sory & Cornelis 1994). Infection of a monolayer of eukaryotic cells by a recombinant *Y. enterocolitica* producing a hybrid protein made of the N-terminus of YopE and the catalytic domain of the adenylate cyclase of *Bordetella pertussis* (YopE-Cya protein) led to an accumulation of cyclic AMP in the cells. Since

the cyclase is not functional in the bacterial cell and in the culture medium because of a lack of calmodulin, this accumulation of cAMP signified the internalization of YopE-Cya into the cytosol of eukaryotic cells (Sory & Cornelis 1994). Thus extracellular *Yersinia* inject YopE into the cytosol of eukaryotic cells by a mechanism that involves at least one other Yop protein, YopD. YopH was later demonstrated to be also injected into the target cells cytosol (Persson *et al*. 1995; Sory *et al*. 1995) and YopB was shown to be required for delivery of YopE and YopH, like YopD. These observations led to the present concept that Yops are a collection of intracellular effectors (including YopE and YopH) and proteins required for translocation of these effectors across the plasma membrane of eukaryotic cells (including YopB and YopD) (Cornelis & Wolf-Watz 1997). Delivery of effector Yops into eukaryotic cells appears to be a directional phenomenon in the sense that the majority of the Yop effector molecules produced are directed into the cytosol of the eukaryotic cell and not to the outside environment (Rosqvist *et al*. 1994; Persson *et al*. 1995).

This model of intracellular delivery of Yop effectors by extracellular adhering bacteria is now largely supported by a number of other results, including immunological observations. While antigens processed in phagocytic vacuoles of phagocytes are cleaved and presented by MHC class II molecules, epitope 249–257 of YopH produced by *Y. enterocolitica* during a mouse infection is presented by MHC class I molecules, such as cytosolic proteins (Starnbach & Bevan 1994). This prompted some authors to convert *Salmonella* (Russmann *et al*. 1998) or *Y. enterocolitica* (Chaux *et al*. 1999) into antigen-presenting vectors.

As already mentioned, translocation across the cell membrane requires other secreted proteins, including YopB and YopD (Rosqvist *et al*. 1994; Sory & Cornelis 1994; Persson *et al*. 1995; Boland *et al*. 1996). These two Yops contain hydrophobic domains suggesting that they could act as transmembrane proteins (Håkansson *et al*. 1993). In agreement with this, *Yersinia* has a contact-dependent lytic activity on sheep erythrocytes, depending on YopB and YopD (Håkansson *et al*. 1996*b*; Neyt & Cornelis 1999). All this suggests that the translocation apparatus involves some kind of a pore in the target cell membrane by which the Yop effectors pass through into the cytosol. This YopB- and YopD-dependent lytic activity is higher when the effector *yop* genes are deleted suggesting that the pore is normally filled with effectors (Håkansson *et al*. 1996*b*). Osmoprotectants can inhibit YopB- and YopD-mediated sheep erythrocyte lysis, provided they are large enough so that they cannot traffic through the pore. This allowed Håkansson *et al*. (1996*b*) to estimate the inner diameter of the putative pore to be between 1.2 nm and 3.5 nm. The idea of a translocation pore was further documented in macrophages: infection of PU5–1.8 macrophages with an effector polymutant *Y. enterocolitica* leads to complete flattening of the cells, similar to treatment with the pore-forming streptolysin O from *Streptococcus pyogenes* (Neyt & Cornelis 1999). When the macrophages are pre-loaded with the low molecular weight fluorescent marker BCECF (623 Da), prior to the infection, they release the fluorescent marker but not cytosolic proteins, indicating that there is no membrane lysis but rather insertion of a pore of small size into the macrophage plasma membrane. Macrophages infected with the same polymutant strain also become permeable to extracellular lucifer yellow CH (443 Da) but not to Texas red-X phalloidin (1490 Da), supporting further the hypothesis of a pore. The hypothesis of a channel was recently reinforced by the observation that artificial liposomes that have been incubated with *Yersinia* also contain channels detectable by electrophysiology (Tardy *et al*. 1999). The observed channel has a conductance of 105 ± 5 pS and no ion selectivity. In agreement with the findings on translocation, all these events are dependent on translocators YopB and YopD. These two hydrophobic Yops seem thus to be central for the translocation of the effectors and for the formation of a channel in lipid membranes. Whether the two events are linked is very likely but not formally proven, so far.

