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The Virulence Plasmid of *Yersinia*, an Antihost Genome

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INTRODUCTION

Invasive pathogenic bacteria have in common the capacity to overcome the defense mechanisms of their animal host and to proliferate in its tissues. They each have their own life-style and target organs and cause a variety of symptoms and diseases, which suggested the existence of great diversity among the bacterial virulence strategies. However, recent data contradict this view and reveal the existence of major virulence mechanisms in various pathogenic bacteria. One is the release of A-B toxins as exemplified by *Bordetella pertussis* and *Bacillus anthracis*. Another was discovered more recently in a number of bacterial pathogens. By this mechanism, sometimes referred to as type III, extracellular bacteria that are in close contact with a eukaryotic cell deliver bacterial proteins into the cytosol of this cell. The Yop system of *Yersinia* spp., which we describe in this review, represents an archetype for this new mechanism. The other animal pathogens with related systems are *Salmonella* spp., *Shigella* spp., enteropathogenic *Escherichia coli* (EPEC), *Pseudomonas aeruginosa*, *Chlamydia psittaci* (165), and *Bordetella* spp. (383a). Related systems are 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 4 and 351). The literature on all the type III systems is now so abundant that an exhaustive description could no longer fit in one review. This review is thus specifically dedicated to the *Yersinia* type III system. However, homologs of the various *Yersinia* proteins in the other bacteria are mentioned and even described when appropriate. To integrate the Yop virulon in the general context of cross talk between bacterial pathogens and their host, the reader may refer to broader reviews (94, 95, 107). More information on *Yersinia* virulence in general is also available in recent reviews (51, 255). Less exhaustive reviews dealing with the type III system (201, 352) or, more specifically, the Yop virulon (75, 98, 335–337) are also available.

Yersinia Life-Style

The genus *Yersinia* includes three species that are pathogenic for rodents and humans; *Yersinia pestis* causes plague, *Yersinia pseudotuberculosis* causes mesenteric adenitis and septicemia, and *Yersinia enterocolitica*, the most prevalent in humans, causes gastrointestinal syndromes ranging from an acute enteritis to mesenteric lymphadenitis (76). *Y. pestis* is generally inoculated by a flea bite, while the two others are food-borne pathogens. In spite of these differences in the infection routes, all three have a common tropism for lymphoid tissues and a common capacity to resist the nonspecific immune response, in particular phagocytosis and killing by macrophages and polymorphonuclear leukocytes (PMNs). *Y. pestis* and *Y. pseudotu-*

berculosis are natural rodent pathogens. Although this does not seem to be the case for *Y. enterocolitica*, experimental infection of mice reproduces some of the symptoms observed in humans, in particular those related to invasion of the lymphoid tissues. After orogastric inoculation of mice, *Y. enterocolitica* selectively invades the Peyer's patches via M cells (15, 131, 140). This invasion leads to an enormous recruitment of PMNs, formation of microabscesses comprising extracellular *Yersinia*, and, finally, complete destruction of the cytoarchitecture of the Peyer's patches. Later, abscesses appear in mesenteric lymph nodes, suggesting that *Y. enterocolitica* disseminates via the lymphatic vessels (15). Anatomopathological examination of mice experimentally infected with *Y. pseudotuberculosis* also concluded that these bacteria are largely extracellular (309). In accordance with these in vivo observations, *Yersinia* manifests some resistance to phagocytosis in vitro, both by macrophages (87, 281) and by PMNs (53, 65, 291, 361). Once they are phagocytosed, *Y. pseudotuberculosis* and *Y. enterocolitica* generally do not survive. These observations led to the concept that *Y. pseudotuberculosis* and *Y. enterocolitica* are extracellular pathogens and that their survival strategy basically consists in avoiding the nonspecific immune response. *Y. pestis* has the same capacity as the other *Yersinia* spp. to resist phagocytosis. However, if it has been phagocytosed, it probably has a better capacity to resist killing. Early work by Straley (333, 334) showed that indeed *Y. pestis* can grow in the phagolysosome of cultured murine resident peritoneal macrophages. The reason for this capacity is not clearly established, but it does not depend on the type III system.

From Ca^{2+} Dependency to a Comprehensive View of the System

It has been known since the mid-1950s that *Y. pestis* is unable to grow at 37°C in Ca^{2+}-deprived media (157). It has also been known for decades that this unusual property can be lost and that its loss correlates with a loss of virulence. This Ca^{2+} dependency phenotype offered an extraordinary clue to the pathogenicity arsenal because nonvirulent mutants could be easily detected and even selected for. It appeared that virulence and Ca^{2+} dependency are encoded by a 70-kb plasmid (112, 390), sometimes called pYV (200). Under conditions of growth restriction, this plasmid governs the synthesis of a set of about 12 proteins called Yops (for "*Yersinia* outer membrane proteins"), which were originally designated by a letter, a number, or their molecular weight, according to the authors (42, 44, 73, 74, 97, 100, 110, 220, 256, 267, 330, 331, 377). The LcrV protein, an antigen of *Y. pestis* that had already been discovered in the mid-1950s (53), turned out to be one of these Yops (97, 239, 331). Most of the *yop* genes have been identified and sequenced, and they appeared to be almost identical in the three species. A uniform nomenclature has been introduced for YopB, YopD, YopE, YopH, YopM, and LcrV. YopN is sometimes still called LcrE (360). A few other Yops do not benefit from a common nomenclature because they were discovered or characterized more recently: YopO, YopP, and YopQ in *Y. enterocolitica* (74, 229) are called YpkA, YopJ, and YopK, respectively, in *Y. pseudotuberculosis* (111). The YopJ nomenclature is also used in *Y. pestis* (330). YopR (8) turned out to be the product of *yscH*. A *Y. pestis* YopL has been mentioned (329, 332), but its gene has not yet been identified and sequenced and it is not known whether it corresponds to a Yop described in *Y. enterocolitica* or *Y. pseudotuberculosis*. Finally, YopT was described only very recently (170). The "S" has been skipped to avoid confusion with Yop in the plural.

Although initially described as outer membrane proteins, the Yops could also be recovered from the culture supernatant (149, 151), and it was later found that they were actually secreted proteins (229). Their secretion occurs by a new pathway (now called type III) and requires a specific apparatus (called Ysc for "Yop secretion"), which is also encoded by the pYV plasmid (228, 229).

To trigger Yop secretion in vitro, *Yersinia* is generally grown at 28°C in a medium depleted of Ca^{2+} and then transferred to 37°C. Ca^{2+} depletion (or contact with a eukaryotic cell [see below]) and temperature both control transcription of the *yop* genes. The best-characterized regulator is VirF (LcrF in *Y. pestis* and *Y. pseudotuberculosis*), a transcriptional activator of the AraC family (72). It controls transcription of most of the genes involved in Yop synthesis and secretion (199).

Genetic analysis indicated that most of the Yop proteins are essential for virulence. In particular, YopE turned out to be responsible for a cytotoxic activity (282) that had been described earlier (119, 266). YopH was found to inhibit the phagocytosis of bacteria by macrophages (281) and later was shown to be a protein tyrosine phosphatase (PTPase) related to eukaryotic counterparts (132). However, three observations were enigmatic: (i) Yops form large and insoluble aggregates in the culture medium, which is unusual for virulence effectors; (ii) YopE has no toxic activity on its own (119, 287); and (iii) what would be the role of an extracellular PTPase?

A major advance occurred when Rosqvist et al. (283) showed that Yop preparations elicit a cytotoxic response when microinjected into HeLa cells, indicating that the target of the YopE protein was intracellular. A *yopD* mutant was unable to affect HeLa cells, while a preparation of Yops secreted by the very same mutant was cytotoxic when microinjected into the cytosol of HeLa cells. Rosqvist et al. logically concluded from this that the YopD protein should play a role in translocating the YopE protein across the plasma membrane of the eukaryotic target cell to reach the cytosolic compartment (283).

The evidence for YopD-mediated translocation of the YopE protein was essentially genetic. In 1994, this elegant hypothesis was confirmed by two different approaches. The first was based on immunofluorescence and confocal laser-scanning microscopy examinations. Rosqvist et al. (285) showed that the YopE protein appeared in the cytosol of HeLa cells infected with wild-type *Y. pseudotuberculosis*. In contrast, when cells were infected with a mutant strain of *Y. pseudotuberculosis* unable to produce YopD, YopE was no longer internalized, showing that the YopD protein was essential for the translocation of YopE across the target cell membrane (285). The second approach was based on a reporter enzyme strategy introduced by Sory and Cornelis (321) (Fig. 1). The reporter system consisted of the calmodulin-activated adenylate cyclase domain (called Cya) of the *Bordetella pertussis* cyclolysin (118). The rationale was as follows: the Yop-Cya hybrid enzyme introduced into the cytosol of eukaryotic cells would produce cyclic AMP (cAMP) while the intrabacterial Yop-Cya hybrid would not, because of the absence of calmodulin in the bacterial cytoplasm. Since the catalytic domain of cyclolysin is unable to enter eukaryotic cells by itself, accumulation of cAMP would essentially reflect Yop internalization. Infection of HeLa cells with recombinant *Y. enterocolitica* producing a hybrid YopE-Cya protein resulted in a marked increase in the level of cAMP even when internalization of the bacteria themselves was prevented by cytochalasin D. Infection with a *Y. enterocolitica* mutant unable to produce both the YopD and YopB proteins did not lead to cAMP accumulation, confirming the involvement of YopD and/or YopB in translocation of the YopE protein across eukaryotic membranes (321).

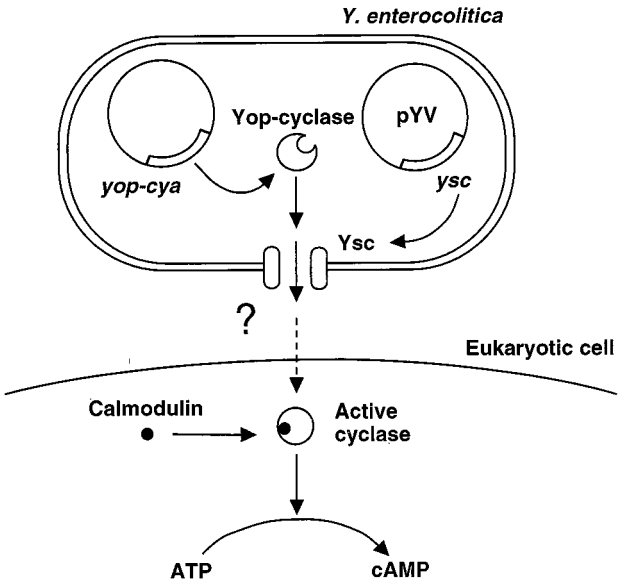

FIG. 1. The Yop-Cya reporter strategy used to study translocation of Yop proteins into the cytosol of eukaryotic cells. Reprinted from reference 321 with permission of the publisher.

In light of these results, a coherent model could be established. According to this model (Fig. 2), the Yops form two distinct groups of proteins. Some Yops are intracellular effectors delivered inside eukaryotic cells by extracellular *Yersinia* organisms adhering at the cell surface, while other Yops (translocator Yops) form a delivery apparatus. This model is now largely supported by a number of other results that will be presented in this review. 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 249-257 of YopH produced by *Y. enterocolitica* during a mouse infection is presented by major histocompatibility complex class I molecules, like cytosolic proteins (328).

The virulence plasmid thus encodes an integrated antihost system allowing the delivery of a set of effector Yops into the cytosol of eukaryotic cells by a delivery apparatus and a specialized secretion system. The virulence plasmid has now been completely sequenced in *Y. enterocolitica* W22703 (pYV227) (171) and in *Y. pestis* KIM (pCD1) (165a, 257a). Most of the sequence of plasmid piB1 from *Y. pseudotuberculosis* YPIII is also available. The genetic maps are given in Fig. 3. About 50 genes are involved in virulence, and they occupy three-quarters of the plasmid. A total of 35 genes encoding the secretion and translocation machineries form a continuous block flanked on both sides by more dispersed genes encoding effectors and their chaperones.

We first review the effects of this virulence apparatus on eukaryotic cells and then analyze in detail the fate of the Yops, from secretion to delivery and action in eukaryotic cells. We then describe the adhesin YadA and, finally, review the genetic aspects, regulation of gene expression, and plasmid organization.

