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Abstract
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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 adaptive 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 strategic 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 septicemia 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 & Sansonetti 1999) and pathogenic E. coli (enteropathogenic E. coli (EPECs) and enterohaemorrhagic E. coli (EHECs)) (Jarvis et al. 1993; Elliott et al. 1998; reviewed by Frankel et al. 1998; Goosney et al. 1999). It has also been described in Pseudomonas aeruginosa.
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 Gijsegem 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

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.
archetype of the type III secretions. The ysc gene nomenclature has been transposed in EPECs (psc 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 psc 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 PIIV 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 F1F1 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 Ca2+-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 secretory system, 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 secretory apparatus 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 secretory system. 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 secretory system 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, hrpN 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 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 secretin 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-between’ 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 cytoxic effect on cultured cells, although live extracellular T. maritima 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 cytoxic when microinjected into the cells. This observation led to the hypothesis that YopE is a cytoxin 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 E. coli 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.
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 *L. enterocolitica* during a mouse infection is presented by MHC class I molecules, such as cytotoxic proteins (Starnbach & Bevan 1994). This prompted some authors to convert *Salmonella* (Rusmann et al. 1998) or *L. 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. 1996b; Næt & 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. 1996b). 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. (1996b) to estimate the inner diameter of the putative pore to be between 1.2 nm and 3.3 nm. The idea of a translocation pore was further documented in macrophages: infection of PU5–1.8 macrophages with an effector polynutant *L. enterocolitica* leads to complete flattening of the cells, similar to treatment with the pore-forming streptolysin O from *Streptococcus pyogenes* (Næt & 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 (490 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; Pettersson et al. 1999). Finally, the 11kDa 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 bacteria remains elusive (Boyd et al. 1998). These four proteins are encoded by the same large operon lcrG/YscDyopBD, which also encodes SycD, the chaperone of YopB and YopD (Wattiau et al. 1994; Næt & 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, PobB 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 SPII 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 LcraV. 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 undistinguishable 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 cytotoxic chaperones

A hallmark of type III secretion is that normal secretion of some substrate proteins requires the presence of small cytotoxic 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 V. cholerae: SycE, SycH, SycT and SycV, one in S. marcescens (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 V. sheringtoni, 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 dispensable 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 V. cholerae 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 intracellular 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 Yop 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

<table>
<thead>
<tr>
<th>protein</th>
<th>kDa</th>
<th>pI</th>
<th>assisted protein</th>
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<tr>
<td>SycE</td>
<td>14.7</td>
<td>4.55</td>
<td>YopE (<em>Yersinia</em>) binds to amino acids 15–50</td>
<td>ORF1 (<em>P. aeruginosa</em>)</td>
<td>Wattiau &amp; Cornelis 1993</td>
</tr>
<tr>
<td>SycH</td>
<td>14.7</td>
<td>4.88</td>
<td>YopH (<em>Yersinia</em>) binds to amino acids 20–70</td>
<td>Scc1 (<em>C. psittaci</em>)</td>
<td>Wattiau et al. 1994</td>
</tr>
<tr>
<td>SycT</td>
<td>15.7</td>
<td>4.4</td>
<td>YopT (<em>Yersinia</em>)</td>
<td>PCR2 (<em>P. aeruginosa</em>)</td>
<td>Iriarte &amp; Cornelia 1998</td>
</tr>
<tr>
<td>SycN</td>
<td>15.1</td>
<td>5.2</td>
<td>YopN (<em>Yersinia</em>)</td>
<td>Day &amp; Plano 1998</td>
<td></td>
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<tr>
<td>YscB</td>
<td>15.4</td>
<td>9.3</td>
<td>YopN (<em>Yersinia</em>)</td>
<td>Jackson &amp; Plano 1998</td>
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<td>SycD family</td>
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<tr>
<td>SycD</td>
<td>19.0</td>
<td>4.53</td>
<td>YopB and YopD (<em>Yersinia</em>)</td>
<td>PcrH (<em>P. aeruginosa</em>)</td>
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<td>CesD</td>
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<td>(EPECs)</td>
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<td>IpgC</td>
<td>18.0</td>
<td></td>
<td>IpaB and IpaC (<em>Shigella</em>)</td>
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<td>SicA</td>
<td>19</td>
<td>4.61</td>
<td>SipB and SipC (<em>Salmonella</em>)</td>
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<td>Kaniga et al. 1995</td>
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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 & Schneeewind (1997, 1999) 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 & Schneeewind (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 & Schneeewind 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 secretions’ can secrete their substrate *in vitro* under given conditions, such as Ca2+-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 ‘directinality’ (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**