Translocation of the effectors also requires the secreted LcrV protein, which interacts with YopB and YopD and is surface-exposed before target-cell contact (Sarker *et al*. 1998; Petterson *et al*. 1999). Finally, the 11 kDa LcrG protein is also required for efficient translocation of *Yersinia* Yop effector proteins into the eukaryotic cells but it is not required for pore formation. LcrG was shown to bind to heparan sulphate proteoglycans, suggesting that it could play a role in the control of release by contact but its exact localization in the bacterium remains elusive (Boyd *et al*. 1998). These four proteins are encoded by the same large operon *lcrGVsycDyopBD*, which also encodes SycD, the chaperone of YopB and YopD (Wattiau *et al*. 1994; Neyt & Cornelis 1999). This genetic organization reinforces the idea that YopB, YopD, LcrV and LcrG act together as 'translocators'. This does not necessarily exclude that some of them could themselves end up in the eukaryotic cytoplasm as was shown for YopD (Francis & Wolf-Watz 1998). *P. aeruginosa* has a translocation apparatus consisting of PcrG, -V, PopB and -D, that is very similar to the LcrG,-V, YopB, -D apparatus of *Yersinia*. However, the other type III systems diverge somehow at this level. *Shigella* and SPI1 have very similar apparatus made of IpaB, -C, -D and SipB,-C, -D, respectively. IpaB and SipB could be considered as the counterparts of YopB, but IpaC, -D and SipC, -D are not similar to either YopD or LcrV. The translocators of the EPECs are called EspB and EspD. The latter could be considered as a counterpart of YopB (Wachter *et al*. 1999).

A central question is, of course, do the translocators belong to the 'needle' or the 'pilus'? It is difficult to answer for *Yersinia* since their 'needle' or 'pilus' has not been seen yet but there are clues in *Shigella*. *Shigella* also has a contact-dependent haemolytic activity and this activity requires IpaB, a secreted protein that has similarities with YopB. Blocker *et al*. (1999) examined under the electron microscope the 'needle' of a mutant deficient in IpaB and found it to be undistinguishible from that of the wild-type, suggesting that the needle probably does not comprise the translocators or, at least, that the translocators are not an abundant element of the 'needle'. *Salmonella* secretes a homologue to IpaB, called SipB. Surprisingly, IpaB and SipB were the two first proteins to be found to have an apoptotic activity by reacting with the cytosolic macrophage protein ICE (Chen *et al*. 1996; Hersh *et al*. 1999). Similarly, EspB, which somehow resembles YopD was also found to be translocated, in an

Esp-dependent manner, into eukaryotic cells (Wolff *et al*. 1998). These observations indicate that the translocators are not restricted to the area of contact between bacteria and eukaryotic cells but that they are themselves trafficking in the eukaryotic cell, possibly associated with membranes, but this has not been determined yet. Thus, although there is a general agreement on the fact that the hydrophobic secreted proteins of the YopB, YopD family are involved in the translocation of the effectors, the understanding of their situation in the structure and of their exact role still deserves a long-standing effort.

(d) *The cytosolic chaperones*

A hallmark of type III secretion is that normal secretion of some substrate proteins requires the presence of small cytosolic chaperones of a new type (Wattiau *et al*. 1994, 1996; Ménard *et al*. 1994). Generally, these chaperones are encoded by a gene located close to the gene encoding the protein they serve and this is a useful indication for recognizing such chaperones. However, there are examples of gene reshuffling such as in *Y. pseudotuberculosis* where the gene encoding the chaperone of YopH was separated from the *yopH* gene by a large inversion. The latest observations suggest that these chaperones may not form a single homogeneous group but rather could belong to two different subfamilies.

