MINIREVIEW

The *Yersinia* Deadly Kiss

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INTRODUCTION

Invasive pathogenic bacteria overcome the defense mechanisms of their animal host and proliferate at its expense. They all have their own lifestyle and target organs, leading to a variety of symptoms and diseases, which could suggest the existence of great diversity among the bacterial virulence strategies. However, there are only a very restricted number of basic virulence mechanisms while genetic reshuffling introduces endless modulations and combinations. One of these basic mechanisms was unravelled only in the last few years. By this mechanism, sometimes referred to as "type III," extracellularly located bacteria that are in close contact with a eukaryotic cell deliver toxic bacterial proteins into the cytosol of this cell. The animal pathogens sharing this type of system are *Yersinia* spp., *Salmonella* spp., *Shigella* spp., enteropathogenic and enterohemorrhagic *Escherichia coli*, *Pseudomonas aeruginosa*, *Chlamydia psittaci* (43), and *Bordetella* spp. (123). This mechanism is also found in the plant pathogens that elicit the so-called "hypersensitive response," such as *Erwinia amylovora*, *Pseudomonas syringae*, *Xanthomonas campestris*, and *Ralstonia solanacearum* (for reviews, see references 1, 112, and 113). Finally, a related system is encountered in *Rhizobium* spp., where it serves not pathogenic but symbiotic purposes (115).

In this minireview, I will describe the *Yersinia* "Yop virulon," which represents a paradigm for these type III systems. I will first give an overview of the system, and then I will describe the complex secretion-translocation apparatus made up of the Syc cytosolic chaperones, the Ysc secretion channel, the Yop translocators, and some proteins involved in the control of Yop release. I will finally deal with the "effector" Yops, the reason for the whole system. The aim is not to be exhaustive but rather to present our current view of this fast-evolving topic and to discuss ideas in a lively, stimulating, and perhaps also provocative way. The emphasis will be on bacteriology rather than on eukaryotic cell biology. I apologize for any involuntary bias the reader may discover in favor of the species *Yersinia enterocolitica*, with which I am working. More information on *Yersinia* virulence in general is available in exhaustive reviews (17, 20, 76), and a complete review of type III systems appeared recently (45).

AN OVERVIEW OF THE SYSTEM

The *Yersinia* lifestyle. The Yop virulon, a weapon common to *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, endows

these three pathogens with the capacity to resist the nonspecific immune response. In particular, it protects them from the macrophage by destroying its phagocytic and signalling capacities and, finally, inducing its apoptosis. In agreement with these in vitro observations, pathological examinations of experimentally infected animals indicate that yersiniae are largely extracellular (100).

Unravelling the basic model. When placed at 37°C in a medium deprived of Ca^{2+} ions, *Yersinia* spp. cease growing and, instead, secrete a set of proteins called Yops. This unusual capacity, strictly correlated with virulence, is encoded by a very conserved 70-kb plasmid that has been completely sequenced in *Y. enterocolitica* (49) and *Y. pestis* (44, 78).

Purified secreted Yops have no cytotoxic effect on cultured cells, although live extracellular yersiniae have such an activity (91). 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 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 (92). In 1994, this hypothesis was demonstrated by two different approaches. The first one was based on immunofluorescence and confocal laser scanning microscopy examinations (93). The second approach was based on a reporter enzyme strategy introduced by Sory and Cornelis (104). The reporter system consisted of the calmodulin-activated adenylate cyclase domain (called Cya) of the *Bordetella pertussis* cyclolysin (35). Infection of a monolayer of eukaryotic cells by a recombinant *Y. enterocolitica* producing a Yop-Cya hybrid enzyme led to accumulation of cyclic AMP in the cells. Since the enzyme is not functional in the bacterial cell and in the culture medium because of a lack of calmodulin, this accumulation of cyclic AMP signified the internalization of YopE-Cya into the cytosol of eukaryotic cells (104). Thus, extracellular yersiniae 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 cell cytosol (80, 103), 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) (Fig. 1).

Delivery into eukaryotic cells of less abundant Yop proteins such as YopO/YpkA, YopP/YopJ, and YopT turned out to be more difficult to demonstrate because of the limited sensitivity of the methods used. However, the difficulty could be circumvented by the use of mutant strains of yersiniae in which most of the genes encoding effectors have been knocked out, facilitating the traffic of the remaining Yops (10, 38). Most of the

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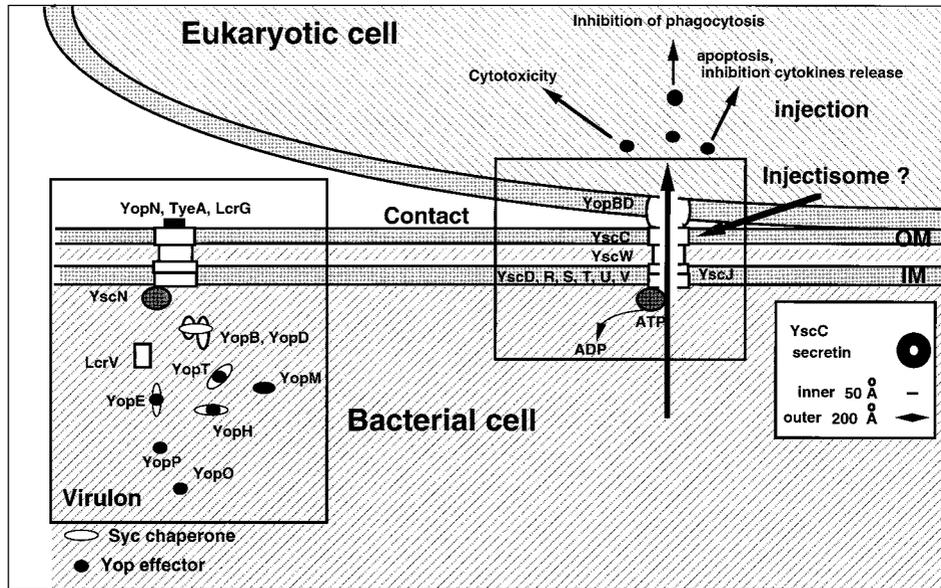
FIG. 1. The basic model. When yersiniae are placed at 37°C in a rich environment, the Ysc secretion apparatus is installed and a stock of Yop proteins is synthesized. As long as there is no contact with a eukaryotic cell, a stop valve, possibly made of YopN, TyeA, and LcrG, blocks the Ysc secretion channel. Upon contact with the eukaryotic target cell, a sensor interacts with a receptor on the cell surface, which results in the opening of the secretion channel at the zone of contact. The Yops are then transported through the secretion channel, and the Yop effectors are translocated across the plasma membrane guided by YopB and -D. During their intrabacterial stage, Yops are capped with their specific chaperones, presumably to prevent premature associations. The rectangle on the left encloses the virulon, while that on the right encloses the injectisome.