EFFECTS ON HOST CELLS

Macrophages

Macrophages are part of the first line of defense against invading organisms, and several elements of the virulon allow

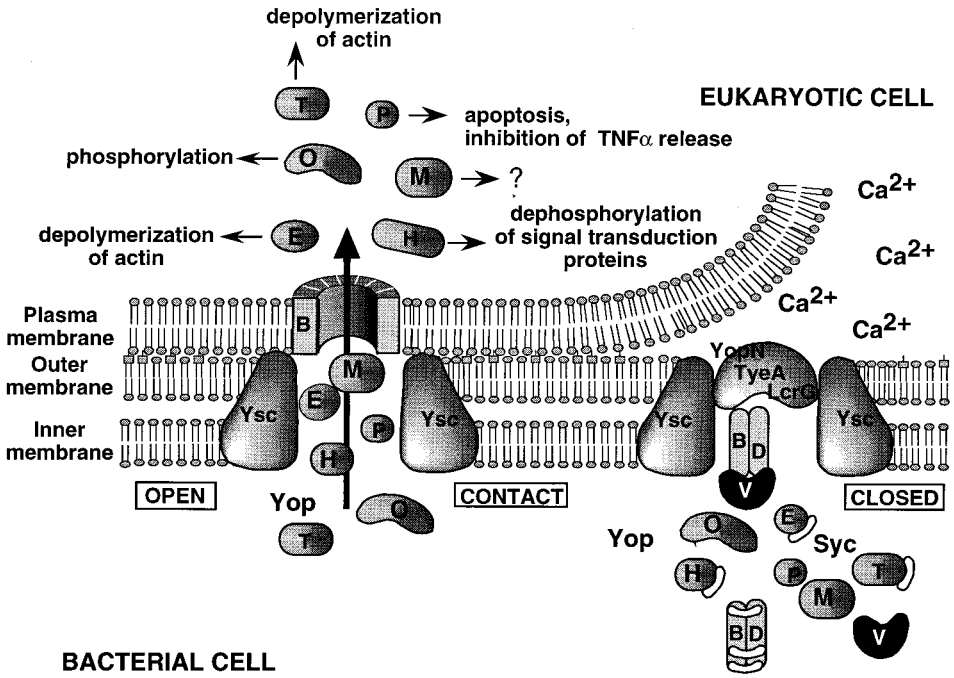

FIG. 2. A tentative model for the interaction between *Yersinia* and a macrophage. When *Yersinia* is placed at 37°C in a rich environment, the Ysc secretion apparatus is installed and a stock of Yop proteins is synthesized. Some of these proteins are capped with their specific Syc chaperones, which presumably prevent premature associations. As long as there is no contact with a eukaryotic cell, the YopN-TyeA-LcrG plug blocks the Ysc secretion channel. Upon Ca^{2+} depletion or contact with the eukaryotic target cell, the secretion channel opens and the YopB translocator inserts in the eukaryotic cell with the help of YopD and LcrV. The Yop effectors (YopE, YopH, YopM, YopO/YpkA, YopP/YopJ, and YopT) are then transported through the secretion channel and translocated across the plasma membrane, guided by the translocators. YopE and YopT act on the cytoskeleton, while YopP/YopJ induces apoptosis and inhibits the release of TNF-α.

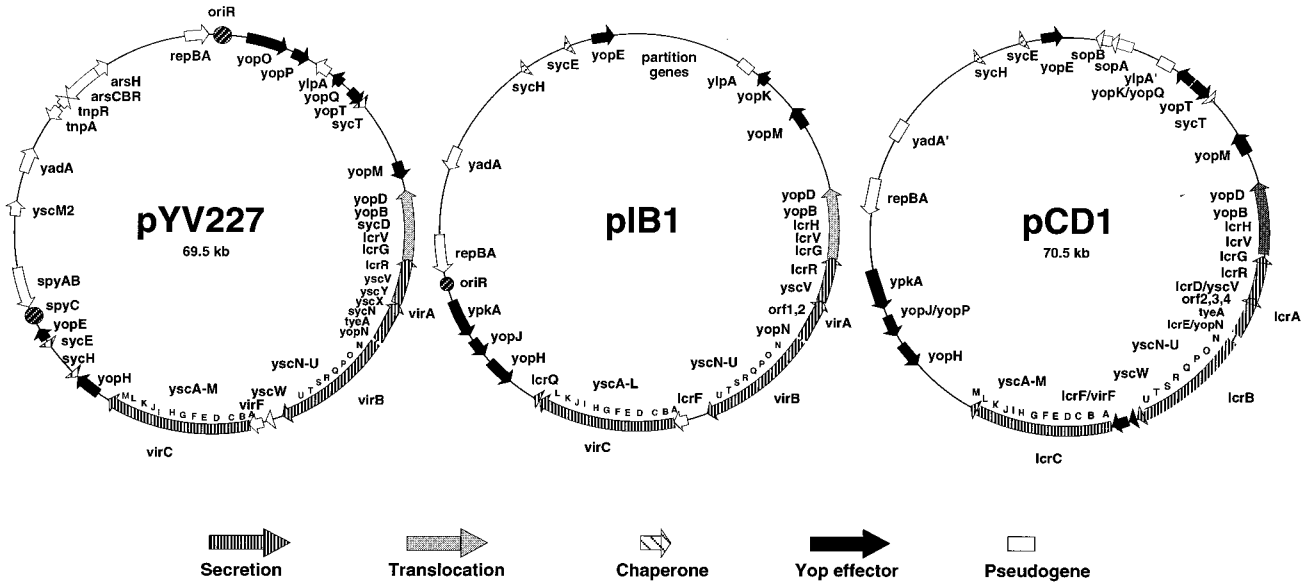

FIG. 3. The genetic maps of pYV227 from *Y. enterocolitica* W227 (serotype O:9) (redrawn from reference 170), pIB1 from *Y. pseudotuberculosis* YPIII (redrawn from reference 259), and pCD1 from *Y. pestis* KIM (redrawn from references 165a and 257a). Note that none of these maps is complete. For pCD1, the plasmid has been sequenced twice (165a, 257a) and only the genes that are identified in the two sequences or in one sequence and in *Y. enterocolitica* are shown. Plasmid pYV227 has also been completely sequenced (171), but only the genes described in this review are included here. Shading of the genes has been done on the basis of the data presented in this review.

Yersinia to circumvent the microbicidal action of these phagocytes. Upon interaction with macrophages, *Yersinia* has the capacity to impair phagocytosis, to inhibit the respiratory burst, to trigger apoptosis, and to suppress the normal release of tumor necrosis factor alpha (TNF-α). Each of these four aspects is treated individually below.

Inhibition of phagocytosis. One of the simplest ways to resist killing by macrophages is to avoid being ingested. It has been known for a long time that *Yersinia* spp. are endowed with the capacity to resist phagocytosis by macrophages and that this property depends on the presence of the pYV plasmid (58, 59).

Working in vitro with *Y. pseudotuberculosis* and resident mouse peritoneal macrophages, Rosqvist et al. (281) showed by a double-immunofluorescence technique (153) that a strain unable to express YopH has a reduced ability to resist phagocytosis. The ability to resist phagocytosis could be complemented in *trans* by introduction of a plasmid carrying only the *yopH* gene, demonstrating that YopH is indeed involved in the antiphagocytic effect. However, mutation of *yopH* did not completely abolish the resistance to phagocytosis. Macrophages phagocytosed 80% of *yopH* mutant bacteria, in comparison to 95% of pYV^- and 35% of pYV^+ bacteria. This intermediate level of phagocytosis inhibition by the *yopH* mutant suggested that another virulence factor was involved in this phenomenon. This second factor turned out to be YopE, since a double *yopH yopE* mutant showed the same low level of phagocytosis resistance as a plasmid-cured strain (282). YopE and YopH thus act in concert to enable *Yersinia* to inhibit its own uptake by macrophages and hence to proliferate in the Peyer's patches as extracellular microcolonies (140).

Fällman et al. (87) undertook more detailed study of the uptake of *Y. pseudotuberculosis* by the macrophage-like cell line J774A.1. Both nonopsonized bacteria and bacteria opsonized with rabbit anti-*Yersinia* immunoglobulin G were able to inhibit their uptake by J774A.1 macrophages, indicating that *Yersinia* can resist specific uptake via Fc receptors. Pretreatment of J774A.1 cells with wild-type bacteria prevented the uptake of nonrelated prey (immunoglobulin G-opsonized yeast particles), while preincubation with mutants impaired in resistance to phagocytosis had no effect; the *Yersinia* antiphagocytic effect thus involves the blocking of a general phagocytic mechanism and is not restricted to the uptake of *Yersinia* organisms themselves (87). Further studies with J774A.1 macrophages suggested that YopH, in addition to inducing an overall dephosphorylation of host cell proteins (34, 36, 128, 147), is able to interfere with early tyrosine phosphorylation signals that occur in the cell during phagocytosis. Andersson et al. (11) showed that exposure of J774A.1 macrophages to *yopH* mutant *Y. pseudotuberculosis* resulted in a transient increase in tyrosine phosphorylation of a number of proteins, including paxillin, which is known to be tyrosine phosphorylated upon Fc receptor-mediated signaling associated with phagocytosis in macrophages (129). This transient tyrosine kinase activity, which probably constitutes part of an early phagocytic signal, was impaired by $yopH^+$ bacteria (11). Recently, two eukaryotic cell proteins, focal adhesion kinase (FAK) and $p130^{CAP}$, have been identified as YopH targets in epithelial cells (see below); this activity of YopH results in disruption of the focal adhesion structures and correlates with an impaired ability of the target cell to carry on the invasion-mediated internalization of the bacteria (31, 258). The role of FAK and $p130^{CAP}$ in phagocytic cells remains to be elucidated.

Inhibition of the respiratory burst. It was suspected for a long time that *Yersinia* interferes with the normal respiratory burst of macrophages, since the oxidative burst occurring after interaction with *Y. pestis* is much lower than that seen after phagocytosis of *E. coli* (59). More recently, Hartland et al. (147) infected bone marrow-derived macrophages with various *Y. enterocolitica* mutant strains before stimulation of the respiratory burst by the addition of zymosan, which triggers the CR3 receptor. They measured the intensity of the respiratory burst by assaying the amount of reduced cytochrome *c* produced during the generation of O_2^- (127). This showed that *Y. enterocolitica* also has the capacity to inhibit the respiratory

burst and that this capacity depends on the pYV plasmid. Loss of the effectors YopE, YopH, and YopO/YpkA did not affect this capacity, but loss of the translocator YopD did. This property thus probably depends on an effector different from YopE, YopH, and YopO.

Since tyrosine phosphorylation is an important component of the signaling pathways responsible for the activation of the macrophage respiratory burst, Green et al. (128) investigated the possible link between the YopH tyrosine phosphatase activity and the inhibition of the respiratory burst. They infected bone marrow-derived macrophages with *Y. enterocolitica* and monitored both tyrosine phosphorylation and respiratory burst in response to zymosan (127). Infection with pYV^+ *Y. enterocolitica* suppressed both phenomena. However, loss of YopH abolished the suppressive effect on tyrosine phosphorylation but not on the respiratory burst. This observation agrees with those of Hartland et al. (147) and confirms that the inhibition of the zymosan-induced macrophage respiratory burst by *Y. enterocolitica* involves a plasmid-encoded virulence protein other than YopH, possibly in addition to YopH.

However, these conclusions differ from those of Bliska and Black (33), who showed that YopH is responsible for the inhibition of the Fc receptor-mediated oxidative burst in macrophages infected by *Y. pseudotuberculosis*. The reasons for these discrepancies are not known, but it is important to note that the experimental procedures used in the two studies are different and thus difficult to compare. Indeed, the pathways used to trigger the respiratory burst involved either the macrophage complement receptors or the Fc receptors; in addition, the type of macrophages, the *Yersinia* species, and the quantification methods were different (33, 82, 127, 128, 147).

In conclusion, *Yersinia* spp. are able to impair the oxidative burst of the macrophages, and so far, the only Yop effector protein that has been shown to be involved in the phenomenon is YopH. However, the role of YopH in the inhibition of the respiratory burst remains a matter of debate, since the detection of this role depends on the pathway used to trigger the respiratory burst.

Induction of apoptosis. In 1986, Goguen et al. (119) reported that *Y. pestis* and *Y. pseudotuberculosis* have a cytotoxic effect on the mouse macrophage cell lines IC21 and P388D1 as well as on mouse resident peritoneal macrophages. They observed that cells infected with a wild-type strain change shape, acquire a granular aspect, and detach easily from the culture dish. This effect, which was dependent on the presence of the pYV plasmid, evokes apoptosis, although it was not described as such at that time. Recently, three groups, two working with *Y. enterocolitica* (232, 290) and one working with *Y. pseudotuberculosis* (237), showed that *Yersinia* triggers apoptosis of cultured macrophages. Infected macrophages displayed general features of apoptosis, such as membrane blebbing (apoptotic body formation), cellular shrinkage (232, 290), and DNA fragmentation (Fig. 4). Infection of macrophages with secretion and translocation mutants of *Y. enterocolitica* did not lead to apoptosis, showing that a translocated Yop effector is involved. Screening of a library of *yop* mutants showed that the YopE cytotoxin is not involved and identified YopP as the effector responsible for apoptosis (232) (Fig. 4). In an independent study, Monack et al. (237) came to the conclusion that YopJ, the *Y. pseudotuberculosis* homolog of YopP, is required for the induction of the cell death process. The phenomenon displays some cell specificity, since epithelial cells (232, 237, 290) and fibroblasts (237) do not undergo apoptosis upon infection with *Yersinia*. The mechanism by which *Yersinia* induces macrophage apoptosis remains to be elucidated, but it parallels that used by cytotoxic T lymphocytes to kill their target cells; cytotoxic T cells inject granzyme B into the cytosol of their target cells, thereby inducing apoptosis (308). One of the virulence functions of *Yersinia* organisms thus appears to mimic a physiological process of their host.