SycE, the chaperone of YopE, is the archetype of the first family (Wattiau & Cornelis 1993). There are four typical representatives of this family in *Yersinia*: SycE, SycH, SycT and SycN, one in *Salmonella* (SicP), one in *P. aeruginosa* (SpcU), one in EPECs (CesT) and two in the *Proteus* flagellum assembly system (table 1). One could also add to this list the less typical YscB from *Yersinia*, acting as a co-chaperone for YopN (Day & Plano 1998; Jackson *et al*. 1998). All these chaperones are small (14–15 kDa) proteins with a putative C-terminal amphiphilic α-helix and most of them are acidic (pI 4.4–5.2). They specifically bind only to their partner Yop. The main feature is that, in the absence of these chaperones, secretion of their cognate protein is severely reduced, if not abolished. However, the exact role of these chaperones remains elusive. Research on Syc chaperones focused first on SycE and SycH. They both bind to their partner Yop at a unique site spanning roughly residues 20 to 70 (Sory *et al*. 1995). Surprisingly, when this site is removed, the cognate Yop is still secreted—though maybe in reduced amounts—and the chaperone becomes dispensible for secretion (Woestyn *et al*. 1996). This suggests that it is the binding site itself that creates the need for the chaperone and thus that the chaperone somehow protects this site from premature associations which would lead to degradation. In agreement with this hypothesis, SycE has indeed an anti-degradation role: the half-life of YopE is longer in wild-type bacteria than in *sycE* mutant bacteria (Frithz-Lindsten *et al*. 1995; Cheng *et al*. 1997). In addition to this putative role of bodyguard, SycE also acts as a secretion pilot leading the YopE protein to the secretion locus (see §2(e)). Finally, both SycE and SycH are required for efficient translocation of their partner Yop into eukaryotic cells (Sory *et al*. 1995). However, when YopE is delivered by a *Yersinia* polymutant strain that synthesizes an intact secretion and translocation apparatus but no other effector, it appears that YopE is delivered without the chaperone and the chaperone-binding site (Boyd *et al*. 2000). Thus, the SycE chaperone appears to be needed, only when YopE competes with other Yops for delivery. This suggests that the Syc chaperones could be involved in some kind of a hierarchy for delivery. This new hypothesis about the role of the Syc chaperones fits quite well with the observation that only a subset of the effectors seem to have a chaperone. Little is known about the role of SycT and SycN. However, there is an unexpected complexity for the latter: SycN apparently requires YscB working as a co-chaperone (Day & Plano 1998).

SycD is the archetype of the second group of 'type III chaperones'. It serves both YopB and YopD (Wattiau *et al*. 1994; Neyt & Cornelis 1999) and in its absence, YopD and YopB are less detectable inside the bacterial cell. SycD appears to be different from SycE and SycH in the sense that it binds to several domains on YopB, which evokes SecB, a molecular chaperone in *E. coli* that is dedicated to the export of newly synthesized proteins and also has multiple binding sites on its targets (reviewed by Fekkes & Driessen 1999). IpgC, the related chaperone from *S. flexneri* has been shown to prevent the intrabacterial association between translocators IpaB and IpaC (Ménard *et al*. 1994). The similarity between IpgC and SycD suggested that SycD could play a similar role and would thus prevent the intrabacterial association of YopB and YopD. However, Neyt & Cornelis (1999) observed that intrabacterial YopB and YopD are associated even in the presence of SycD. Since YopB and YopD also have the capacity to bind to LcrV, one could speculate that SycD prevents a premature association, not between YopB and YopD but rather between YopB, YopD and LcrV, but this hasn't been shown yet. CesD, the homologue from the EPECs, has also been shown to be required for full secretion of the translocators EspB and EspD, but it was only shown to bind to EspD, the translocator that is closest from YopB and IpaB. Like SycD and IpgC, CesD is present in the bacterial cytosol, but a substantial amount of this protein was also found to be associated with the inner membrane of the bacterium (Wainwright & Kaper 1998).

(e) *Recognition of the transported proteins*

Effectors delivered by the type III secretion systems have no classical cleaved N-terminal signal sequence (Michiels *et al*. 1990). However, it appeared very clearly that Yops are recognized by their N-terminus but that no classical signal sequence is cleaved off during Yop secretion (Michiels *et al*. 1990). The minimal region shown to be sufficient for secretion was gradually reduced to 17 residues of YopH (Sory *et al*. 1995), 15 residues of YopE (Sory *et al*. 1995) and 15 residues of YopN (Anderson & Schneewind 1997).