Yop proteins have been sorted out by the two different methods. Six Yops, YopE, YopH, YpkA/YopO, YopM, YopP/YopJ, and YopT, appeared to be intracellular effectors (11, 38, 47, 50, 80, 93, 103–105). The role of LcrV, a Yop with a different name (see below), is not so clear. One recent report indicates that it is required for the extrusion of YopB and YopD from the bacterial cell (97), while another concludes that it is necessary for the deployment of YopB (70). Whatever the exact role, both reports conclude that LcrV is indirectly necessary for the delivery of the effectors. YopB, YopD, and LcrV will thus be considered translocator Yops. Not surprisingly, they are encoded by three neighboring genes arranged as an operon. Another Yop, YopN, behaves as a plug closing the secretion channel (see below). Although it was first believed that YopN is not translocated (11), more recent data indicate that this Yop also ends up in the cytosol of the target cell (57).

This model of intracellular delivery of Yop effectors by extracellular adhering bacteria is largely supported by a number of other results. Among others, it is supported by immunological observations. While antigens processed in phagocytic vacuoles of phagocytes are cleaved and presented by major histocompatibility complex class II molecules, epitope 249 to 257 of YopH produced by *Y. enterocolitica* during a mouse infection is presented by major histocompatibility complex class I molecules, like cytosolic proteins (108).

The yersinia virulence plasmid thus encodes a specialized secretion-translocation system and Yop effectors. About 50 genes are involved, and they occupy three quarters of the plasmid. Thirty-five genes encoding the secretion and translocation machineries form a continuous block flanked on both sides by more dispersed genes encoding effectors and their chaperones.

Conditions of operation. Yop delivery requires living bacteria adhering to their target cell. In *Y. pseudotuberculosis*, invasin (Inv) is the main adhesin promoting Yop translocation (80). In *Y. enterocolitica*, the adhesin YadA is more important

than Inv (104), but either YadA or Inv will suffice to initiate the contact between *Y. enterocolitica* and epithelial cells to allow subsequent translocation (16). The situation is not clear for *Y. pestis*, which lacks these two adhesins.

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 (93). There is some disagreement about the degree of "directionality." By using various Cya reporters, Boland et al. (11) determined that roughly only half of the amount of Yops secreted by *Y. enterocolitica* is directed into cultured macrophages. In contrast, Persson et al. (80) reported that *Y. pseudotuberculosis* delivers more than 99% of the YopH-associated protein tyrosine phosphatase activity into HeLa cells. Whether the discrepancy originates from the bacterium or from the system used for the study is not known. Whatever the degree, both groups observed that more Yops are delivered into eukaryotic cells than into the culture supernatant, which implies that contact with eukaryotic cells triggers the release.

Finally, translocation occurs not from intravesicular yersiniae but exclusively from extracellular yersiniae. Indeed, selective killing of extracellular bacteria by gentamicin inhibits cytotoxicity (80, 91) and translocation of YopH. Moreover, the use of cytochalasin D, which inhibits the entry of bacteria into cells, does not greatly affect the amount of YopE that is internalized (104). Translocation of Yops is thus the feat of extracellular yersiniae.

THE Syc CYTOSOLIC CHAPERONES

In the first stages of the process, Yops appear to be cytosolic, and some of them are associated with specific chaperones called Sycs (specific Yop chaperones) (117).
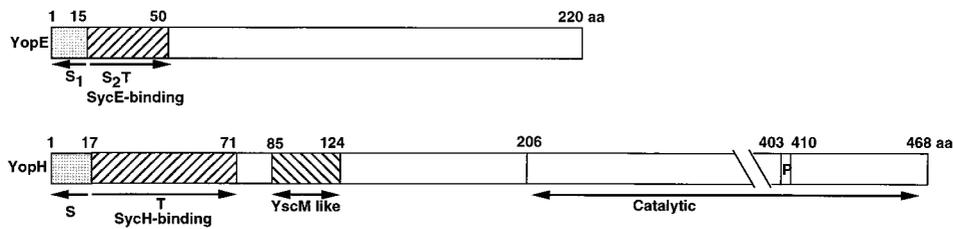
FIG. 2. Schematic representation of YopE and YopH. S_1, first secretion domain; S_2/T, second secretion domain and translocation domain; P, catalytic P-loop site. Residues 85 to 124 of YopH present a significant but unexplained similarity to the hypothetical regulator LcrQ/YscM. aa, amino acids.