Although apoptosis is obvious in vitro, its physiopathological role is not yet clear. A *yopP/yopJ* mutant is not affected in virulence, at least in a mouse model (111, 330), and moreover, the induction of apoptosis by *Yersinia* has never been shown in vivo. The only known element is that in *Y. enterocolitica*-infected mice there is an increased number of apoptotic cells in the infected Peyer's patches (15). It remains to be demonstrated if this effect is a consequence of a general degradation of the infected tissues or is due to the action of YopP/YopJ. However, the induction of apoptosis of target cells is undoubtedly a master strategy used by several invasive pathogens (for reviews, see references 394 and 395). *Yersinia* spp. are not the only pathogens endowed with a type III system that have been found to induce apoptosis. Apoptosis induction has also been reported for *Shigella* (393) and *Salmonella* (60, 238). For *Shigella*, it has been shown that apoptosis is mediated by IpaB (392), which binds to and activates the interleukin-1β (IL-1β)-converting enzyme (caspase 1) (62, 158, 346). SipB, the *Salmonella* homolog of IpaB, is likely to induce apoptosis (60, 236) by a similar mechanism (236, 391). The mechanism by which *Yersinia* induces apoptosis is probably different. First, *Yersinia* induces apoptosis from the outside of the host cell (232), which is different from what has been described for *Shigella* (393). Second, YopB, which is the *Yersinia* counterpart of IpaB and SipB, is not the effector of the phenomenon, although it is indirectly involved in the induction of apoptosis through its role of translocator. Third, an inhibitor of caspase 1 does not prevent *Yersinia*-induced apoptosis (290). However, a broad-spectrum caspase inhibitor blocks the completion but not the onset of *Yersinia*-induced apoptosis, suggesting that *Yersinia* might initiate apoptosis at a level upstream from caspases (290). Recently, Ruckdeschel et al. (288) showed that *Y. enterocolitica* inhibits activation of the transcription factor NF-κB in murine J774A.1 and peritoneal macrophages; analysis of different *Y. enterocolitica* mutants revealed a striking correlation between the abilities to inhibit NF-κB activation and to trigger apoptosis. Several reports showed that apoptosis can be prevented by the expression of NF-κB, suggesting that the induction of NF-κB may be part of a survival mechanism (21, 23, 215, 341, 349, 350, 364). These results suggest that *Yersinia* could trigger apoptosis by suppressing the cellular activation of NF-κB (288) (Fig. 5).

Inhibition of TNF-α and IFN-γ release. TNF-α is a proinflammatory cytokine that plays a central role in the development of the immune and inflammatory responses to infection. Secreted mainly by macrophages, TNF-α acts on various cell types involved in the host defense mechanisms. It stimulates both macrophage and PMN microbicidal activity and acts on natural killer cells together with IL-12 to provoke the release of gamma interferon (IFN-γ), which further increases the microbicidal activity of macrophages. Moreover, TNF-α induces expression of adhesion molecules on endothelial cells and is chemotactic for monocytes, thus contributing to the amplification of the inflammatory response (for a review, see reference 356). The importance of the cytokines TNF-α and IFN-γ in the host immune response against a *Yersinia* infection was first illustrated by the fact that treatment of mice with antibodies directed against TNF-α or IFN-γ exacerbates infection by *Y. enterocolitica* (16). Moreover, an immunohistological study showed that administration of anti-TNF-α antibodies to mice before and after orogastric infection with *Y. enterocolitica* leads to complete destruction of Peyer's patches and to a dramatic

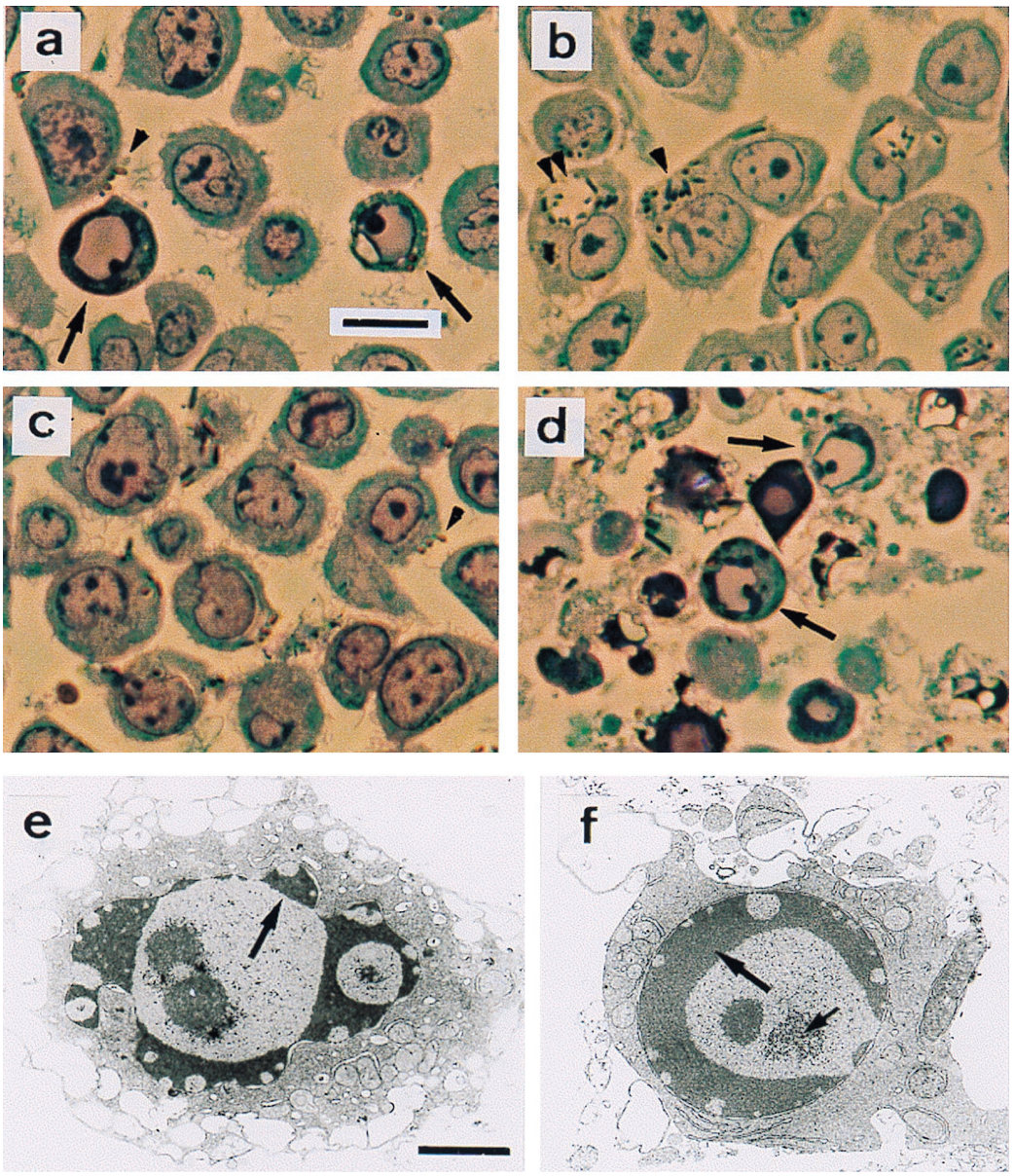

FIG. 4. YopP-induced apoptosis. Semithin sections were stained with toluidine blue and examined by light microscopy (a to d). (a) Wild-type *Y. enterocolitica* E40. Apoptotic nuclei (arrows) and cell surface-associated bacteria (brown particles, arrowhead) are visible. (b) *yscN* secretion mutant. No apoptotic cells are detected. Internalized bacteria either in tight (single arrowhead) or spacious (double arrowhead) phagosomes are abundant. (c) *yopP* effector mutant. No apoptotic cells are detected. Bacteria are seen at the cell surface (arrowhead). (d) $yopP^{+++}$ (*yopP* cloned in a multicopy vector). Apoptotic cells are visible (arrows). (e and f) Ultrastructural analysis of cells infected with $yopP^{+++}$ from panel d is shown. Typical features of apoptosis include (i) peripheral chromatin condensation in crescents, except in the vicinity of nuclear pores (large arrows); (ii) bulging of nuclear crescents into the cytoplasm (best seen in panel e); and (iii) appearance of central clusters of small particles of unknown nature, typical of apoptosis (small arrow in panel f). Nuclear and plasma membrane alterations contrast with a good ultrastructural preservation of cytoplasm, particularly of endoplasmic reticulum and mitochondria. Bars, 10 μm (a to d) and 2 μm (e and f). Reprinted from reference 232 with permission of the publisher.

increase of bacterial counts in Peyer's patches, mesenteric lymph nodes, and spleen, even though phagocytes were normally recruited in Peyer's patches and mesenteric lymph nodes (17). This suggests that TNF-α plays an essential role in the local host defense mechanism in the intestinal tissues, possibly by activating phagocytes (17).

Interestingly, the levels of TNF-α and IFN-γ in mice infected with wild-type *Y. pestis* are much lower than those observed in mice infected with a pYV^- strain, suggesting that the pYV plasmid encodes a factor suppressing TNF-α and IFN-γ synthesis (240). Further studies with *Y. pestis* suggested a role for LcrV in this process, based on the observations that passive immunization with anti-LcrV antibodies or active immunization with purified protein A-LcrV hybrid protein protected mice against lethal doses of *Y. pestis* (240, 241). Another group working with mouse peritoneal macrophages and *Y. enterocolitica* confirmed the suppressive effect of virulent *Yersinia* on TNF-α release and claimed that YopB was responsible for this phenomenon (26). More recently, Ruckdeschel et al. (289), working with the mouse monocyte-macrophage cell line J774A.1 and *Y. enterocolitica*, showed that a functional type III secretion machinery is required for the phenomenon to occur

and suggested a correlation between this inhibition of TNF-α release and inhibition of the ERK1/2, p38, and JNK mitogen-activated protein kinase (MAPK) activities. Several reports already described a link between MAPK activation and TNF-α production (202, 203, 273, 280, 347, 388). It has been shown recently both for *Y. enterocolitica* (39) and for *Y. pseudotuberculosis* (251) that the *Yersinia*-induced inhibition of TNF-α release requires not only the type III secretion apparatus but also a functional Yop translocation apparatus and the effector YopP (*Y. enterocolitica*)/YopJ (*Y. pseudotuberculosis*). No other translocated effector seem to be involved in the phenomenon (39, 251). In addition, a strain secreting only YopB, YopD, YopN, YopE, YopH, and LcrV does not impair TNF-α release in vitro, indicating that these proteins are not, or at least not solely, responsible for the phenomenon (289). Taken together, these results suggest that YopB and LcrV presumably act indirectly as part of the translocation machinery required to deliver YopP/YopJ inside the macrophages (41, 138, 294). However, it must be added here that Brubaker and collegues provided evidence for a direct immunosuppressive effect of purified LcrV injected into mice (241). Thus, although LcrV is undoubtedly an element of the virulon and as such is required for the intracellular delivery of effectors, it may also act on its own as a protein released during the infection. The same could apply to YopB (26, 52a).

In agreement with the results of Ruckdeschel et al. (289), YopP/YopJ is also involved in the inhibition of the ERK2, p38, and JNK MAPK activities in infected macrophages (39, 251), but its actual target and mechanism of action remain unknown. It is noteworthy that YopP/YopJ is also involved in the triggering of apoptosis (see above), and it may well be that the two phenomena are linked. The link between apoptosis and MAPK activation is not clear, but the *Y. enterocolitica*-induced inhibition of NF-κB activation mentioned previously (288) is correlated not only to the induction of apoptosis but also to the inhibition of TNF-α production (288). One can thus speculate that YopP/YopJ could act upstream or at the junction of cascades leading to apoptosis on one hand and to inhibition of TNF-α on the other hand; alternatively, the initial role of YopP/YopJ could be to induce the death of the macrophage by triggering apoptosis, thereby impairing the synthesis and release of TNF-α (Fig. 5).

Inhibition of TNF-α production is not only encountered during *Yersinia* infection of macrophages. Other bacteria such as *Brucella* spp. (55), *Listeria monocytogenes* (78), *Bacillus anthracis* (163), and *Mycobacterium avium* (297) also possess the capacity to disturb the normal cytokine production. In *Brucella* spp., inhibition of TNF-α expression is due to the release of a specific, protease-sensitive bacterial factor (54). Parasites such as *Leishmania donovani* (79) and viruses (122, 317) also interfere with TNF-α production, showing that this defense mechanism is widely used by pathogens.

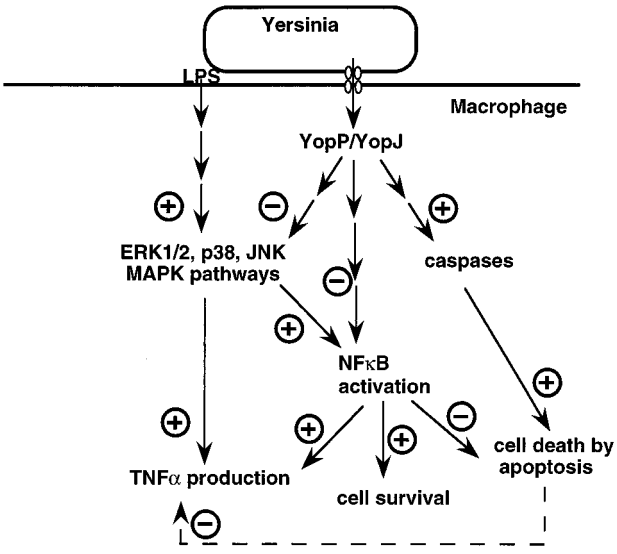

FIG. 5. Model showing the effects of *Yersinia* spp. on the macrophage intracellular cascades. Lipopolysaccharide (LPS) activates the ERK1/2, JNK, and p38 MAPK pathways, leading to increased TNF-α production. Activated MAPKs can lead to NF-κB activation; activated NF-κB can, in turn, enhance TNF-α transcription. Translocated YopP/YopJ induces macrophage apoptosis by a mechanism involving caspase activation. It also downregulates MAPKs and impairs NF-κB activation, two effects that could explain the YopP/YopJ-induced reduction of TNF-α production. See the text for details and references.