There is no similarity between the secretion domains of the Yops, which suggested recognition of a conformational motif of the nascent protein (Michiels *et al*. 1990). To explain that proteins with no common signal could be recruited by the same secretion apparatus, Wattiau & Cornelis (1993) suggested that the Syc chaperones could serve as pilots. However, this hypothesis was questioned when it appeared that YopE could be secreted even if its chaperone-binding domain had been deleted (Woestyn *et al*. 1996). It was then

Table 1. *Type III cytosolic chaperones*

	protein	kDa	pI	assisted protein	strong similarities	references
SycE family	SycE	14.7	4.55	YopE (*Yersinia*) binds to amino acids 15–50	ORF1 (*P. aeruginosa*) Scc1 (*C. psittaci*)	Wattiau & Cornelis 1993
	SycH	14.7	4.88	YopH (*Yersinia*) binds to amino acids 20–70		Wattiau *et al*. 1994
	SycT	15.7	4.4	YopT (*Yersinia*)		Iriarte & Cornelis 1998
	SycN	15.1	5.2	YopN (*Yersinia*)	Pcr2 (*P. aeruginosa*)	Day & Plano 1998 Iriarte & Cornelis 1999
	YscB	15.4	9.3	YopN (*Yersinia*) (co-chaperone)		Jackson & Plano 1998 Day & Plano 1998
	SicP	13.6	4.0	SptP (*Salmonella*)		Fu & Galan 1998
	SpcU		4.4	ExoU (*P. aeruginosa*)		Finck-Barbançon *et al*. 1998
	CesT		7.1	Tir (EPECs and EHECs)		Abe *et al*. 1999 Elliott *et al*. 1999
	FlgN	16.5		FlgK and FlgL (*Proteus flagellum*)		Fraser *et al*. 1999
	FliT	14		HAP2 (*Proteus flagellum*)		Fraser *et al*. 1999
SycD family	SycD	19.0	4.53	YopB and YopD (*Yersinia*)	PcrH (*P. aeruginosa*)	Wattiau *et al*. 1994
	CesD			(EPEcs)		Wainwright & Kaper 1998
	IpgC	18.0		IpaB and IpaC (*Shigella*)		Ménard *et al*. 1994
	SicA	19	4.61	SipB and SipC (*Salmonella*)		Kaniga *et al*. 1995

concluded that secretion was dependent only on the short N-terminal signal but secretion of a Yop lacking only this N-terminal signal had never been tested.

A systematic mutagenesis of the secretion signal by Anderson & Schneewind (1997, 1999*b*) led to doubts about the proteic nature of this signal. No point mutation could be identified that specifically abolished secretion of YopE, YopN and YopQ. Moreover, some frameshift mutations that completely altered the peptide sequences of the YopE, YopN signals also failed to prevent secretion. Anderson & Schneewind (1997, 1999) concluded from these observations that the signal that leads to the secretion of these Yops could be in the 5′-end of the messenger RNA rather than in the peptide sequence. Translation of *yop* mRNA might be inhibited by a property either of its own RNA structure or as a result of its binding to other regulatory elements. If this is correct, one would expect that no Yop could be detected inside bacteria. However, while this is reported to be true for YopQ (Anderson & Schneewind 1999), it is certainly not true for other Yops such as YopE.

To determine whether this N-terminal (or 5′-terminal) signal is absolutely required for YopE secretion, Cheng *et al*. (1997) deleted codons 2–15 and they observed that 10% of the hybrid proteins deprived of the N-terminal secretion signal were still secreted. They inferred that there is a second secretion signal and they showed that this second, and weaker, secretion signal corresponds to the SycE-binding site. Not surprisingly, this secretion signal is only functional in the presence of the SycE chaperone (Cheng *et al*. 1997), rejuvenating the pilot hypothesis of Wattiau & Cornelis (1993). The Syc chaperone could ensure stability and proper conformation of the protein and target it to the secretion channel. At the moment of secretion, the chaperone must be released from the partner Yop to allow secretion.

Thus, the effectors that have a chaperone, such as YopE, YopH, YopN and YopT, are likely to have two secretion signals that operate during *in vitro* secretion, one linked to translation and one post-translational. What the relative importance of these two systems is *in vivo* remains to be elucidated. For the other Yops, for instance YopQ, the N-terminal secretion signal would be the only one. This non-cleavable N-terminal or 5′mRNA signal seems to be a hallmark of type III secretion systems.