Syc cytosolic chaperones SycD, SycE, SycH, SycT, and SycN. Normal secretion of YopE, YopH, YopN, and YopT by a wild-type yersinia requires the presence of SycE, SycH, SycN, and SycT, respectively (47, 48, 116, 117). All of these chaperones are small (14 to 15 kDa), acidic (pI 4.4 to 5.2) proteins with a putative C-terminal amphiphilic α-helix, and each binds only to its partner Yop. SycE and SycH possess a conserved leucine repeat motif in this α-helix structure, where most of the hydrophobic residues, essentially leucines, are present on the same side of the helix. A consensus sequence was derived by Wattiau et al. (118) from the alignment of this conserved leucine repeat of SycE, SycH, and their homologs (LLWxRxPL xxxxxxxLxxxLExLVxxAExL). In *Y. enterocolitica*, each of these chaperones is encoded by a gene located close to the gene encoding the corresponding Yop (49). SycD is slightly different from this group of chaperones, and it will be treated separately. No chaperone has been found for YopO/YpkA, YopP/YopJ, YopK/YopQ, YopR, LcrV, or YopM. Although the sequence of pYV227 reveals the existence of an additional putative orphan chaperone (ORF155) (49), it is likely that not every Yop has a chaperone.

Role of SycE and SycH. Research on Syc chaperones focused first on SycE and SycH. Each of these two chaperones binds to its partner Yop at a unique site spanning roughly residues 20 to 70 (103) (Fig. 2). Surprisingly, when this site is removed, the Yop is still secreted—though maybe in reduced amounts—and the chaperone becomes dispensable for secretion (121). 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 lead to degradation. In agreement with this hypothesis, SycE has an antidegradation role, since the half-life of YopE is longer in wild-type bacteria than in *sycE* mutant bacteria (19, 32). The antidegradation role of SycH is not as clear as that of SycE, because YopH can be detected in the cytosol of *sycH* mutant bacteria (116). In addition to this putative bodyguard role, SycE also acts as a secretion pilot, leading the YopE protein to the secretion locus (5). Finally, both SycE and SycH are also required for efficient translocation of their partner Yops into eukaryotic cells (57, 103, 121) (see below).

SycD, the chaperone of YopB and YopD. SycD (called LcrH in *Y. pseudotuberculosis* and *Y. pestis*) (7, 86) is a chaperone serving both YopB and YopD (69, 116). In the absence of SycD, YopD and YopB are less detectable inside the bacterial cell (69, 116). SycD appears to be somewhat different from SycE and SycH in that it binds to several domains of YopB (69). This evokes SecB, a molecular chaperone in *E. coli* which is dedicated to the export of newly synthesized proteins (54) and also has multiple binding sites on its targets (51).

SycD resembles the chaperone IpgC from *Shigella flexneri*, which has been shown to prevent the association between IpaB and IpaC (60). 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. Sur-

prisingly, intrabacterial YopB and YopD seem to be associated in the bacterium, even in the presence of SycD (69). Since YopB and YopD also have the capacity to bind to LcrV (97), one could speculate that SycD prevents premature association of YopB, YopD, and LcrV (69).

RECRUITMENT FOR EXPORT

To be secreted, Yops need to be specifically recognized by the secretion apparatus. Two secretion signals have been identified.

A first secretion signal on Yops. When Yop secretion was established, it appeared that Yops are recognized by their N terminus but that no classical signal sequence is cleaved off during Yop secretion (64). The minimal region shown to be sufficient for secretion was gradually reduced to 17 residues of YopH (103), to 15 residues of YopE (103) (Fig. 2), and to 15 residues of YopN (5).

There is no similarity between the secretion domains of the Yops, which suggested recognition of a conformational motif of the nascent protein (62). To explain why proteins with no common signal could be recruited by the same secretion apparatus, Wattiau and Cornelis (117) 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 (32, 121). It was then 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.

Systematic mutagenesis of the secretion signal by Anderson and Schneewind (5) led to doubts about the proteic nature of this signal. No point mutation could be identified that specifically abolished the secretion of YopE or YopN. Moreover, some frameshift mutations that completely altered the peptide sequences of the signals also failed to prevent secretion. Anderson and Schneewind (5) concluded from these observations that the signal that leads to the secretion of Yops could be in the 5′ end of the mRNA rather than in the peptide sequence.

A second secretion signal. To determine whether this N-terminal (or 5′-terminal) signal is absolutely required for YopE secretion, Cheng et al. (19) deleted codons 2 to 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 functional only in the presence of the SycE chaperone (19), rejuvenating the pilot hypothesis (117). The Syc chaperone could ensure the 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, YopE and YopH (possibly also YopN and YopT) are likely to have two secretion signals (Fig. 2). For the other Yops,

the N-terminal secretion signal would be the only one. No signal sequence is removed from YopB, YopD, and LcrV upon secretion, but their secretion signal has not been identified.

THE Ysc SECRETION MACHINERY: A BIOLOGICAL SYRINGE

Global composition. The Ysc secretion apparatus is complex and far from being completely characterized. Some elements have been localized in the inner membrane, while others are inserted in the outer membrane. It is thought to form a continuous channel across the two bacterial membranes, but this has not been formally proven. It is encoded by four contiguous loci that are called *virA* (*yopN*, *tyeA*, *sycN*, *yscXYV*, *lcrR*), *virB* (*yscNOPQRSTU*), *virG*, and *virC* (*yscABCDEFGHIJKLM*) in *Y. enterocolitica* (3, 6, 23, 27, 48, 63). The *yscV* gene was initially described in *Y. pestis* as *lcrD* (*lcr* for low calcium response) (81, 83). In total, 29 genes have been identified within these loci. Knockout mutants have been constructed for most of them, and with very few exceptions, they are deficient in Yop secretion.