Polymorphonuclear Leukocytes

PMNs constitute the second group of professional phagocytes that are encountered by *Yersinia* bacteria invading the lymphoid tissues of their host. The interaction between *Yersinia* and PMNs has been studied for more than a decade, essentially with human PMNs and *Y. enterocolitica*.

Resistance to phagocytosis and killing. The interaction between *Y. enterocolitica* and PMNs was first studied by monitoring the luminol-enhanced chemiluminescence (CL) response (211), which is a measure of the intensity of the oxidative burst (82). A pYV^+ *Y. enterocolitica* strain grown at 37°C (Yop-inducing conditions) induced four- to sixfold less CL than did the same strain grown at 25°C or a plasmidless, isogenic strain grown at either temperature. This demonstrated for the first time the involvement of pYV-encoded proteins in the inhibition of the PMN oxidative burst (211). Since the CL response is a sensitive, indirect measure of the degree of phagocytosis in human neutrophils (126), this also suggested that *Y. enterocolitica* may resist phagocytosis by PMNs. Indeed, Lian et al. (210) showed that wild-type pYV^+ bacteria are resistant to phagocytosis by PMNs while pYV^- bacteria are not. This effect was seen not only in vitro but also in vivo; after intradermal inoculation into rabbits, histological examination of the inflammatory lesions by light or electron microscopy revealed that numerous bacteria of the pYV^- strain were located intracellularly in vacuoles of PMNs and mononuclear cells while pYV^+ bacteria were extracellular and surrounded by inflammatory cells without being phagocytosed (209).

To identify the pYV gene(s) encoding these capacities to inhibit the oxidative burst and to resist phagocytic uptake by PMNs, China et al. (65) tested the CL response of PMNs to various well-characterized *Y. enterocolitica* pYV mutants opsonized with normal human serum (NHS). They came to the conclusion that the YadA outer membrane protein, also encoded by the pYV plasmid (see below), is involved in both inhibition of the CL response and resistance to phagocytosis. The mechanism by which *Y. enterocolitica* resists phagocytosis could involve a reduction of complement-mediated opsonization due to the YadA protein (65). YadA binds complement factor H (66) and thus reduces opsonization by C3b molecules (66), and there is a correlation between the lack of an oxidative burst and the reduction of opsonization by C3b molecules (344).

Experiments carried out with human PMNs and various *Y. enterocolitica* strains opsonized with rabbit immune serum instead of NHS confirmed that plasmid-bearing bacteria resist phagocytosis and killing by PMNs while plasmid-cured bacteria

are readily ingested and killed by these cells (361). However, under the latter conditions, YadA did not play a major role; *Y. enterocolitica* mutants expressing YadA but lacking Yops were ingested by PMNs to the same extent as were pYV-cured bacteria, while mutants lacking YadA but secreting Yops were poorly ingested. Thus, in the presence of anti-*Yersinia* antibodies and complement, some Yop rather than YadA is responsible for the inhibition of phagocytosis of *Y. enterocolitica* by human PMNs. This difference in the observed mechanisms probably results from different opsonization conditions and possibly from different uptake mechanisms.

The differential contribution of YadA and Yops to evasion of the antibacterial activities of PMNs (oxidative burst, phagocytosis, killing) was further studied by Ruckdeschel et al. in an attempt to clarify the situation (291). It could be concluded that in the presence of NHS, (i) the YadA protein is essential for the protection of *Y. enterocolitica* from PMNs, since a *yadA* mutant induces a CL response stronger than that induced by the wild-type strain; (ii) that expression of YadA alone does not have any effect, since a secretion mutant that still produces YadA induced the same CL response as a pYV$^-$ strain; (iii) YopH also plays an important role, since a strain affected in YopH secretion (*sycH* mutant) was highly susceptible to phagocytosis and killing by PMNs; (iv) the strain impaired in YopH secretion also failed to inhibit a secondary zymosan-induced CL response, indicating that YopH is also involved in the oxidative burst inhibition; and (v) YopE is also involved, since a strain producing both YopE and YopH was more efficient in reducing the oxidative burst and in preventing phagocytosis and killing than a strain producing YopH only. Taken together, these results indicate that YopH, YopE, and YadA act in concert to resist antibacterial activities of PMNs under opsonizing conditions with NHS. The hypothesis of Ruckdeschel et al. (291) is that the adhesin YadA favors the adherence of bacteria to PMNs and that inhibition of the bactericidal functions is caused predominantly by YopH and, to a certain extent, also by YopE.

Resistance to antimicrobial peptides. As described above, pYV$^+$ *Y. enterocolitica* strains impede to some extent their phagocytosis by PMNs. However, when ingested, most of the pYV$^+$ bacteria are not killed whereas pYV$^-$ bacteria are killed almost instantly (86, 362), implying that plasmid-encoded factors can interfere with the killing mechanisms. These involve oxygen-dependent mechanisms (oxidative burst) and oxygen-independent mechanisms, which include acidification of the phagosome and attack by antimicrobial polypeptides. Antimicrobial polypeptides present in azurophilic granules of human granulocytes include bactericidal permeability-increasing protein, cathepsin G, elastase, proteinase 3, azurocidin, lysozyme, and defensins. These antimicrobial polypeptides are released into the phagolysosome through fusion of cytoplasmic granules with the phagosomes. Using a gel overlay assay (205), Visser et al. (362) showed that pYV$^-$ *Y. enterocolitica* strains are more susceptible to these granule-antimicrobial polypeptides than are wild-type *Yersinia* strains. Similarly, a *yadA* mutant was also more sensitive than wild-type bacteria, and introduction of a plasmid encoding only YadA in a pYV$^-$ strain restored, at least partially, the bacterial protection against the microbicidal activity of the granule extracts. YadA is thus involved in the resistance of *Y. enterocolitica* to the antimicrobial activity of polypeptides from human granulocytes, although the involvement of other plasmid-encoded factors could not be completely ruled out (362).

Epithelial Cells

The cell types that are the actual targets of the Yop effector proteins in vivo are not known at the moment, and although macrophages and PMNs are obvious in vivo targets, one can speculate that endothelial cells and epithelial cells of the gastrointestinal tract may also be targets of the Yop virulon. Endothelial cells play an important role in the development of the immune and inflammatory responses, by recruiting PMNs through expression of adhesion molecules. Epithelial cells not only constitute a barrier against bacterial invasion but also synthesize and secrete a number of cytokines.

Cytotoxicity. HeLa cells have been very important in the discovery of injection of Yop effectors inside eukaryotic cells by extracellular adhering bacteria (137, 259, 285, 321). HEp-2 cells (267, 358, 359) and HeLa cells (287) are very sensitive to the cytotoxic effect of YopE. This cytotoxic effect consists in rounding up of the cells and detachment from the extracellular matrix (119, 282). Rosqvist et al. (283) showed that the YopE-induced cytotoxicity is due to disruption of the actin microfilament structures of the target cell and that this effect is mediated by intracellularly located YopE. In addition to YopE, three other Yops, namely YopH, YopO, and YopT, have a cytotoxic effect on cultured epithelial cells (see "Yop effectors and their targets" for details).

Cytokine response. The cytokine response of epithelial cells to *Yersinia* infection has been investigated by using the HEp-2 human laryngeal epithelium cell line (13) and various human colon epithelial cell lines (181, 303). The capacity of HEp-2 cells to release cytokines is modified upon *Yersinia* infection, and although these cells do not originate from the gastrointestinal tract, this observation suggests that epithelial cells may participate in the modulation of the immune response against infection by *Yersinia* via the release of cytokines (13). In agreement with this idea, infection of monolayers of human colon epithelial cells (T84, HT29, and Caco-2) with invasive bacteria, including *Y. enterocolitica*, results in the coordinate expression and upregulation of a specific array of four proinflammatory cytokines, namely, IL-8, monocyte chemotactic protein-1, granulocyte-macrophage colony-stimulating factor, and TNF-α, as assessed by mRNA levels and cytokine secretion (181). The same cytokines, as well as IL-6, are also expressed by freshly isolated human colon epithelial cells and upregulated upon infection with invasive bacteria including *Y. enterocolitica* (181). These cytokines play a role in the initiation or amplification of the inflammatory response; IL-8 and monocyte chemotactic protein-1 act as potent chemoattractants and activators of neutrophils and monocytes, respectively; TNF-α activates neutrophils and mononuclear phagocytes, while granulocyte-macrophage colony-stimulating factor prolongs the survival of neutrophils and monocytes and increases the response of those cells to other proinflammatory stimuli, which can further amplify the inflammatory response. Colon epithelial cells thus appear to be programmed to provide a set of chemotactic and activating signals to adjacent and underlying immune and inflammatory cells in the earliest phases after microbial infection (181). Interestingly, virulent *Y. enterocolitica* strains induce a significantly lower level of IL-8 secretion by T84 cells than do nonvirulent *Y. enterocolitica* strains and the YopB and YopD proteins are required for this suppressive effect (303). It is easily conceivable that this effect favors *Yersinia*, especially during the early phase of infection, by delaying a massive influx of PMNs into the site of infection.

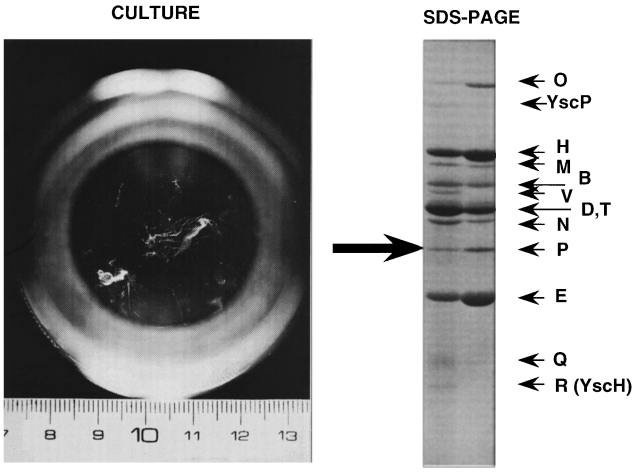

FIG. 6. Yops secreted by *Y. enterocolitica* W22703. Bacteria were grown at 28°C in a conical flask (seen from the top) containing oxalated brain heart infusion and then transferred to 37°C. The photograph showing the Yop filaments was taken 4 h after the temperature shift. SDS-PAGE of the filaments (right lane) and of Yops precipitated from the supernatant by ammonium sulfate (left lane) is shown on the right. Adapted from reference 229.

Yop SECRETION

Yop Secretion Pathway

Discovery of Yop secretion. The Yops were initially described as outer membrane proteins (44, 267, 331). Later, Heesemann et al. (149, 151) showed that Yops could also be recovered from the culture supernatant. Some of the Yops (LcrV, YopM, YopQ/YopK, and YopR) are soluble in the culture supernatant, but others (YopH, YopE, YopO/YpkA, YopB, YopD, YopP/YopJ, and YopN/LcrE) have a propensity to aggregate as visible filaments (229) (Fig. 6). This led Michiels et al. (229) to question the outer membrane localization of the Yops. These authors studied the kinetics of transcription and appearance of the Yops in the different compartments and observed the following. (i) Yops are detected first in the supernatant and later in the membrane fraction. (ii) The appearance of Yops in the membrane fraction is concomitant with the decrease of the corresponding protein in the supernatant. (iii) Disappearance of the less soluble Yops from the supernatant is not a consequence of degradation. (iv) There is a correlation between the propensity of a given Yop to aggregate in the supernatant and the presence of that Yop in the membrane fraction. (v) Yops still accumulate in the membrane fraction after 3 h of induction, whereas transcription of the *yop* genes at that time is dramatically reduced. (vi) Yops are separated from the cell fraction upon treatment with hydrophobic agents such as xylene or hexadecane, whereas chromosome-encoded integral membrane proteins and YadA are not. On the basis of these observations, Michiels et al. (229) concluded that Yops are not membrane-anchored proteins but true secreted proteins that copurify with membranes when they are prepared as centrifugation pellets. The name YOP, introduced by the group of Wolf-Watz (44) for yersinia outer membrane protein, could thus be questioned, but it is so popular that it was decided, during the Keystone 1990 meeting on *Yersinia*, to keep it but to write it Yop(s) rather than YOP(s) to indicate that it is not a set of initials. The name "*Yersinia* outer proteins" fits with the acronym but is not particularly elegant.

In vitro and in rich Ca^{2+}-deprived medium, Yops are very abundant. Michiels et al. (229) calculated that 10^7 *Y. enterocolitica* W22703 cells secrete 1 μg of Yops, which corresponds roughly to 20% of the total bacterial proteins. A peculiarity of *Y. pestis* must be mentioned here: in the supernatant of *Y. pestis* cultures, Yops are rapidly degraded by the membrane-associated Pla protease, which is encoded by a small bacteriocinogenic plasmid (318, 319). YopM, YopN/LcrE, and LcrV are relatively resistant to this proteolysis (208, 329).

No classical signal sequence is cleaved off. When Bölin and Wolf-Watz (46) and Michiels and Cornelis (226) sequenced the *yopH* gene (then known as *yop2b* and *yop51*), they noticed that the N-terminal end of the predicted YopH protein does not resemble a typical signal sequence. In 1990, Michiels et al. (229) determined the sequence of the N terminus of the secreted YopH and found the same sequence as that deduced from translation of the 5′ end of the gene, including the terminal methionine. Reisner and Straley (274) showed that the 13 N-terminal residues of YopM are also identical to those deduced from the nucleic sequence. The same observation was made later for YopN by Forsberg et al. (99): residues 2 to 9 obtained by the Edman degradation procedure were those encoded by codons 2 to 9. Håkansson et al. (136) reported the same for YopD. Finally, the 7 N-terminal residues of YpkA/YopO and the 11 N-terminal residues of YopJ/YopP have also been found to match the translated nucleic sequence (111). Hence, secretion of YopH, YopN, YopP/YopJ, YpkA/YopO and YopD occurs without removal of an N-terminal signal sequence. This presumably also applies to the other Yops. Indeed, no typical signal was found in the sequence of YopE (100, 229), YopQ (229), YopM (208), LcrV (25), YopB (136), YopR (8), or YopT (170).