(f) *Control of the injection*

We have seen that 'type III secretons' can secrete their substrate *in vitro* under given conditions, such as Ca^{2+}-chelation for instance. What is the triggering signal *in vivo*? Most probably contact with a eukaryotic cell. Several reports in *Yersinia* have shown that Yops delivery is a 'directional' phenomenon in the sense that most of the load is delivered inside the eukaryotic cell and that there is little leakage (Persson *et al*. 1995). According to the assays used, there is some discrepancy on the degree of 'directionality' (Boland *et al*. 1996) but there is no doubt that the majority of the released Yops load ends up in the eukaryotic cell and thus that contact must be the signal. Pettersson *et al*. (1996) provided a nice visual demonstration of the phenomenon. By expressing luciferase under the control of a *yop* promoter, they showed indeed that active transcription of *yop* genes is limited to bacteria that are in close contact with eukaryotic cells. Release of Ipa proteins from *Shigella* was also shown to depend on contact between bacteria and epithelial cells (Watarai *et al*. 1995).

3. EFFECTOR PROTEINS AND HOST RESPONSES

(a) *A panoply of enzyme activities*

Delivery of effectors across the plasma or vacuolar membrane appears to be the object of type III secretion. We have seen in § 2 that some of the translocators, namely IpaB, SipB, EspB (Wolff *et al*. 1998) and YopD (Francis &

Table 2. *Type III effectors*

	effector	enzymatic activity	target	similarity	effect	reference
Yersinia	YopE	unknown	unknown	ExoS, SptP	cytotoxin, actin filaments disruption, antiphagocytic	Michiels *et al*. 1990; Rosqvist *et al*. 1994; Sory & Cornelis 1994
	YopH	PTPase	P130cas FAK	SptP	disruption of peripheral focal complexes, antiphagocytic	Persson *et al*. 1997
	YopM	unknown	unknown	IpaH	migrates to the nucleus	Skrzypek *et al*. 1998; Boland *et al*. 1996
	YpkaA/ YopO	serine, threonine kinase	unknown	—	unknown	Håkansson *et al*. 1996*a*
	YopP/ Yop	unknown	unknown	AvrA AvrRxv	inhibition of TNFα release, apoptotic	Mills *et al*. 1997; Monack *et al*. 1997; Schesser *et al*. 1998
	YopT	unknown	RhoA	—	cytotoxin, actin filaments disruption, antiphagocytic	Iriarte & Cornelis 1998
Salmonella SPI1	AvrA	unknown	unknown	YopP/YopJ AvrRxv		Hardt & Galan 1997; Schesser *et al*. 2000
	SipA	unknown	actin	SipA	enhances actin polymerization, macropinocytosis	Zhou *et al*. 1999
	SipB		caspase-1		induction of apoptosis	Hersh *et al*. 1999
	SopB/ SigD	InsP phosphatase	various		intestinal chloride secretion	Norris *et al*. 1998; Jones *et al*. 1998
	SopE	GDP-GTP exchange factor	CDC42,Rac			Hardt *et al*. 1998
	SptP	PTPase	unknown	YopE, YopH		Kaniga *et al*. 1996
	SopD					Jones *et al*. 1998
Salmonella SPI2	SpiC				inhibition of fusion between phagosomes and lysosomes	Uchiya *et al*. 1999
Pseudomonas aeruginosa	ExoS	ADP-ribosyl-transferase	Ras	YopE, ExoT		Finck-*Barbançon et al*. 1997; Frithz-Lindsten *et al*. 1997; McGuffie *et al*. 1998; Pederson *et al*. 1999
	ExoT	ADP-ribosyl-transferase	unknown	ExoS		Finck-Barbançon *et al*. 1997
	ExoU/ PepA				cytotoxin	FinckBarbançon *et al*. 1997; Hauser *et al*. 1998
	ExoY	adenylate cyclase				Yahr *et al*. 1998
Shigella	IpaA	unknown	vinculin	SipA		Van Nhieu *et al*. 1997
	IpaB		caspase-1	SipB	induction of apoptosis	Hilbi *et al*. 1998
	IpaC				activation of Cde42, entry of *Shigella*	Van Nhieu *et al*. 1999
EPEC/ EHEC	Tir/ Esp E	receptor			receptor for intimin	Kenny *et al*. 1997; Deibel *et al*. 1998