We are still very far from assigning a role to each of the 29 components of the Ysc machine. However, I suspect that one of them, still unidentified, is some kind of endoglucanase. Indeed, the mesh of the peptidoglycan is presumably too small to allow installation of the Ysc apparatus and needs to be locally dismantled.

Characterized elements. YscC (52, 63, 83) 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. Like the other secretins, it exists as a very stable multimeric complex of about 600 kDa (52, 83) that forms a ring-shaped structure with an external diameter of about 200 Å and an apparent central pore of about 50 Å (52). In comparison, the pIV secretion of phage f1 has an internal diameter of about 80 Å, allowing the passage of the filamentous capsid with a diameter of 65 Å (59). Lipoprotein YscW (previously called VirG) (2) is required for efficient targeting of the YscC complex to the outer membrane (52). The Ysc apparatus also contains another lipoprotein called YscJ (Fig. 1).

Four proteins have been shown to span the inner membrane (Fig. 1): YscD (83), YscR (27), YscU (4), and YscV (formerly LcrD) (82). According to their sequences, two other proteins, YscS and YscT, are probably also anchored in the inner membrane. Finally, 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 (120). It probably energizes the secretion process.

Secreted Ysc proteins. Somewhat unexpectedly, the loci encoding the secretion apparatus also encode a few proteins that are themselves secreted by the machine. These are LcrQ/YscM, YopR (the product of *yscH*) (3), YopN, YscO (74), and YscP (75, 106). LcrQ/YscM is thought to be a regulator (see below), and YopN is considered the plug closing down the channel and will be described below. YscO is necessary for secretion of all of the Yops (74), but this is not the case for YscP (75) or YopR (3).

Overlapping of the secretion and translocation functions. We will see below that YopN, TyeA, and LcrG are required to keep the secretion channel closed. They must thus be somehow associated with the secretion channel. YopN appeared to be dispensable for translocation of the effectors across the eukaryotic cell membrane (11, 93). However, TyeA is necessary for translocation of YopE and YopH (50) and LcrG is required for efficient translocation of all of the known Yop effectors into macrophages (98). These observations suggest that secretion

and translocation are tightly coupled operations carried out by the same complex apparatus.

A supramolecular structure resembling the flagellum: the injectisome? The Ysc machinery, like any type III secretion machinery, contains several pieces that have homologues in the flagellar assembly apparatus, which suggests some relationship between the two structures. This assumption has been strikingly supported by the recent electron microscopic observation of the type III apparatus of *Salmonella typhimurium* (53). This apparatus, evoking a syringe, resembles the basal body of a flagellum extended by a straight rod. This rod extends outside the bacterial cell, which indicates that the elements that allow secretion across the two bacterial membranes are only one part of a more complex supramolecular structure. In this context, it is not surprising that some Ysc proteins appear to be "secreted." The core of the Ysc apparatus would be the syringe, while secreted Ysc proteins or the translocator Yops would form the needle.

To the question of whether the flagellum has a type III secretion system, the answer for me is undoubtedly yes, but the flagellum is obviously much more than a secretion apparatus. The flagellum is an organelle including a type III secretion apparatus. Thus, we should no longer call the virulence organelles type III but should rather introduce a new name evoking an organelle, perhaps something like injectisome, and consider that this organelle includes a type III secretion apparatus. The injectisome itself would only be a part of the whole virulon, which also includes the effector Yops.

To the question of which one is the ancestor, my answer is the flagellum because swimming must have been the first necessity before aggressing eukaryotes. Thinking along this line, it would be interesting to look at whether or not such organelles would also be used by environmental bacteria to protect themselves against protozoa.

THE TRANSLOCATION MACHINERY: THE NEEDLE?

Translocators YopB, YopD, and LcrV. Among the 12 secreted Yops, only 2, YopB and YopD, have hydrophobic domains (37), suggesting that they could interact with membranes. YopD is a 33.3-kDa protein with a central 31-amino-acid-long hydrophobic region (37). The Eisenberg plot analysis (25) suggests that it is a transmembrane protein (37). YopB is a 41.8-kDa protein (37) with two central hydrophobic regions separated by only 15 amino acids (37). It has a moderate level of similarity to proteins of the RTX family of α-hemolysins and leukotoxins (8, 111). YopB and YopD are encoded by the large *lcrGV-sycD-yopBD* operon (7, 67, 86), which also encodes LcrG, LcrV, and SycD/LcrH, the chaperone of YopB and YopD.

YopB and YopD are both required for translocation of effector Yops across the eukaryotic cell membrane (11, 38, 92, 93, 104). The fact that YopB resembles proteins of the RTX toxin family suggests that the translocation apparatus could involve some kind of pore, in which YopB would be the main element. The observation of Håkansson et al. (39) that *Yersinia* has a YopB- and contact-dependent lytic activity on sheep erythrocytes supports this hypothesis. This YopB-dependent lytic activity is higher when the effector *yop* genes are deleted, suggesting that the pore is normally filled with effectors during contact (39). By analysis of the protective effect of sugars of increasing size, Håkansson et al. (39) estimated the internal diameter of the putative pore to be between 12 and 35 Å.