The N-terminal (or 5′ mRNA) secretion signal. Analysis of the secretion of hybrid proteins composed of the N terminus of YopH or YopE and various prokaryotic or even eukaryotic proteins indicated that the information necessary for Yop secretion is nevertheless contained in the N terminus (227, 321–323). The minimal region shown to be sufficient for secretion of YopH was gradually reduced from 48 residues in a YopH-PhoA hybrid (227) to 17 residues in a YopH-Cya hybrid (320). Similarly, the minimal sequence required for secretion of YopE was reduced to 15 residues by gradual deletions of YopE-Cya hybrids (320) and later even to 11 residues, still by the same approach (300). By analysis of translational fusions to neomycin phosphotransferase (Npt), Anderson and Schneewind (10) localized the YopN secretion signal in the first 15 codons of the gene. The minimal domain of YopM sufficient for secretion of YopM-Cya was found to be shorter than 40 residues (41). For YopO/YpkA and YopP/YopJ, it is shorter than 77 and 43 residues, respectively (324).

There is no similarity between the secretion domains of the Yops with respect to amino acid sequence, hydrophobicity profile, distribution of charged residues, or prediction of secondary structure, which suggested recognition of a conformational motif of the nascent protein (227). To explain that proteins with no common signal could be recruited by the same secretion apparatus, Wattiau and Cornelis (366) suggested that the Syc chaperones (discussed below) 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 (106, 376). 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.

A systematic mutagenesis of the secretion signal by Anderson and Schneewind (10) led to doubts about this signal being of purely proteic nature. No point mutation could be identified that specifically abolished the secretion of YopE or YopN. Moreover, frameshift mutations that completely altered the

peptide sequences of the signals also failed to prevent secretion. Anderson and Schneewind (10) concluded that the signal that leads to the secretion of Yops could be in the mRNA rather than in the peptide sequence. However, some point mutations in the YopE signal do abolish Yop secretion (300).

A second secretion signal? The experiments of Sory et al. (320) demonstrated that the first 15 codons of YopE contain a signal that is sufficient to promote secretion in rich culture medium. They did not show that this N-terminal signal is absolutely required for YopE secretion. To address this question, Cheng et al. (63) deleted codons 2 to 15 and monitored secretion of the hybrid YopE-Npt. They observed that 10% of the hybrid proteins deprived of the N-terminal secretion signal were still secreted in M9 medium supplemented with 1% Casamino Acids. They inferred that there is a second secretion signal and showed that this second, weaker secretion signal corresponds to the SycE-binding site (see below). Not surprisingly, it is functional only in the presence of the SycE chaperone (63), rejuvenating the pilot hypothesis of Wattiau and Cornelis (366) for SycE. As discussed below, this second signal, binding the chaperone, is required for translocation of YopE into eukaryotic cells (204a, 320).

What has been shown for YopE might also apply to YopH, since it also has a chaperone (365) (see below). However, this should be checked, because some older observations suggest that the N-terminal signal sequence is absolutely required for secretion. Indeed, Michiels and Cornelis (227) replaced the first six codons of a truncated YopH by 12 codons of *lacZ′* and did not observe secretion.

Conclusion. There are two different signals driving the export of YopE by the type III secretion apparatus. The first is the structure of the 5′ mRNA, and the second, built into the protein, uses the chaperone as a pilot. The same could apply to the effectors YopH and YopT. Some other effector Yops do not seem to have a chaperone, in which case they would be recognized only by their N- or 5′-terminal signal. Finally, it must be stressed that we know less about secretion of the translocators. No signal sequence is removed from YopB, YopD, and LcrV, but their secretion signal has not yet been identified. Some observations tend to suggest that secretion of YopB and YopD could proceed by a mechanism slightly different from that used by the effector Yops. First, LcrV appears to be necessary for secretion of YopB and YopD (294). Second, mutations in some genes such as *virG* (7), *yscF* (8), or *yscM*/*lcrQ* (275, 327) lead to phenotypes in which YopB, YopD, and LcrV are secreted differently from the other Yops.

Ysc Secretion Apparatus

In 1991, Michiels et al. (228) established that, like the Yops, the Yop secretion apparatus is encoded by the pYV plasmid itself and in particular by genes that they called *ysc* (for "Yop secretion"). Some of these genes had previously been considered regulatory genes; this misinterpretation can be explained by the fact that there is a strong regulatory feedback that blocks Yop synthesis as soon as secretion is compromised (see "Regulation of transcription of the virulon genes," below).

The *ysc* genes are contained in four contiguous loci that were initially called *virA*, *virB*, *virG*, and *virC* (for "virulence") in *Y. enterocolitica* (73) (Fig. 3). *lcrD* (for "low calcium response"), initially described in *Y. pestis* (263), turned out later to be one of these secretion genes (265), and it will probably be called *yscV* in the future. In total, 28 genes have been identified within these loci. Knockout mutants have been constructed for most but not all of them. The information available on these genes and their products is detailed in the next paragraphs and summarized in Table 1. For the sake of clarity, the four loci are treated separately.

YscC secretin and other products of the *virC* operon. The *virC* locus of *Y. enterocolitica* consists of a large operon, *yscAB CDEFGHIJKLM*, encoding 13 proteins (228, 327). Parts of the *virC* locus have also been analyzed in *Y. pestis* (134) and *Y. pseudotuberculosis* (275), where the counterparts of *yscH*, *yscI*, *yscJ*, *yscK*, *yscL*, and *yscM* have been initially called *lcrP*, *lcrO*, *lcrKa*, *lcrKb*, *lcrKc*, and *lcrQ*, respectively. Apart from *yscM1*, which is called *lcrQ* in *Y. pseudotuberculosis* (275), the *ysc* nomenclature has now been adopted in the three species. Nonpolar mutations in *yscC*, *yscD*, *yscE*, *yscF*, *yscG*, *yscI*, *yscJ*, *yscK*, and *yscL* completely abolish Yop secretion (8, 265). In contrast, nonpolar *yscA*, *yscH*, and *yscM* mutants are not impaired in Yop secretion (8, 296).

yscC encodes an outer membrane protein (194, 228, 265) that 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 (113, 212b, 292). All the secretins have a conserved domain in the C terminus, whereas the N-terminal domains are conserved only between proteins of related systems (113). Several members of this family (61, 142, 212a, 242, 307), including YscC (265), form large multimers. Koster et al. (194) showed that the 600-kDa very stable YscC complex forms a ring-shaped structure with an external diameter of about 200 Å and an apparent central pore of about 50 Å. As a matter of comparison, the PIV secretin of phage f1 has an internal diameter of about 80 Å, allowing the passage of the filamentous capsid with a diameter of 65 Å (212b). Lipoprotein VirG (7), described below, is required for efficient targeting of the YscC complex to the outer membrane (194), a situation reminiscent of that of secretin PulD and lipoprotein PulS (143).

Relatively little is known about the other proteins of the *virC* operon that are required for secretion. YscB is a 15.4-kDa protein which has neither a putative signal sequence nor a hydrophobic domain (228). YscD is an inner membrane protein (265). Complete inactivation of *yscF* abolishes Yop secretion. However, truncation of YscF reduces the secretion only of YopB and YopD and not that of the other Yops, suggesting that YopB and YopD are secreted via a slightly different mechanism (see the previous section) or that secretion of YopB and YopD is more sensitive to small alterations in the secretion machinery (8). YscJ is a 27.0-kDa lipoprotein (228). YscL has no obvious membrane-spanning domain, but it could be membrane associated (228).

yscH encodes the 18.3-kDa secreted protein that was called YopR (8). YopR is not required for secretion of the other Yops, but it could be involved in pathogenesis, since the 50% lethal dose of the *yscH* mutant was 10-fold higher than that of the wild-type strain (8).

Finally, *yscM*, the last gene of the *virC* operon, is not required for Yop secretion but is involved in the feedback inhibition of Yop synthesis (8, 327) (see below).

The order of the 13 genes is the same in the three *Yersinia* species (134, 228, 275), but there could be minor differences in their transcriptional organization. In *Y. enterocolitica*, the *virC* locus consists of only one large operon extending from *yscA* to *yscM* (8, 228, 327), while primer extension analysis suggested the existence of a Ca^{2+}-regulated promoter within *yscF* in *Y. pestis* (134). *lcrQ* in *Y. pseudotuberculosis* has been reported to be monocistronic (275).

Several proteins encoded by the *virC* operon have sequence homology to components of other type III secretion systems and also to proteins involved in the assembly of flagella (Table 2). Homologs to all the proteins encoded by the *virC* operon,

TABLE 1. Ysc secretion apparatus

Protein	Size (kDa)	Features[a]	Localization in bacteria[a]	Role in Yop secretion[a]	Reference(s)
YscA	3.8	Hydrophobic C-terminal domain	Unknown	−	228, 296
YscB	15.4		Unknown	NT	228
YscC	67.1	Signal sequence of 26 residues; member of the secretin family; forms pores of 200 Å with a central channel of 50 Å	OM	+	194, 228
YscD	46.7	Hydrophobic domain (aa 120–130)	IM	+	228, 265
YscE	7.4	Hydrophobic C-terminal domain	Unknown	+	8, 228
YscF	9.4		Unknown	+	8, 228
YscG	12.9	Hydrophobic N-terminal domain	C/M	+	8, 228, 265
YopR	18.3	Encoded by *yscH*	Secreted	−	8, 228
YscI	12.6		Unknown	+	8, 228
YscJ	27.0	Lipoprotein; hydrophobic C-terminal domain followed by 3 positively charged residues; previously called YlpB	Unknown	+	8, 64, 228
YscK	23.9	One hydrophobic domain	Unknown	+	8, 228
YscL	24.9		Unknown	+	228, 326
YscM1/LcrQ	12.3	Resembles YscM2 and YopH	Secreted	−	8, 228, 327
YscN	47.8	Contains Walker box A and B; putative ATPase	IM/C	+	24, 93, 375
YscO	19.0		Secreted	+	24, 93, 252
YscP	50.4		Secreted	+	24, 93, 252a, 326a
YscQ	34.4		Unknown	+	24, 93
YscR	24.4	Four transmembrane domains and a large central cytoplasmic region	IM	+	24, 93
YscS	9.6	Two putative transmembrane domains	Unknown (probably IM)	+	24, 93, 261
YscT	28.4	Six putative transmembrane domains	Unknown (probably IM)	NT	24, 93
YscU	40.3	Four transmembrane domains at the N terminus with a large cytoplasmic C-terminal region	IM	+	9, 24, 93
YscX	13.6		Unknown	+	170a
YscY	13.1		Unknown	+	170a
LcrD/YscV	77.8	Eight potential transmembrane domains; hydrophobic N-terminal half; hydrophilic C terminus predicted to protrude into the cytoplasm	IM	+	263
VirG/YscW	14.6	Lipoprotein ancillary to YscC secretin	Probably OM	+ (YopB, YopD, LcrV)	7

[a] IM, inner membrane; OM, outer membrane; C, cytosolic; M, membrane-associated protein; NT, not tested; +, required for secretion; −, not required for secretion; aa, amino acids.

except YscA and YscM/LcrQ, have been identified in *P. aeruginosa* (for a review, see reference 102). YscC and YscJ have counterparts in the *Shigella*, *Salmonella*, and EPEC type III secretion systems. Homologs to YscF have been identified in *Shigella* and *Salmonella* spp. The identity between YscJ and YscF and the corresponding genes in the *Shigella* system, MxiJ and MxiH, is 26 and 24%, respectively, but the genes from the two species are not functionally interchangeable (8). For a more complete review of type III secretion homologs, see references 4, 201, and 352.

VirG/YscW lipoprotein. *virG* is a small, monocistronic gene situated immediately upstream from the *virC* operon and downstream from the regulatory gene *virF* (7) (Fig. 3). It encodes a polypeptide of 131 amino acids with a predicted molecular mass of 14.7 kDa and a calculated isoelectric point of 11.1. The signal sequence of VirG ends with Leu-Xaa-Gly-Cys, a motif characteristic of the processing site of lipoproteins. While attempting to show that VirG is a lipoprotein that can be labelled by [^{3}H]palmitate, Allaoui et al. (7) encountered the difficulty that gram-negative bacteria produce several lipoproteins in the range of 10 to 30 kDa. To circumvent this, they labeled three strains containing different *virG-phoA* gene fusions and detected the larger VirG-PhoA hybrid proteins among the proteins labelled with [^{3}H]palmitate. VirG is thus a small lipoprotein. Allaoui et al. (7) constructed a nonpolar *virG* mutant and observed that secretion of some Yops, in particular YopB, YopD and LcrV, was severely impaired. The function of VirG became more clear when the YscC secretin was characterized by Koster et al. (194). It appeared that VirG is required for proper insertion of YscC in the outer membrane, but more work is needed for an understanding of its exact function (194). The correlation between the role of VirG in the installation of the secretin and the requirement of VirG for secretion of YopB, YopD, and LcrV suggests that these Yops could be the most bulky ones to be transported through the YscC channel. Since lipoprotein VirG belongs to the Ysc secretion apparatus, we suggest that it be renamed YscW.