Wolf-Watz 1998), have been shown to be delivered themselves into the eukaryotic cell. In addition to these proteins, 18 effectors have been described in the various animal pathogen systems and this relatively large list is increasing very fast. The effectors and their activity are detailed in table 2. Six effectors have been characterized in *Yersinia*: YopE, YopH, YopM, YopJ/P, YopO/YpkA and YopT (Cornelis *et al*. 1998). Five effectors are delivered by the *Salmonella* SPI1-encoded apparatus: AvrA, SipA, SopB, SopE and SptP, and one, SpiC, has been identified for SPI2 (Uchiya *et al*. 1999). Four are delivered by the Psc apparatus *of Pseudomonas aeruginosa*: ExoS (Frithz-Lindsten *et al*. 1997), ExoT (Vallis *et al*. 1999), ExoU (Finck-Barbencon *et al*. 1998) and ExoY (Yahr *et al*. 1998). *Shigella* delivers IpaA and IpaC (Van Nhieu *et al*. 1997, 1999). Finally, EPECs or EHECs deliver their own receptor, Tir (Kenny *et al*. 1997) or EspE (Deibel *et al*. 1998). No effector has been characterized yet for the other systems.

Five different enzymatic activities could be identified so far in the panoply of type III effectors: phosphotyrosine phosphatases (YopH, SptP), serine-threonine kinase (YpkA/YopO), inositol phosphate phosphatase (SopB) (Norris *et al*. 1998), ADP-ribosyltransferases (ExoS, ExoT) and an adenylate cyclase (ExoY). It is worthwhile noticing that the two latter activities are classical in A-B toxins. However, although ExoY resembles the toxins of *Bordetella pertussis* and *Bacillus anthracis*, it does not require calmodulin for its activity. The similarity between activities of type III effectors and A-B toxins suggest that these type III effectors could be considered as some kind of toxins that need a very sophisticated apparatus for

their delivery. Some of the type III effectors are hybrid proteins composed of two domains that display different activities. SptP from *S. enterica* appears to be a hybrid between YopE and YopH from *Yersinia*: the C-terminal part is a phosphotyrosine phosphatase homologous to YopH while the N-terminal part is homologous to YopE (Kaniga *et al*. 1996). This YopE-like domain also occurs in the N-terminal part of ExoS from *P. aeruginosa* (Frithz-Lindsten *et al*. 1997).

(b) *The cytoskeleton is a major target*

There is also a great diversity among the targets and the effects induced by the effectors. However, two major themes emerge. The first one is the cytoskeleton. Several effectors stimulate the cytoskeleton activity, which leads to macropinocytosis of *Salmonella* and *Shigella* (SipA, SopE, IpaA), while others disrupt the actin filaments, which leads to cytotoxicity and inhibition of phagocytosis of *Yersinia* and *P. aeruginosa* (YopE, YopH, YopT, ExoS). Small GTP-binding proteins such as Rho, Rac and CDC42 are essential in the control of the cytoskeleton movements. These GTP-binding proteins can cycle between two states: a GDP-bound (inactive) and a GTP-bound (active) form capable of engaging different effector molecules. Not surprisingly, several of the effectors that affect the cytoskeleton have been shown to act on such small G proteins. SopE is a GDP–GTP exchange factor acting on Cdc42 and Rac-1 (Hardt *et al*. 1998*a*); the ADP-ribosyltransferase domain of ExoS acts on Ras (McGuffie *et al*. 1998) and YopT has just been shown to act on RhoA (Zumbihl *et al*. 1999). SipA has been reported to act directly on actin and to decrease its critical concentration for polymerization while IpaA has been shown to bind to vinculin, which initiates the formation of focal adhesion-like structures required for *Shigella* invasion (Van Nhieu *et al*. 1997).

(c) *Signalling interference*

The second theme for the action of type III effectors is inflammation and cell signalling. Key elements in the induction of the inflammatory response are some cytokines. Central to their synthesis are the transcriptional activator NFκB and the mitogen-activated protein kinases ERK, JNK and p38. Several type III effectors downregulate the inflammatory response. The best example of this is YopP (YopJ in *Y. pestis* and *Y. pseudotuberculosis*). Injection of YopP/YopJ into macrophages leads to a significant reduction in the release of TNFα, a pro-inflammatory cytokine, and to apoptosis (Mills *et al*. 1997; Monack *et al*. 1997; Boland & Cornelis 1998). The two events are probably the consequence of the same early event in a common signalling cascade but reduction in the release of TNFα is not simply the consequence of apoptosis since it occurs even if apoptosis is prevented by caspase inhibitors. Concomitantly with these two events, one can observe the inhibition of NF-κB activation and the inhibition of the ERK1/2, p38 and JNK mitogen-activated protein kinases (MAPKs) activities (Ruckdeschel *et al*. 1998; Schesser *et al*. 1998; Boland *et al*. 1998). Interestingly, YopP and YopJ share a high level of similarity with an Avr protein from *Xanthomonas campestris* and a protein from the nitrogen-fixing *Rhizobium*. Because of this similarity, the *S. enterica* counterpart of YopP/J was called AvrA (Hardt & Galan 1997) but so far, no activity described for YopP/J could be assigned to AvrA.