YopB and YopD bind to each other (69), suggesting that they interact with each other at some stage to fulfill their function. They could be associated within the putative pore, but the pore has been neither purified nor observed by electron
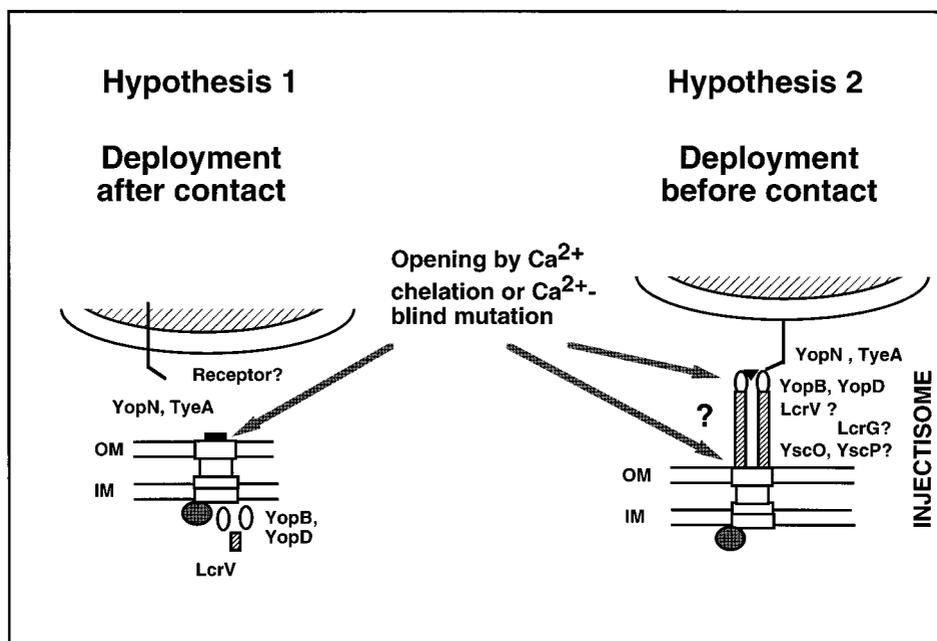
FIG. 3. Two models for translocation. In the first hypothesis, the Ysc secretion apparatus (the syringe) is installed in the bacterial membranes but is closed by YopN and TyeA. Upon contact with a eukaryotic cell, the plug is removed and the translocation apparatus, composed of YopB, YopD, and LcrV (the needle?), grows into the eukaryotic cell. This model is supported by the observation that in vitro, the translocator Yops are secreted only upon Ca^{2+} chelation, like the other Yops. In this hypothesis, Ca^{2+} chelation would remove the YopN-TyeA plug, allowing secretion of all of the Yops. In the second hypothesis, the needle is installed before contact. This model is inspired by the electron microscopy observations of Kubori et al. (53) with *S. typhimurium*. In this model, Ca^{2+} chelation would either remove a cap at the tip of the needle or separate the needle from the basal body, leaving a large hole and inducing massive secretion of all of the Yops. LcrG could be a critical element at the base of the needle. OM, outer membrane; IM, inner membrane.

microscopy. According to Holmström et al. (42), the size of the putative pore is controlled by the 21-kDa YopK/YopQ protein that is encoded outside the *lcrGV-sycD-yopBD* operon (64).

The *lcrGV-sycD-yopBD* operon also encodes the LcrV protein. At variance with YopB and YopD, this Yop exhibits a certain degree of polymorphism (88). LcrV has been described as a regulatory protein involved in the calcium response, since an *lcrV* in-frame deletion mutant was found to be Ca^{2+} independent and downregulated in the transcription of *yop* genes (7, 77, 85, 102, 110). However, other data (97) indicate that LcrV could be a functional element of the translocation apparatus, since deletion of the entire *lcrV* gene abolishes the secretion of LcrV, YopB, and YopD but has no effect on the secretion of the other Yops. Another recent report suggests that LcrV rather facilitates the expression (or stability) and secretion of YopB (70). In agreement with these observations, LcrV interacts with both the YopB and YopD proteins (97), as well as with LcrG (70, 71, 98). From these observations, one can envision LcrV as a third component of the delivery apparatus. One could speculate that it forms some kind of short needle underneath YopB and YopD, but as I have mentioned before, this function could also be fulfilled by the secreted Ysc proteins, such as YscO and YscP.

Is the needle installed before contact with a eukaryotic cell? At variance with most of the Ysc proteins, the translocators, like the Yop effectors, are secreted in vitro upon Ca^{2+} chelation. This led to the hypothesis that the translocation elements (the needle?) are not installed in the presence of Ca^{2+} or prior to contact with cells (Fig. 3, hypothesis 1). However, several observations indicate that the Ysc secretion apparatus (the syringe) is installed at 37°C in the presence of Ca^{2+} ions (52). One could thus wonder whether or not some translocators would also be installed on top of the syringe prior to cellular

contact (Fig. 3, hypothesis 2). Like the Ysc syringe, the needle would also require sensitive immunoblotting methods to be detected because this situation would be very different from the massive release observed upon Ca^{2+} chelation. Electron microscopy will probably be the method of choice to settle that question.

CONTROL OF Yop RELEASE

In vivo control of Yop release by cell contact. When yersiniae are incubated at 37°C in a Ca^{2+}-containing eukaryotic cell culture medium, they do not secrete Yops. Nevertheless, they inject Yops into the eukaryotic cells. This implies that contact with these cells somehow triggers the system and suggests that a particular bacterial ligand could be involved in this contact.

If such a ligand exists, its loss might well result in a permanently open secretion channel and in permanent secretion at 37°C. In other words, such mutants would be insensitive to Ca^{2+} repression and, hence, be Ca^{2+} blind. Such mutants have been isolated.