VirG/YscW shows extensive similarity (26.2% identity in a 126-amino-acid overlap) to ExsB, a 137-amino-acid putative polypeptide from *P. aeruginosa*, encoded by a *trans*-regulatory locus controlling exoenzyme S synthesis (ExoS) (103). However, *exsB* does not seem to be expressed in *P. aeruginosa* (122a).

Products of the *virB* operon. The *virB* operon consists of eight genes, *yscN* to *yscU* (24, 93). Among the proteins encoded by these genes, YscN, YscR, and YscU are the best characterized so far. YscN is a 47.8-kDa protein with ATP-binding motifs (Walker boxes A and B) resembling the β catalytic subunit of F_0F_1 proton translocase and related ATPases. A pYV derivative encoding an YscN protein deprived of Walker

TABLE 2. Homologs to the Ysc proteins

Yersinia protein	Homolog in:								
	Shigella spp.[a]	*Salmonella* spp.		EPEC[d]	*Pseudomonas aeruginosa*[e]	*Chlamydia psittaci*[f]	Phytopathogenic bacteria (HR reaction)[g]	*Rhizobium* spp.[h]	Flagella[i]
		SPI I[b]	SPI II[c]						
YscA									
YscB					PscB				
YscC	MxiD	InvG		EscC	PscC		HrcC		
YscD				EscD	PscD		HrpQ*		
YscE					PscE				
YscF	MxiH	PrgI	SsaH	EscF	PscF				
YscG					PscG				
YscH					PscH				
YscI					PscI				
YscJ	MxiJ	PrgK	SsaJ	EscJ	PscJ		HrcJ	NolT	FliF
YscK					PscK				
YscL			SsaK		PscL		HrpF**		
YscM									
YscN	Spa47/SpaL	SpaL/InvC	SsaN	EscN	PscN		HrcN	HrcN	FliI
YscO	Spa13	SpaM/InvI	SsaO		PscO				FliJ
YscP	Spa32	SpaN/InvJ							
YscQ	Spa33/SpaO	SpaO/InvK	SsaQ				HrcQ	HrcQ	FliN/Y
YscR	Spa24/SpaP	SpaP/InvL	SsaR	EscR			HrcR	HrcR	FliP
YscS	Spa9/SpaQ	SpaQ/InvM	SsaS	EscS			HrcS	HrcS	FliQ
YscT	Spa29/SpaR	SpaR/InvN	SsaT	EscT			HrcT	HrcT	FliR
YscU	Spa40/SpaS	SpaS	SsaU	EscU		Cds1	HrcU	HrcU	FlhB
YscX					Pcr3				
YscY					Pcr4				
LcrD/YscV	MxiA	InvA	SsaV	EscV		Cds2	HrcV/HrpI		FlhA/FlbF
VirG/YscW					ExsB				

[a] References 5, 6, 12, 299, and 357.
[b] SPI, *Salmonella* pathogenicity island. References 68, 69, 83, 108, 116, 130, 182, 253, and 389.
[c] SPI, *Salmonella* pathogenicity island. References 154, 306, and 348.
[d] References 178 and 201.
[e] References 201, 378, and 379.
[f] Reference 165.
[g] *Erwinia amylovora*, *Ralstonia solanacearum*, *Pseudomonas syringae*, and *Xanthomonas campestris*. References 4, 37, 91, 92, 124, 166–168, 212, 352, 353, and 370. *, only in *E. amylovora*; **, only in *P. solanacearum*.
[h] Reference 104. Accession no. L12251 (222).
[i] *Bacillus*, *Salmonella*, *Caulobacter crescentus*, *Campylobacter jejuni*, and *Helicobacter pylori*. References 3, 29, 30, 56, 57, 192, 201, 218, 219, 231, 233, 272, 293, 302, and 363.

box A is impaired in Yop secretion, indicating that YscN is a necessary component of the secretion machinery and possibly acts as an energizer (375). It has not been shown that YscN is an ATPase, but this was shown for InvC, the YscN homolog in *S. typhimurium* (83). It is thus reasonable to assume that YscN acts as an ATPase. YscR is a 24.4-kDa inner membrane protein with four transmembrane regions and a large central hydrophilic domain, as suggested by the analysis of *yscR-phoA* translational gene fusions (93). The 40.3-kDa YscU is a second inner membrane protein with four transmembrane segments anchoring a large cytoplasmic C-terminal domain (9). Not surprisingly, mutations in *yscR* and *yscU* abolish Yop secretion (9, 93). Interestingly, the products of *yscO* (251) and *yscP* (252a, 326a) are secreted by the Ysc apparatus under low-Ca^{2+} conditions. YscO (251) is required for secretion of all the Yops, while YscP (252a) is required for normal secretion of some Yops. Less is known about YscQ (93) and YscS (261), but they have been shown to be required for Yop secretion. Finally, the importance of YscT has not been determined.

The *virB* locus as a whole is remarkably well conserved in other type III secretion systems such as those of *Shigella* spp., *Salmonella* spp., EPEC, *P. aeruginosa*, *Chlamydia psittaci*, *Rhizobium* spp., and phytopathogenic bacteria (Table 2). The degree of identity varies between 20 and 50% for each individual protein, but every gene is found at the same relative position. There is also a significant homology between the products of the *virB* operon and proteins implicated in the building of the flagellum in various species (Table 2). The *virB* locus is thus the most highly conserved part of the type III secretion machinery.

***virA* locus: *yopN*, *tyeA*, *sycN*, *yscXY*, *lcrD*/*yscV*, and *lcrR*.** The *virA* locus encodes first YopN, TyeA, and SycN, which are described later in this review. The next two genes, *yscX* and *yscY*, encode small proteins that are required for Yop secretion (170a). The next gene, *lcrD*/*yscV*, encodes a 77-kDa inner membrane protein that is required for Yop secretion (263, 264). LcrD/YscV is the archetype of a family of proteins encountered in every known type III system (Table 2). The predicted overall secondary structure of these proteins is quite well conserved and consists of a hydrophobic N terminus with six to eight potential transmembrane domains and a hydrophilic C terminus protruding into the cytoplasm. All the members of this family can be aligned over the entire length of the amino acid sequence, with the highest degree of homology occurring in the N terminus (108, 263, 264). At least some of the members have interchangeable functions. For instance, MxiA from *Shigella* is able to complement the eukaryotic cell entry defect of a *Salmonella invA* mutant, but LcrD/YscV from *Yersinia* cannot. However, a chimeric protein consisting of the N-terminal part of LcrD/YscV and the C-terminal end of InvA can replace InvA, suggesting that the C-terminal end of these

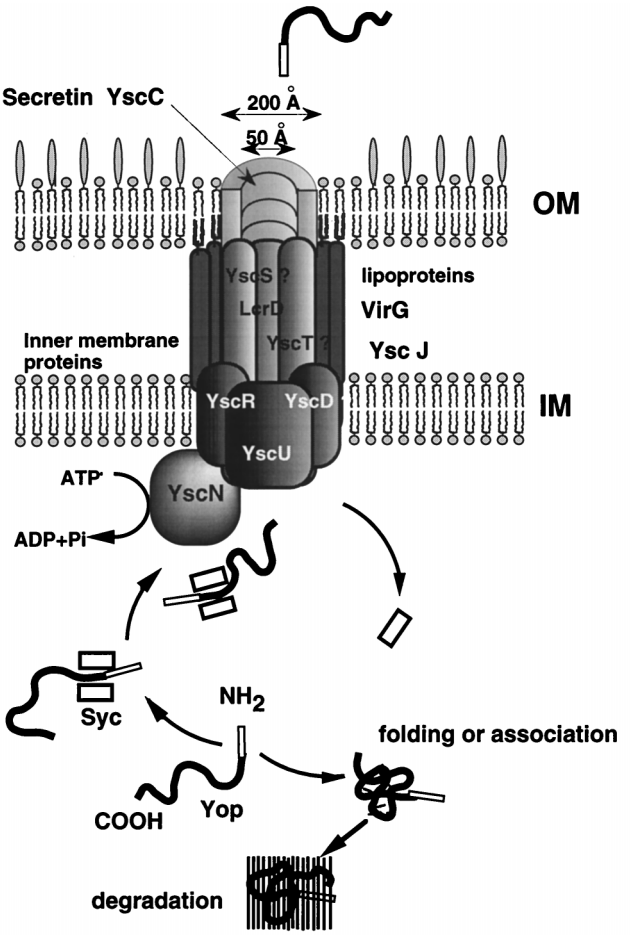

FIG. 7. Model for Yop secretion. OM, outer membrane; IM, inner membrane.

proteins may determine the specificity for each of the secretory systems (115).

lcrD forms an operon with *lcrR*. The latter gene encodes a hydrophilic, basic protein of 16.4 kDa whose function remains unknown (22).

Conclusion. Secretion of the Yop proteins requires a complex secretion machinery made of at least 28 proteins. These proteins, especially those encoded by the *virB* operon, are quite well conserved in the type III secretion system of plant and animal pathogens. Four proteins, LcrD, YscD/YscV, YscR, and YscU, span the inner membrane. The YscC secretin forms a large pore in the outer membrane, presumably stabilized by lipoprotein VirG. The YscN ATPase energizes the transfer of Yops, but the proton motive force could also be involved (Fig. 7).

Syc Cytosolic Chaperones, SycE, SycH, SycT, SycN, and SycD

Discovery of the *Yersinia* chaperones. Genetic analysis of the *yopE* region of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* revealed the existence of a conserved 620-bp region upstream of the *yopE* gene (101). This region contains a 130-codon open reading frame encoding a 14.7-kDa protein, transcribed divergently from the *yopE* gene. A mutant with an insertion in this region showed a reduced intracellular level of YopE protein as compared to the wild type (101), which suggested that this locus plays a regulatory role and inspired the name *yerA*, for "*yopE*-regulating gene A" (101). In 1993, Wattiau and Cornelis (366) observed that the counterpart of YerA in *Y. enterocolitica* is required for YopE secretion but not for its synthesis and that it binds specifically to YopE. Hence, they concluded that it is a chaperone specific for YopE, and they called it SycE (for "specific Yop chaperone E").

The discovery of SycE prompted a search for other chaperones (365). A *Y. enterocolitica* gene located immediately downstream of *yopH* and transcribed divergently encodes a putative protein of 141 residues with a calculated molecular mass of 15.7 kDa, an acidic pI (4.88), and no classical N-terminal signal sequence. Its sequence does not resemble that of SycE, but its predicted physicochemical properties (pI, hydrophobicity, and hydrophobic moment) and secondary structures (α-helices) strikingly evoke those of SycE/YerA. This made it a likely candidate to be the YopH chaperone, and it was called SycH. In agreement with this hypothesis, SycH turned out to be necessary for the secretion of YopH and to bind specifically to YopH (365).

SycT (170), the chaperone of YopT, has 69.7% similarity to SycE/YerA and 23% similarity to SycH. It has the same properties as the other chaperones (pI of 4.40, molecular mass of 15.1 kDa), it is necessary for efficient secretion of the YopT protein (see below), and it binds to YopT (170).

SycN, encoded next to YopN and TyeA, appears to be a chaperone of the same family, dedicated to YopN (170a).

The SycD protein is encoded by the *lcrGV sycD yopBD* operon (25, 270). It was first described as a regulatory gene (271), which explains its designation, LcrH (for "low-calcium-response gene H") in *Y. pestis* and *Y. pseudotuberculosis*. Indeed, an *lcrH*/*sycD* mutant of *Y. pestis* showed only limited growth at 37°C when Ca^{2+} was present (271), while the parental *Y. pestis* strain showed full growth in these conditions (271). Moreover, Price and Straley (271) showed that LcrH/SycD is required for normal Yop expression. Bergman et al. (25) also assigned a negative control function to LcrH/SycD because they observed that overproduction of LcrH/SycD in polar insertion *lcrV* and *lcrH*/*sycD* mutants resulted in the loss of Ca^{2+} dependency and in a downregulation of the expression of the *lcrGV sycD yopBD* operon and of *yopE*. However, on the basis of its gene location, size, and pI, Wattiau et al. (365) considered that SycD/LcrH could be a specific chaperone serving YopB and/or YopD. They constructed a nonpolar *sycD*/*lcrH* mutant, and from its analysis, they concluded that SycD is required for YopB and YopD secretion (365). It was also shown that SycD binds to YopD (365) and to YopB (243), thus acting as a bivalent chaperone. In the absence of SycD, YopD and YopB are less detectable inside the bacterial cell (243, 365). SycD thus appears to be a chaperone, which does not exclude the possibility that it also plays a regulatory role but that this regulatory role is an indirect consequence of its role in YopB and YopD secretion.

Finally, a gene upstream from *yopO*/*ypkA* encodes a putative protein (called ORF 155) that has clear characteristics of a Syc chaperone (171). It is orphan up till now.

In summary, six chaperones have been identified so far. In *Y. enterocolitica*, their genes are located close to the gene encoding the corresponding Yop. In *Y. pseudotuberculosis*, however, the *sycH* gene has been separated from *yopH* by some genetic rearrangement (259) (Fig. 3). No chaperone has been described so far for YopM, YopO/YpkA, YopP/YopJ, YopQ/YopK, YopR, and LcrV. Although putative chaperone ORF 155 is still orphan, it seems reasonable to believe that not every Yop has a chaperone.