In contrast to the *Yersinia* Ysc system, the *S. enterica* SPI1 system tends to induce a profound inflammatory response in the intestinal epithelium. The exact effector(s) responsible for this have not been identified yet, but again MAPKs are involved (Hobbie *et al*. 1997). JNK MAPK is also activated as a consequence of SopE-induced activation of Rho (Hardt *et al*. 1998*a*).

(d) *Intracellular trafficking of the effectors*

Not very much is known so far on the intracellular traffic of the effectors. Most of them are presumed to be cytosolic but two of them have been shown to follow a different route. YopM is a strongly acidic protein containing LRRs whose action and target remain unknown. However, it has been shown to traffick to the cell's nucleus by means of a vesicle-associated pathway that is strongly inhibited by brefeldin A, perturbed by monensin or bafilomycin (Skrzypek *et al*. 1998). Tir from EPECs (EspE in EHECs) is particularly interesting in the sense that it inserts in the plasma membrane of the target enterocytes and serves as a receptor for intimin, a powerful adhesin of EPECs. Thus EPECs and EHECS insert their own receptor into mammalian cell surfaces, to which they then adhere to trigger additional host signalling events and actin nucleation (Kenny *et al*. 1997; Deibel *et al*. 1998).

(e) *Intracellular action of translocators*

The *Shigella* IpaB and its *Salmonella* counterpart SipB, bind caspase 1 (Casp-1) and, by doing this, they induce apoptosis (Chen *et al*. 1996; Hilbi *et al*. 1998; Hersh *et al*. 1999), bypassing signal transduction events and caspases upstream of Casp-1. *Shigella*-induced apoptosis is thus distinct from other forms of apoptosis and seems uniquely dependent on Casp-1. Binding studies show that SipB associates with the pro-apoptotic protease Casp-1. This interaction results in the activation of Casp-1, as seen in its proteolytic maturation and the processing of its substrate interleukin-1 beta. Functional inhibition of Casp-1 activity by acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone blocks macrophage cytotoxicity, and macrophages lacking Casp-1 are not susceptible to *Salmonella*-induced apoptosis. Taken together, the data demonstrate that the *Shigella* IpaB and the *Salmonella* SipB function not only as translocators but also as effectors inducing apoptosis. Thus, type III systems of *Yersinia*, *Salmonella* and *Shigella* all induce apoptosis but it must be stressed that they do it by two totally distinct pathways.

Finally, IpaB and SipB are not the only bifunctional translocators: purified IpaC was recently shown to nucleate and to bundle actin filaments (Hayward & Koronakis 1999). How this discovery correlates with the role of SipA (Zhou *et al*. 1999) remains to be clarified.

4. COMPARISON OF THE VARIOUS TYPE III SYSTEMS

(a) *Three major groups of systems among the animal pathogens*

A superficial comparison of the sequences of the secretion–translocation systems encountered in the animal pathogens

suggests the existence of at least three families: the Psc system of *Pseudomonas aeruginosa* is extremely close to the Ysc system of *Y. enterocolitica*, which is quite surprising given the long evolutionary distance between these two bacterial species. The system of *S. typhimurium* encoded by centisome 63 and the Mxi/Spa system of *Shigella*, both involved in bacterial invasion of epithelial cells, are also very similar. Finally, the second system of *S. typhimurium*, encoded by centisome 30 (Ochman *et al*. 1996; Shea *et al*. 1996; Hensel *et al*. 1997) seems to be rather related to the system found in EPECs and EHECs. Several attempts have been made to trans-complement mutations in secretion genes using the homologue from another but these were generally unsuccessful. However, the *pcrV* gene from *P. aeruginosa* can complement an *lcrV* mutation in *Y. pseudotuberculosis* (Petterson *et al*. 1999). It seems thus that, apart from the couple *Yersinia–Pseudomonas*, it is impossible to mix the pieces of various injectisomes.