Ca^{2+}-blind mutants. The isolation of Ca^{2+}-blind mutants, i.e., mutants that secrete Yops even in the presence of Ca^{2+} (122), allowed the identification of three proteins that are required to keep the channel closed in the presence of Ca^{2+}: YopN (29), LcrG (98, 101), and TyeA (50).

YopN is a 32.6-kDa protein encoded together with *ysc* genes (29, 48, 50, 114). Under low-Ca^{2+} conditions, most of the YopN produced is released into the culture supernatant, while in the presence of Ca^{2+}, YopN is not released but is exposed at the bacterial cell surface (29, 50).

TyeA is a 10.8-kDa protein encoded immediately downstream of *yopN* (29, 50, 114). Like YopN, TyeA is loosely associated with the membrane. It has the capacity to bind to

YopN (50) and has been named TyeA by Iriarte et al. because it plays a role in the translocation of some Yop effectors (50). It is probably meaningful that YopN and TyeA are encoded by genes that are buried among *ysc* genes. It suggests that these two proteins belong to the Ysc syringe and could represent some kind of external cap.

At variance with YopN and TyeA, LcrG is encoded by the operon devoted to the translocators. It is an 11.0-kDa protein that has been shown to be primarily cytosolic (71, 101), and in spite of efforts with both *Y. pestis* and *Y. enterocolitica*, it remains uncertain whether some LcrG is surface exposed. One argument in favor of the notion that LcrG is surface exposed is based on the facts that it binds heparan sulfate proteoglycans and that heparin interferes with translocation of YopE into HeLa cells (15) (see below).

To summarize, YopN, TyeA, and LcrG are necessary to keep the secretion channel closed or intact. They could be part of the physiological cap, but it is not necessarily so. Indeed, Ca^{2+}-blind (*yopN*, *tyeA*, and *lcrG*) mutants could have an "open" apparatus, but it is equally possible that they have a "broken" apparatus instead (Fig. 3, hypothesis 2). Finally, it is reasonable to assume that YopN and TyeA are associated with each other, but there is no evidence that LcrG is associated with YopN and TyeA.

Effect of chelating Ca^{2+} ions. Ca^{2+} chelation results in a massive release of Yops. It is likely that Ca^{2+} chelation specifically displaces one of the three proteins mentioned above, but again, this protein does not need to be the physiological cap. In the hypothesis that the needle is normally installed at 37°C (Fig. 3, hypothesis 2), one could envision Ca^{2+} chelation either uncapping the needle or breaking it at another site. In both hypotheses, Ca^{2+} chelation would open the channel and trigger massive Yop secretion. Now, let us come back to the basic question.

Is YopN, TyeA, or LcrG the sensor involved in eukaryotic cell recognition? As mentioned previously, it seems reasonable to assume that a mutant lacking the sensor would be constitutively open and thus have the Ca^{2+}-blind phenotype. In that hypothesis, one of the three proteins could be the sensor. The observation that LcrG binds to heparan sulfate proteoglycans (15) is consistent with that hypothesis, but the significance of this binding is not clear (see below). As far as YopN and TyeA are concerned, it is not known whether they interact with eukaryotic cells. Finally, one cannot exclude the possibility that a mutant lacking the sensor would be constitutively closed. In that hypothesis, the phenotype would be "nonsecreting," a phenotype shared by most of the *ysc* mutants.

Is there a eukaryotic cell receptor? The fact that LcrG binds to heparin-agarose beads and to heparan sulfate proteoglycans (15) suggests that there could be a specific cell receptor. In agreement with this observation, the addition of exogenous heparin decreases the level of YopE-Cya translocation into HeLa cells, indicating that the interaction between LcrG and proteoglycans favors translocation. However, proteoglycans cannot be the only cell receptor because heparin does not affect translocation into macrophages (16) and does not affect Yop secretion in vitro (15). The question of a specific receptor is thus still open.

ACTING IN THE EUKARYOTIC CELL: THE POISON

Disturbing the eukaryotic cell in order to avoid the reaction that would clear yersiniae is what it is all about. We have seen in the Introduction that six effectors have been identified. Taking into account the information supplied by the sequence, it seems reasonable to assume that the list is now complete. We will briefly mention the action of each of them.

YopE. YopE (30, 64) is a 23-kDa cytotoxin that leads to disruption of the microfilament structure (92). Its exact enzyme activity and target are still unknown. Along with YopH, it blocks phagocytosis.

YopH. YopH (14, 61) is a powerful protein tyrosine phosphatase with a molecular mass of 51.0 kDa (36). It contributes to the inhibition of bacterial uptake by dephosphorylation of p130^{Cas} and FAK and disruption of peripheral focal complexes (9, 79, 90). YopH inhibits phagocytosis by polymorphonuclear neutrophils and macrophages, mediated by complement receptors (96) or Fc receptors, respectively (26).

YopM. YopM (11, 24, 28, 58, 67) is a strongly acidic protein with a molecular mass of approximately 40 kDa. Unlike the other Yop effector proteins that are well conserved among different *Yersinia* species, YopM is somewhat heterogeneous (12). Due to the presence of leucine-rich repeats, YopM shows similarity to a great number of eukaryotic proteins, but its intracellular target and action remain unknown.

YopO/YpkA. YpkA is a protein kinase (33) with some similarity to the COT (cancer Osaka thyroid) oncogene product, a cytosolic serine/threonine protein kinase expressed in hematopoietic cells and implicated in signal transduction by growth factors (41). YpkA catalyzes autophosphorylation of a serine residue in vitro. Infection of HeLa cells with a multiple *yop* mutant overproducing YpkA leads to a morphological alteration of the cells different from those mediated by YopE and YopH. The cells round up but do not detach from the extracellular matrix. Inside HeLa cells, the YpkA protein is targeted to the inner surface of the plasma membrane (38). No target protein of YpkA/YopO has been identified.