TABLE 3. Syc cytosolic chaperones

Protein	Size		pI	C-terminal domain	Role	Relevant similarities	Reference(s)
	aa[a]	kDa					
SycD/LcrH	168	19.0	4.53	Amphipathic α-helix	Needed for secretion of YopB and YopD; no specific binding domain	IpgC (*Shigella*), SicA (*Salmonella*), PcrH (*P. aeruginosa*)	183, 225, 243, 365, 379
SycE/YerA	130	14.7	4.55	Amphipathic α-helix, Leu repeat	Needed for secretion of YopE; binds to aa 15–50; pilot for YopE	SycT, ORF1 (*P. aeruginosa*), Scc1 (*C. psittaci*)	63, 106, 165, 170, 366, 376
SycH	141	15.7	4.88	Amphipathic α-helix, Leu repeat	Needed for secretion of YopH; binds to aa 20–70	OrfU (EPEC)	179, 365
SycT	130	15.1	4.40	Amphipathic α-helix	Needed for secretion of YopT	SycE	170
SycN	123	13.6	5.2	Amphipathic α-helix	Needed for secretion of YopN	Pcr2 (*P. aeruginosa*)	170a
ORF155	155	17.2	4.5	Amphipathic α-helix	Unknown	ORF1 (*Y. pseudotuberculosis*), ORF1 (*P. syringae*)	4a, 171

[a] aa, amino acids.

Common properties of the Syc chaperones. Although the Syc chaperones seem to play a common role in protein secretion, they are distantly or even not related in terms of amino acid sequence. However, they have some common features: an acidic pI, a size in the range of 15 to 19 kDa, and a C-terminal amphiphilic α-helix (Table 3). 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. (368) from the alignment of this conserved leucine repeat of SycE and SycH and their homologs (LLWxRxPLxxxxxxxLxxxLExLVxxAExL) (Table 3) (see below). The C-terminal potential amphiphilic α-helix of SycD, however, does not include a leucine repeat.

Role of SycE and SycH. The three chaperones SycE/YerA, SycH, and SycD/LcrH were first thought to constitute a single family of new protein chaperones (365), but this hypothesis appears questionable today. SycE and SycH appear to play related roles, and they could belong to the same new family, but SycD/LcrH could be quite different. Therefore, they are discussed separately. However, the role of SycT is not discussed, since it has not yet been completely analyzed.

The Syc chaperones were first thought to be necessary mainly for the secretion of their cognate Yop, and Wattiau and Cornelis hypothesized that they could act as some kind of secretion pilots to drive nascent Yops to the secretion machinery (365, 366). This hypothesis was first questioned by Frithz-Lindsten et al. (106), who showed by confocal microscopy that when a double *yerA*/*sycE yopD* mutant strain infects HeLa cells, YopE localizes at discrete spots at the zone of contact between the bacterium and the HeLa cell, as it does in *yopD* mutants (285). This showed that YerA/SycE is not required for the targeting of YopE to these translocation sites. Moreover, residual secretion of YopE or YopH was observed in the absence of SycE/YerA or SycH, respectively (365, 366). However, these observations were made in the presence of the other chaperone, and one could hypothesize that this second chaperone could partially take over from the missing one. Woestyn et al. (376) then mapped the regions of YopE and YopH that bind to the cognate chaperone (see below) and showed that hybrid YopE-Cya or YopH-Cya proteins devoid of the SycE or SycH chaperone-binding site are normally secreted in the presence and in the absence of both chaperones. Similarly, YopH devoid of its chaperone-binding site is normally secreted in the presence and in the absence of SycH. This suggested that SycE/YerA and SycH are probably not targeting factors. However, at that time, no experiments were done with YopE and YopH deprived of their N-terminal secretion signal. Later, Cheng et al. (63) deleted the N-terminal domain from YopE and showed that YopE could still be secreted but only if SycE was present. As we have seen before, this indicated that SycE/YerA can act as a kind of secretion pilot, as initially suggested by Wattiau and Cornelis (366). This is probably not the only role for the Syc chaperones.

YopH and YopE have a discrete domain (residues 15 to 50 for YopE and residues 20 to 70 for YopH) that is specifically required for their translocation into eukaryotic cells (320) (see "Translocation signal on Yop effectors"). Woestyn et al. (376) showed that the Syc-binding domain is unique for both chaperones and that the very same region of YopE and YopH is required for translocation. In addition, they showed that in a *sycH* mutant, YopH secretion is more efficient in the absence of YopB and YopD than in their presence (376). This result suggests that SycH could prevent the association of YopH with YopB and/or YopD, but this hypothesis still awaits a confirmation.

SycE/YerA also plays an antidegradation role, since the half-life of YopE is longer in wild-type bacteria than in *sycE*/*yerA* mutant bacteria (63, 106). SycH does not play such a clear antidegradation role, since YopH can be detected in the cytosol of *sycH* mutant bacteria (365).

Finally, SycE/YerA and SycH could also play an antifolding role, to maintain the Yop proteins in a conformation which is adequate for secretion.

In summary, it appears that the SycE and SycH chaperones fulfill several roles concerning YopE and YopH stability, secretion, and possibly conformation and interaction between translocators and effectors. One could imagine that the chaperone first acts as a secretion pilot, leading the Yop protein to the secretion locus and simultaneously preventing premature association with the translocators. By binding the Yop, the Syc chaperone could ensure the stability and proper conformation of the protein. At the moment of secretion, the chaperone is released from the partner Yop and the translocation domain would be free to interact with the translocation machinery, which then leads the Yop to the host cell cytoplasm.

Role of SycD/LcrH. As mentioned above, it was first thought that SycD/LcrH was a regulatory protein, involved in Ca^{2+} responsiveness (25, 271). Now it appears that SycD is a chaperone for the YopD and YopB proteins and that its presumed regulatory role is probably indirect. However, this chaperone is somewhat different from SycE and SycH in terms of its role and the way it behaves. First, it serves two Yops rather than one; second, it serves translocators whereas SycE and SycH

serve effector Yops. Third, SycD binds to several domains on YopB (243) whereas SycE and SycH bind their cognate Yop at a unique site. The last property is similar to that of SecB, a molecular chaperone in *E. coli* which is dedicated to the export of newly synthesized proteins (196) and which also has multiple binding sites on these proteins, such as the maltose-binding protein (191).

YopB and YopD are less detectable inside *Yersinia* in the absence of SycD than in its presence (243, 365), while YopE and YopH can still be detected in the bacterial cytosol in the absence of their cognate chaperone. This suggests that in the absence of their chaperone, the hydrophobic YopB and YopD proteins could be toxic for the bacterium and are thus probably degraded by a bacterial "housekeeping" protease. This hypothesis is supported by the work of Neyt and Cornelis (243), who showed that overexpression of YopB in *E. coli* leads to cell lysis and death whereas overexpression of YopB in the presence of the SycD chaperone does not. This protective role of SycD is unprecedented for Syc chaperones, but it is not necessarily the only role of SycD/LcrH. SycD resembles the IpgC chaperone from *Shigella flexneri*, which has been shown to prevent the association between its cognate Ipas, namely, IpaB and IpaC (225). The similarity (51%) between IpgC and SycD/LcrH suggests that SycD could play a similar role and would thus prevent the intrabacterial association of YopB and YopD. Neyt and Cornelis (243) investigated this hypothesis by testing the ability of YopB and YopD to associate in the presence of SycD. Surprisingly, they observed that YopB and YopD, bound to SycD, can still associate. In agreement with this, they observed that YopB and YopD are already associated in the bacterial cell in the presence of SycD (243).

Homologs of the Syc chaperones in other species. As mentioned above, SycD/LcrH is related to the *Shigella* chaperone IpgC (previously called IppI) (298), which binds IpaB and IpaC independently, preventing the formation of a complex between the two Ipa proteins in the bacterial cytoplasm (225). Although SycD/LcrH is 51.0% similar and 26.5% identical to IpgC, we have seen that their roles could be different. IpgC is 57% similar to SicA (*Salmonella* invasion chaperone A) (183). SycD/LcrH also has a homolog in the *P. aeruginosa* Psp system (PcrH, 75% similarity) (379) and in the EPEC Esp system (CesD, 67% similarity) (363a).

The SycE/YerA chaperone is homologous to open reading frame 1 (ORF1) from *P. aeruginosa* (44% identity). ORF1 is adjacent to the gene encoding the ADP-ribosyltransferase ExoS (see below) (105), and its importance is controversial. Yahr et al. (378) showed that the secretion of ExoS expressed from a plasmid in *P. aeruginosa* PA103 does not require ORF1. However, when the *exoS* gene is expressed in *Yersinia* (see below), the absence of an intact ORF1 results in a 10-fold reduction in the secretion of ExoS (105), which suggests that ORF1 serves a similar function in the secretion of ExoS to that of SycE for YopE. SycE/YerA also has a homolog in *Chlamydia psittaci*, which is called Scc1 (54% similarity) (165).

The SycH chaperone has a homolog called OrfU in EPEC (179). The similarity is rather weak (19.8% identity), but the C-terminal amphipathic helix seems to be conserved. This protein is encoded by a gene adjacent to *eaeA*, which encodes intimin. It is unlikely that this putative chaperone serves intimin, because intimin is exported by the Sec pathway (121), but we would like to hypothesize that it serves the protein Tir, which is encoded by a gene located upstream of *orfU*. Transfer of Tir to the host cell is dependent on the type III secretion apparatus (189).

Conclusion. In conclusion, the Syc chaperones probably constitute two different families. SycE/YerA, SycH, and presumably SycT appear to be both secretion/translocation pilots and anti-association factors, while SycD/LcrH is more likely to play a protective role covering the YopB-YopD cytoplasmic association until secretion. Concerning the antidegradation role of the Syc chaperones, it is unclear whether the Yops are degraded in the absence of their chaperone because they are not secreted or because the chaperone plays a direct antidegradation role.

DELIVERY OF EFFECTOR Yops INTO EUKARYOTIC CELLS

Translocation across the Eukaryotic Cell Plasma Membrane

Identification of intracellular effectors. As discussed above, translocation of YopE across the eukaryotic cell membrane was demonstrated in 1994 by two different approaches: confocal microscopy (285) and the Yop-Cya reporter enzyme strategy (321). The same methods were applied to demonstrate translocation of YopH and YopM across the plasma membrane of epithelial cells and macrophages (41, 259, 320).

Delivery into eukaryotic cells of less abundant Yop proteins such as YopO/YpkA, YopP/YopJ, and YopT turned out to be more difficult to monitor. Håkansson et al. (137) constructed a mutant of *Y. pseudotuberculosis* that was unable to produce the more abundant Yop effectors (YopE, YopH, and YopM) as well as YopK (see below), and this strain allowed the visualization of translocation of YpkA/YopO into HeLa cells by confocal microscopy (88, 137, 161). A similar multiple-*yop* mutant of *Y. enterocolitica* was constructed (39), and this mutant allowed the demonstration of the delivery of YopO-Cya, YopP-Cya, and YopT-Cya into macrophages (170, 172, 324), by a system dependent on both YopD and YopB.

Translocation requires adherence of living bacteria to their target. Only *Y. pseudotuberculosis* producing Inv can deliver Yops into HeLa cells (282). However, the presence of Inv is not an absolute requirement, since centrifuged bacteria producing YadA but not Inv still translocate YopH through the plasma membrane of HeLa cells (259). Surprisingly, in vivo, the Inv protein is not required for the full virulence of *Y. pseudotuberculosis*.

In *Y. enterocolitica*, the YadA adhesin is more important than Inv for the delivery of YopE into HeLa cells (321), but adherence is still a requirement for efficient injection of Yops. A double *yadA inv* mutant is indeed devoid of any cytotoxicity (the most sensitive indicator of translocation) in epithelial cells (47). However, either YadA or Inv will suffice to initiate the contact between *Y. enterocolitica* and epithelial cells to allow subsequent translocation.

Goguen et al. had already noticed in 1986 (119) that only live *Y. pestis* bacteria could induce a cytotoxic effect in macrophages. Moreover, *Y. pestis*, which is unable to adhere to HeLa cells, was also inefficient in inducing cytotoxicity in this cell type. However, introduction of a gene encoding either the invasin (Inv)—a large outer membrane protein binding β_1-integrins (206, 308)—or YadA conferred on *Y. pestis* the ability to induce cytotoxicity in HeLa cells (282). Thus, like *Y. pseudotuberculosis* and *Y. enterocolitica*, *Y. pestis* needs to adhere before it can deliver its effector Yops. However, the main element needed for this adherence has not been identified.