(b) *Exchangeability between the effectors of the different systems*

Are the various type III systems functionally interchangeable in the sense that effectors from one system could be secreted or even delivered intracellularly by another system? The N-terminal domain (217 residues) of ADP-ribosyltransferase ExoS from *P. aeruginosa* (453 residues total) is 54% similar to the entire YopE (see § 3) and the protein encoded by the gene next to ExoS (ORF1) is very similar to SycE (Wattiau *et al*. 1996). These observations prompted Frithz-Lindsten *et al*. (1997) to introduce the two genes from *P. aeruginosa*, transcribed from the P_{lac} promoter, into *Y. pseudotuberculosis*. Since they observed that the recombinant *Y. pseudotuberculosis* could secrete ExoS, they pursued their investigation and they wondered whether ExoS would not be delivered by a recombinant *Y. pseudotuberculosis* into HeLa cells, just like YopE. They introduced the *exoS* gene and ORF1 in a non-cytotoxic double *yopE*, *yopH* mutant of *Y. pseudotuberculosis* and they infected HeLa cells. The result was clear cytotoxicity, indicating that ExoS is translocated across the HeLa cell plasma membrane and also that ExoS has a cytotoxic activity. Repeating the experiment with a mutated form of ExoS that has a 2000-fold reduced ADP-ribosyltransferase activity, they still observed cytotoxicity, which indicated that ExoS is a bifunctional protein endowed with a YopE-like cytotoxic activity. These experiments demonstrated that the closely related *Yersinia* and *Pseudomonas* type III systems are functionally interchangeable. Given the taxonomic distance between these two species, the observation is of importance because it strengthens the idea of a horizontal spread of these type III systems.

Wolf-Watz's group also observed that *Y. pseudotuberculosis* can secrete IpaB from *S. flexneri* and that *S. typhimurium* can secrete YopE (Rosqvist *et al*. 1995). The latter recombinant *Salmonella* is also cytotoxic for HeLa cells, suggesting that YopE could even be translocated across the cell plasma membrane.

5. GENETIC SUPPORT

Comparison of the systems and the phylogeny analyses suggest that these systems must have been transferred hortizontally during evolution. Not surprisingly, the genes that encode these systems have been found to be part of elements that are more mobile than most of the other bacterial genes. In *Yersinia* and *Shigella*, the whole systems are plasmid-borne, while they are on pathogenicity islands in *Salmonella* (SPI1 and SPI2) and in EPECs. In general, the genes encoding the secretion–translocation systems appear to be part of large, compact operons, while the genes encoding the effectors are more scattered. Pathogenicity islands are sometimes considered as vestigial phages. Interestingly, in *S. typhimurium*, Hardt *et al*. (1998*a*) observed that SopE, one of the substrates of the system encoded by SPI1 is encoded outside the SPI, but on a cryptic P2-like phage. This observation tends to suggest that the effectors could be horizontally transferred independently from the secretion–translocation systems. This hypothesis is consistent with the observation that the effectors from one system can generally be delivered by another one, provided there is no limitation in their synthesis.

6. PROSPECTS

Since its discovery in 1994, type III secretion has expanded very rapidly, to become a whole field. Study of the type III systems allowed a better understanding of the pathogenesis of Gram-negative bacteria and discoveries with the different pathogens benefited from a constant cross-feed. The recent very fast progress made with *P. aeruginosa*, taking advantage of its similarity with *Yersinia*, is a spectacular example of such cross-feeding. The *Yersinia* lesson was not limited to the understanding of the fate of the known *P. aeruginosa* exotoxins but it extended to promising vaccination attempts. The *Yersinia* translocator LcrV was known to represent a protective antigen against plague since the mid-1950s (Burrows & Bacon 1958). Because of its extensive similarity to LcrV, one could guess that PcrV from *P. aeruginosa* could also act as a protective antigen. This was indeed shown recently by Sawa *et al*. (1999), using lung infection in mice as a model. As appealing as it may be, the development of new vaccines is not the only spin-off of this exciting new field. From a medical point of view, it could lead to the development of 'antipathogenicity molecules'. From a more basic point of view, it could also be beneficial to eukaryotic cell biology, by bringing in new tools if not new concepts.

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