YopP/YopJ. YopP (YopJ in *Y. pestis* and *Y. pseudotuberculosis*) is a 32.5-kDa protein encoded by the same operon as YpkA/YopO (24, 34). It induces apoptosis of murine macrophages (65, 66) but not of other cell types, such as epithelial cells or fibroblasts (65, 66).

Injection of YopP/YopJ into macrophages also leads to a significant reduction in the release of tumor necrosis factor alpha (TNF-α), a proinflammatory cytokine playing a central role in the development of the immune and inflammatory responses to infection (10, 73) (Fig. 4). This reduction probably results from the inhibition of NF-κB activation by YopP/YopJ (94, 99). YopP/YopJ has also been shown to inhibit the ERK1/2, p38, and JNK mitogen-activated protein kinase (MAPK) activities (Fig. 4) (10, 73, 95). One can thus speculate that YopP would act upstream or at the junction of cascades leading to apoptosis on the one hand and to the inhibition of TNF-α on the other hand.

Interestingly, YopP and YopJ share a high level of similarity to *Xanthomonas campestris* AvrRxv (119) and *Rhizobium* y410 (31). No function is known for AvrRxv and y410. However, AvrRxv is one of many avirulence proteins identified in plant pathogens that elicit the hypersensitive response, a process that is likely to result from the activation of a programmed cell death pathway (119). However, no cytotoxic effect has been described for AvrRxv. Animal and plant pathogens, therefore, share a type III secretion-dependent effector to elicit programmed cell death in their respective hosts.

YopT. YopT is a 35.5-kDa protein that has been described and characterized recently (47). YopT induces a cytotoxic effect in HeLa cells and macrophages. The effect on HeLa cells consists of disruption of the actin filaments and alteration of the cell cytoskeleton.
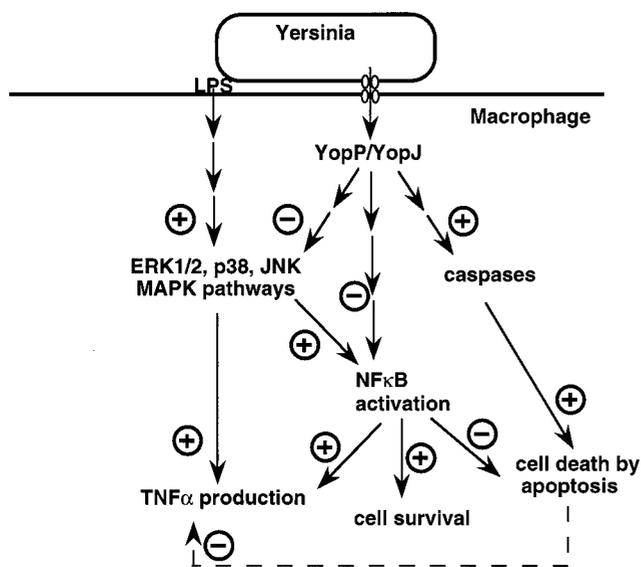
FIG. 4. Model showing the effects of YopP/YopJ on the macrophage intracellular cascades. Lipopolysaccharide (LPS) activates the ERK1/2, JNK, and p38 MAPK pathways, which leads to increased TNF-α production. Activated MAPKs can lead to NFκB activation; activated NFκB can, in turn, enhance TNF-α transcription. Translocated YopP/YopJ induces macrophage apoptosis by a mechanism involving caspase activation. It also downregulates MAPKs and impairs NFκB activation, two effects that could explain the YopP/YopJ-induced reduction of TNF-α production. See the text for details and references.

REGULATION OF TRANSCRIPTION OF THE VIRULON GENES

As we have seen, in vitro, Yop secretion occurs only at 37°C in the absence of Ca^{2+}. Transcription of the *ysc* genes and the *yop* genes is strongly thermoinduced. This thermoregulation results from the interplay between the transcriptional activator VirF (LcrF in *Y. pestis* and *Y. pseudotuberculosis*) and chromatin structure. Transcription of the *yop* genes is repressed by Ca^{2+} or by mutations in the secretion apparatus. This second regulation is a feedback inhibition mechanism exerted by the closed secretion apparatus on transcription of the genes encoding the proteins to be secreted.

The positive loop. VirF/LcrF is a 30.9-kDa protein that belongs to the AraC family of regulators (22). In *Y. enterocolitica*, the *virF* gene itself is strongly thermoregulated and VirF is active only at 37°C. Indeed, when *virF* is transcribed at low temperature from a *tac* promoter, the *yop* genes are only poorly transcribed. By contrast, at 37°C, the response to isopropyl-β-D-thiogalactopyranoside (IPTG) mimics the normal response to thermal induction (56). Thus, the expression of the *yop* regulon is first controlled by temperature, and the expression of some of its genes is reinforced by the action of VirF, the synthesis of which is also temperature controlled. The most likely hypothesis is that temperature could somehow modify the structure of the chromatin, making the promoters more accessible to VirF. Rohde et al. (89) confirmed that temperature alters DNA supercoiling and DNA bending by VirF in *Y. enterocolitica*, and they hypothesized that temperature dislodges a repressor, perhaps histone-like protein YmoA (21), bound on promoter regions of VirF-sensitive genes and of some other thermoregulated genes.