<table>
<thead>
<tr>
<th>Effector</th>
<th>Enzymatic activity</th>
<th>Target</th>
<th>Similarity</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Yersinia</em></td>
<td>YopE</td>
<td>unknown</td>
<td>unknown</td>
<td>ExoS, SptP</td>
<td>cytotoxin, actin filaments disruption, antimycotic</td>
</tr>
<tr>
<td>YopH</td>
<td>PTPase</td>
<td>P130cas, FAK</td>
<td>SptP</td>
<td>disruption of peripheral focal complexes, antimycotic</td>
<td>Persson et al. 1997</td>
</tr>
<tr>
<td>YopM</td>
<td>unknown</td>
<td>unknown</td>
<td>IpaH</td>
<td>migration to the nucleus</td>
<td>Skrzypek et al. 1998; Boland et al. 1996</td>
</tr>
<tr>
<td>YpkaA/</td>
<td>serine, threonine</td>
<td>kinase</td>
<td>—</td>
<td>unknown</td>
<td>Håkansson et al. 1996a</td>
</tr>
<tr>
<td>YopO</td>
<td>unknown</td>
<td>AvrA</td>
<td>AvrRxv</td>
<td>inhibition of TNFα release, apopotic</td>
<td>Mills et al. 1997; Monack et al. 1997; Schesser et al. 1998</td>
</tr>
<tr>
<td>YopP/</td>
<td>unknown</td>
<td>RhoA</td>
<td>—</td>
<td>cytotoxin, actin filaments disruption, antimycotic</td>
<td>Iriarte &amp; Cornelis 1998</td>
</tr>
<tr>
<td>YopT</td>
<td>unknown</td>
<td>unknown</td>
<td>YopP, YopJ</td>
<td>YopE, YopH</td>
<td>Hardt &amp; Galan 1997; Schesser et al. 2000</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>AvrA</td>
<td>unknown</td>
<td>unknown</td>
<td>YopP, YopJ</td>
<td>AvrRxv</td>
</tr>
<tr>
<td>SP11</td>
<td>SipA</td>
<td>unknown</td>
<td>actin</td>
<td>SipA</td>
<td>enhances actin polymerization, macropinocytosis</td>
</tr>
<tr>
<td>SipB</td>
<td>caspase-1</td>
<td>various</td>
<td>—</td>
<td>induction of apoptosis</td>
<td>Norris et al. 1998; Jones et al. 1998</td>
</tr>
<tr>
<td>SopB</td>
<td>InsP phosphatase</td>
<td>CDC54</td>
<td>—</td>
<td>intestinal chloride secretion</td>
<td>Hardt et al. 1998</td>
</tr>
<tr>
<td>SigD</td>
<td>GDP-GTP exchange factor</td>
<td>CDC42, Rac</td>
<td>—</td>
<td>—</td>
<td>Kaniga et al. 1996</td>
</tr>
<tr>
<td>SptP</td>
<td>PTGTPase</td>
<td>unknown</td>
<td>YopE, YopH</td>
<td>SpCo</td>
<td>inhibition of fusion between phagosomes and lysosomes</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>SpIC</td>
<td>unknown</td>
<td>unknown</td>
<td>ExoS</td>
<td>Finck-Barbançon et al. 1997; Frithz-Lindsten et al. 1997; McGuffie et al. 1998; Pederson et al. 1999</td>
</tr>
<tr>
<td>SP12</td>
<td>Pseudomonas aeruginosa</td>
<td>ExoS</td>
<td>ADP-ribosyltransferase</td>
<td>Ras</td>
<td>YopE, ExoT</td>
</tr>
<tr>
<td>ExoT</td>
<td>ADP-ribosyltransferase</td>
<td>unknown</td>
<td>ExoS</td>
<td>—</td>
<td>Yahr et al. 1998</td>
</tr>
<tr>
<td>ExoY</td>
<td>adenylate cyclase</td>
<td>vinculin</td>
<td>SipA</td>
<td>induction of apoptosis activation of Cdc42, entry of <em>Shigella</em></td>
<td>Hilbi et al. 1998; Van Nhieu et al. 1999</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>IpaA</td>
<td>unknown</td>
<td>caspase-1</td>
<td>SipB</td>
<td>receptor for intimin</td>
</tr>
<tr>
<td>IpaB</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>IpaC</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EPEC/</td>
<td>Tir/</td>
<td>receptor</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EHEC</td>
<td>Esp E</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

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, YopO/YpkaA and YopA (Cornelis et al. 1998). Five effectors are delivered by the *Salmonella* SP11-encoded apparatus: AvrA, SipA, SopB, SopE and SptP, and one, SpIC, has been identified for SP12 (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-Barbançon 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 (YpkaA/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

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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 macroinocytosis 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, YopG, 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. 1998a); the ADP-ribose transferase domain of ExoS acts on Rac (McGuffie et al. 1998) and YopG has just been shown to act on RhoA (Zumbibl 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 (Rückdeschel et al. 1998; Schesser et al. 1998; Boland et al. 1998). Interestingly, YopP and YopG 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* SPII 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. 1998a).

(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 traffic 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). Tfr from EPECs (EspE in EHEC) 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-Ile-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 *Yersinia 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 *levR* 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 injectosomes.

**(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-riboosyltransferase 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 SecE (Wattiau et al. 1996). These observations prompted Frithz-Lindsten et al. (1997) to introduce the two genes from *P. aeruginosa*, transcribed from the P7 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-riboosyltransferase 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 IpA 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 horizontally 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. (1998a) 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 in general 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 of 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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