Feedback control of Yop synthesis by the secretion apparatus: LcrQ/YscM. By analogy with the secreted antisigma factor involved in the regulation of flagellum synthesis (46, 55), Rimpiläinen et al. (87) suggested that feedback inhibi-

tion could be mediated by a repressor that is normally expelled via the Yop secretion machinery. In *Y. pseudotuberculosis*, they suggested that LcrQ, a 12.4-kDa secreted protein encoded by the last gene of the *virC* locus, could be this hypothetical regulator because overproduction of this protein abolishes Yop production. In *Y. enterocolitica*, there are two homologues of LcrQ: YscM1 and YscM2 (3, 107). How LcrQ or YscM1 and YscM2 work is still unknown, but their mode of action is probably indirect.

CONCLUSIONS AND FUTURE PERSPECTIVES

Although we have come close to a global picture, it is important to stress that many basic questions remain. Indeed, the system may now become a victim of its popularity, and after a period of excessive suspicion, we might fall into a period of oversimplification. I will thus end with a few questions that will allow us to remember the complexity of the system and the main present issues.

What is a Yop? The term Yop was coined by Wolf-Watz and colleagues (13) to qualify the *Yersinia* outer membrane proteins initially described by Portnoy et al. (84) and Straley and Brubaker (109) and shown by Heesemann and colleagues (40) to be "released" from the bacterium. Later, Michiels, other coworkers, and I showed that these proteins are true extracellular proteins secreted by a new system, called Ysc (for Yop secretion), and encoded by the same plasmid (62–64). Yops can thus be defined as yersinia proteins secreted by the Ysc apparatus under low-Ca^{2+} conditions. By this definition, LcrV, the protective antigen of plague known since the mid-1950s (18), is clearly a Yop. Moreover, it is encoded by the same operon as the translocator Yops and is involved in translocation. LcrV is not the only Yop with a different name; four other proteins (YopR, YscO, YscP, and LcrQ/YscM) that are secreted under the same conditions as Yops appear to be encoded by operons devoted to secretion. Since this is also the case for YopN, there is no reason to exclude these four proteins from the Yop family. Thus, Yop proteins form a family of secreted proteins that includes intracellular effectors and several components of the secretion-translocation apparatus that is released from the bacterium upon Ca^{2+} chelation. In spite of the constant care of yersiniologists, nomenclature remains somewhat unfriendly. For the sake of clarity, new names could be introduced when the functions of all of these proteins become clear.

All of the Yops are thus elements of the complex apparatus described here. However, it must be stressed that this does not exclude the possibility that some Yops have another role, on their own, independent of the system, simply as proteins released in the organism. Indeed, Nakajima et al. provided evidence for a direct immunosuppressive effect of purified LcrV injected into mice (68).

What does the yersinia organelle look like? By analogy with *S. typhimurium*, it is very likely that the Ysc proteins and the translocators encoded by 35 contiguous genes form a unique organelle. In this minireview, it was assumed that it resembles the salmonella organelle. However, it must be clearly stated that this is no more than an assumption. What are the elements of the Ysc syringe, what are the components of the needle, and what is the role of LcrV and LcrG in the organelle seem to be the most haunting questions in that respect. Electron microscopy techniques such as those that provided wonderful images of the assembling flagellum (72) or the *S. typhimurium* type III apparatus (53) will certainly help answer many of these questions.

Is a pore formed in the eukaryotic cell? Although not firmly established, the existence of a pore is very likely, as suggested by the contact hemolysis shown by Håkansson et al. (39), but this pore needs to be characterized. Does it consist of YopB alone or of YopB associated with YopD? What is the role of LcrV in the assembly of this hypothetical pore? Could it form some kind of needle underneath YopB and YopD? What is the function of LcrG in translocation? Do the translocators interact with the effector Yops during translocation?

What does contact control mean? Contact control is one of the most appealing aspects of the system, but as we have seen, several major questions remain. (i) Is the translocation apparatus deployed prior to contact? (ii) Does Ca^{2+} chelation really mimic contact, or does it cause another event leading to massive artifactual leakage? (iii) What is the degree of "directionality"? (iv) Last but not least, how does contact lead to the opening of the channel. Generally, contact means an interaction between a receptor and a ligand. However, in this case, no specific receptor at the cell surface has been identified. Recognition of heparan sulfate proteoglycans by LcrG is a first clue but certainly not the definite answer. To identify a receptor, we first have to guess the identity of the bacterial ligand. Although several pieces of the system have been localized at the bacterial surface, the actual bacterial ligand has not been identified. Is it YopN, YopN-associated TyeA, or LcrG, or could it be a protein that is not even involved in the control of secretion by Ca^{2+} ions?

What is the recruitment signal, and what is the function of the Syc chaperones? The unravelling of the Ysc secretion pathway will also require long collaborative efforts. There are still more than 25 components to localize in the bacterium, and we feel in the situation of the do-it-yourself enthusiast who thinks that there must be extra pieces in his assembly kit! However, in this area, the hottest question is perhaps that of the secretion signal. Does the Ysc apparatus recognize mRNA, and if so, how? What is the exact role of the Syc chaperones? Are they bodyguards, pilots, or both?

Tools and toys for the cell biologist. Finally, cell biology has a lot of answers to give. Why do yersiniae deliver six effectors to the same target cell? Are the six effectors needed to neutralize the same phagocyte? Would the action of some effectors be very rapid (YopH?) but reversible while the action of others would be slower but irreversible (YopP/YopJ)? Or, alternatively, would some effectors be specifically designed for some cell types and others for other cell types?

In conclusion, research on yersiniae has been extremely fruitful in terms of concepts. The Yop virulon being at the leading edge of type III secretion, it probably represents the most suitable system for investigation of its most basic aspects, such as control, secretion, and translocation. Once again, fundamental research on a system with very little, if any, commercial or health interest has led to new ideas, which we believe will pay off sooner or later.

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