The pH 6 antigen of *Y. pestis* (213, 214), the pH 6 antigen of *Y. pseudotuberculosis* (381) and its counterpart, the Myf fibrillae of *Y. enterocolitica* (175), could promote adherence (381). However, synthesis of the pH 6 antigen requires an acidic environment, and the lack of the pH 6 antigen causes only a relatively small decrease in virulence of *Y. pestis* in a systemic

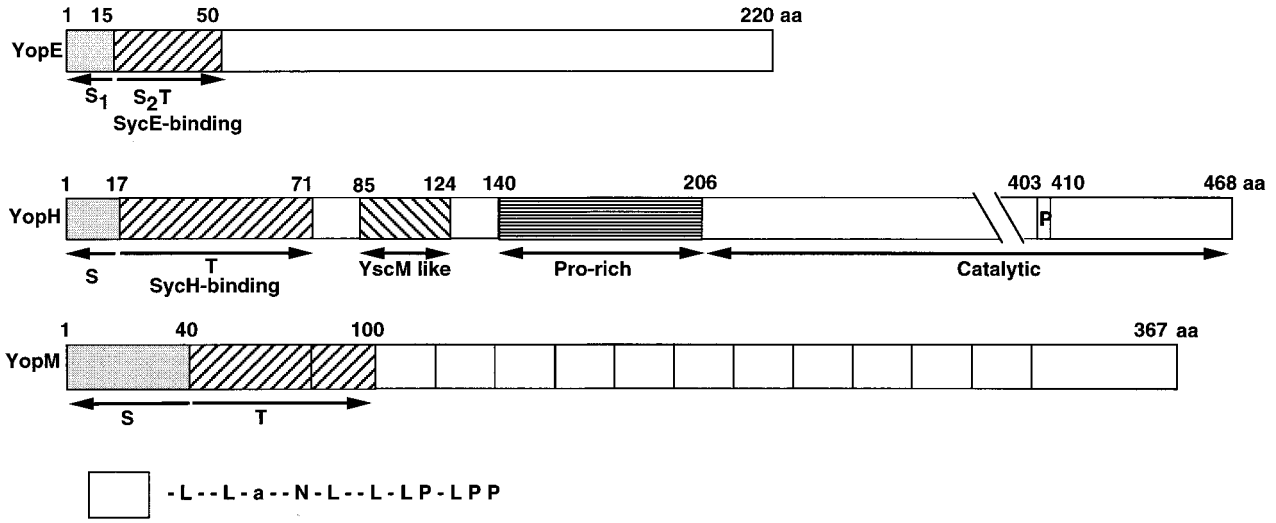

FIG. 8. Schematic representation of YopE, YopH, and YopM. S_1, first secretion domain; S_2/T, second secretion domain and translocation domain. The catalytic domain of YopH includes the P-loop (P). The LRR motifs (193) in YopM are represented by open boxes, and their composition is given below.

challenge, in contrast to a *yopE* or *yopH* mutation. In this context, it is quite surprising that *Y. pestis* produces neither Inv nor YadA (286, 310).

Translocation is the feat of extracellular bacteria. Rosqvist et al. (282) demonstrated that selective killing of extracellular bacteria by gentamicin inhibits cytotoxicity. Killing of extracellular bacteria also prevents translocation of YopH across the plasma membrane of eukaryotic cells, indicating that translocation occurs not from intracellular bacteria but, rather, from extracellular bacteria (259). In agreement with this, Sory and Cornelis (321) showed that the use of cytochalasin D, which inhibits the entry of bacteria into cells, does not greatly affect the amount of YopE that is internalized. Moreover, the same authors showed that *Y. enterocolitica* that is unable to synthesize Inv and thus is unable to invade HeLa cells delivers YopE-Cya almost as efficiently as wild-type bacteria do (321). It thus appears that intracellular bacteria do not translocate Yops across the endosomal membrane, which is in good agreement with the observation that intracellular bacteria produce only small amounts of Yops (282).

Influence of de novo protein synthesis on translocation. It is difficult to clearly report on the role of de novo protein synthesis in translocation, because the experimental conditions used to address this question were highly variable. Protein synthesis was inhibited with chloramphenicol or gentamicin, and translocation was monitored by measuring cytotoxicity, phagocytosis, or even the oxidative burst. As described above, the addition of gentamicin during incubation of eukaryotic cells with *Y. pseudotuberculosis* impaired the YopE-mediated cytotoxicity (282) as well as the inhibition of phagocytosis caused by YopH (87). However, some of the YopH-mediated antiphagocytic activity could still be observed when bacteria were preincubated under conditions favorable for Yop production (37°C in the absence of Ca^{2+}) (87). Also, when *Y. pestis* or *Y. pseudotuberculosis* was preincubated at 37°C in the presence of Ca^{2+} (nonpermissive for Yop secretion) before their contact with macrophages, cytotoxicity or an inhibition of the oxidative burst occurred even in the presence of chloramphenicol (33, 119). In contrast, no cytotoxicity was observed in the presence of chloramphenicol when bacteria were preincubated at 28°C. This suggests that bacteria grown at 37°C are inherently able to deliver Yops inside eukaryotic cells whereas bacteria grown at 28°C require some incubation under conditions that permit Yop protein synthesis. The secretion apparatus and the elements of the translocation apparatus to be deployed are probably present after incubation at 37°C, in any medium, and some Yops await translocation. A signal then settles the secretion and translocation systems, and the delivery of the Yops is amplified by a specific enhancement of Yop synthesis (see "Regulation of transcription of the virulon genes," below). Depending on the conditions used to incubate the bacteria and to the phenomenon recorded (inhibition of phagocytosis, cytotoxicity, etc.), a variable lag phase will thus be observed before the effect on eukaryotic cells is recorded.

Directionality of secretion. When Yop secretion is triggered by eukaryotic cells, it is "polarized" in the sense that the majority of the Yop effector molecules produced are directed into the cytosol of the eukaryotic cell, not to the outside environment (285). The term "polarization" is perhaps confusing, because it may suggest that it occurs at the pole of the bacterium, which has never been shown. To avoid this, one might say that Yop translocation is "oriented" or "directional." Whatever the term, there is some discrepancy in the degree of "directionality." Using various Cya reporters, Boland et al. (41) observed that roughly only half of the amount of Yops secreted by *Y. enterocolitica* is directed into cultured macrophages. In contrast, Persson et al. (259) reported that *Y. pseudotuberculosis* delivers more than 99% of the YopH-associated PTPase activity into HeLa cells. Whether the discrepancy originates from the bacterium or from the system used for the study is not known. As discussed below, YopN, TyeA, and LcrG are involved in this "contact-oriented" phenomenon (41, 98, 172, 259, 285, 295).

Translocation signal on Yop effectors. Taking advantage of the Yop-Cya strategy, Sory et al. (320) identified a domain required for the internalization of YopE and YopH into murine PU5-1.8 macrophages. Starting from hybrids that were readily translocated, they engineered gradual deletions into the *yop* gene, starting from a restriction site at the hinge between *yopE* or *yopH* and *cyaA*. Internalization into macrophages, revealed by cAMP production, required the N-terminal 50 amino acids of YopE and the N-terminal 71 amino acids of YopH. Sory et al. (320) concluded that YopE and YopH are modular proteins composed of a secretion domain, a translo-

TABLE 4. Yop proteins involved in translocation

Protein	Size (kDa)	Structural features[a]	Role(s)	Relevant similarities	References
YopB	41.8	Central hydrophobic domains (aa 168–208 and aa 224–258); coiled coils (aa 103–165 and aa 330–385)	Needed for translocation; needed for contact hemolysis; hypothetical constituent of a pore	RTX toxin (LktA, HlyA, . . .); IpaB (*S. flexneri*); PopB (*P. aeruginosa*); YopD; EspB (EPEC); SipB (*S. typhimurium*)	41, 136, 138, 146, 183, 190, 224, 321, 379
YopD	33.3	Central hydrophobic domain (aa 122–152); coiled coils (aa 249–292); C-terminal amphipathic α-helix	Needed for translocation	PopD (*P. aeruginosa*); YopB	41, 136, 259, 321, 379
LcrV	37.2	Polymorphism	Required for extrusion of YopB and YopD	PcrV (*P. aeruginosa*)	270, 276, 294, 379
YopK/YopQ	20.8	None	Controls translocation by modulating the size of the YopB-induced pore	None	161, 162

[a] aa, amino acids.

cation domain, and an effector domain (Fig. 8). As discussed above, this translocation domain corresponds to the Syc-binding domain (376). The identification of a domain specifically required for translocation does not imply that secretion and translocation are uncoupled. Indeed, the fact that delivery occurs only when translocators and effectors are synthesized by the same bacterium suggests that translocation immediately follows secretion (320).

The same experiments, carried out on YopE from *Y. pseudotuberculosis*, confirmed these observations (300). However, there is a difference in the results. While YopE-Cya hybrids containing between 15 and 47 amino acids of YopE were shown to be secreted by *Y. enterocolitica* (320), YopE-Cya hybrids containing between 11 and 75 residues were exported by *Y. pseudotuberculosis* but remained attached to the bacterial surface, which suggested to the authors that there is a "release domain" in YopE (300). The reason for the discrepancy between these two reports is not known. It could be due to a slight difference between *Y. enterocolitica* and *Y. pseudotuberculosis*, but this seems to be rather unlikely.

More recently, Lee et al. (204a) showed that YopE residues 1 to 100 are necessary and sufficient for the targeting of hybrid neomycin phosphotransferase. As expected, SycE was required for this targeting (204a).

The domain required for translocation of the other effectors has also been shown to reside in the N-terminal domain. For YopM, it is localized within the first 100 residues and extends further than the first 41 residues (41). For YopO/YpkA and YopP/YopJ, the signal is localized within the 77 N-terminal residues and the 99 N-terminal residues, respectively (172, 324). For YopT, it is within the 124 N-terminal residues (170).

Conclusion. So far, six effector proteins, YopE, YopH, YpkA/YopO, YopM, YopP/YopJ, and YopT, are known to be translocated across the eukaryotic membrane by a directional process (41, 137, 170, 172, 259, 285, 320, 321, 324). The YopN plug also appears to be translocated (204a). The process requires bacteria that are alive and endowed with the capacity to adhere to eukaryotic cells. It requires protein synthesis if bacteria were grown at low temperature before their contact with eukaryotic cells. If bacteria were incubated at 37°C, they can probably deliver a load of presynthesized effectors, but if Yop synthesis resumes, larger amounts of Yops will be delivered. Finally, the phenomenon is oriented in the sense that most of the production is delivered into cells and not secreted in the culture supernatant, but the degree of orientation—or leakiness—remains a matter of debate.

Delivery Apparatus

Among the 12 secreted Yops, only 2, YopB and YopD, have hydrophobic domains (136), suggesting that they could interact with membranes. They are encoded by the same large *lcrGV sycD yopBD* operon (25, 239, 270), which also encodes LcrG, LcrV, and SycD/LcrH, the chaperone of YopB and YopD (see above). As discussed in detail in this section, the whole *lcrGV sycD yopBD* operon is involved in translocation. The information is also summarized in Table 4.

YopD. Early on, different observations pointed to the importance of YopD in translocation. First, a *yopD* mutant was unable to induce cytotoxicity in HeLa cells whereas a preparation of Yops obtained from the same strain induced cytotoxicity after microinjection (283). Second, when HeLa cells were infected with a *yopD* mutant and studied by immunofluorescence techniques, no YopE was found in the cytosol (285). Third, a polar *yopB* insertion mutant was unable to translocate a YopE-Cya hybrid into HeLa cells (321). These observations led to the conclusion that YopD is required for translocation of YopE across the eukaryotic cell membrane. Independently, Hartland et al. (147) observed that a *yopD* mutant also has a markedly reduced ability to inhibit the respiratory burst, to disrupt actin filaments, and to resist phagocytosis. This pleiotropic effect indicated that YopD was involved in the translocation of a number of Yop effectors, not just YopE. The importance of YopD in translocation of the effectors YopE, YopH, YopM, YopO/YpkA, and YopP/YopJ was then shown directly using the Cya reporter enzyme system (41, 320, 324) or confocal microscopy (259, 285, 324).

YopD consists of 306 amino acids and has a molecular mass of 33.3 kDa and a neutral pI (136). Analysis of YopD with the Lupas algorithm (217) suggests the presence of a domain that could form coiled coils (structures commonly involved in protein-protein interactions) spanning residues 249 to 292. The same domain could also form an amphipathic helix. The hydropathy analysis identifies a 31-amino-acid hydrophobic region in the middle of YopD (136). The Eisenberg plot analysis (84) suggests that YopD is a transmembrane protein (136). YopD has a homolog in *P. aeruginosa*, PopD, which is 43% identical (379).

YopB. Transcomplementation of a *yopBD* mutant with the *yopD* gene alone was not sufficient to restore the ability of the bacteria to cause cytotoxicity or tyrosine dephosphorylation in cultured cells or virulence in mice (146). However, transcomplementation with both the *yopB* and *yopD* genes restored these functions, indicating that YopB also plays a role in translocation. Recent analysis of nonpolar *yopB* and *yopD* mutants showed that YopB is also individually required for translocation of the effectors across the eukaryotic cell plasma membrane (41, 138).

YopB is a 401-residue protein with a molecular mass of 41.8 kDa and a neutral pI (136). Analysis of YopB with the Lupas algorithm (217) predicts the presence of two putative coiled coils, spanning residues 103 to 165 and residues 330 to 385. The central part of YopB contains two hydrophobic regions, separated by only 15 amino acids, and, as with YopD, the Eisenberg plot of YopB suggests that it is a transmembrane protein (84, 136). YopB has a moderate level of similarity to proteins of the RTX family of alpha-hemolysins and leukotoxins such as LktA of *Pasteurella haemolytica* (338) and HlyA of *E. coli* (27, 90). The homology between YopB and the RTX proteins is limited to the hydrophobic regions. Since, in the RTX proteins, these hydrophobic regions are believed to be involved in disrupting the target cell membrane (371), it seems logical to assume that they play the same role in YopB. The fact that YopB resembles proteins of the pore-forming toxins of the RTX family suggests that the translocation apparatus could be some kind of a pore, where YopB would be the main element. The observation of Håkansson et al. (138) that *Yersinia* has a YopB- and contact-dependent lytic activity on sheep erythrocytes supports this hypothesis. Moreover, purified YopB has the ability to disrupt lipid bilayers (138). 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 (138). The presence of sugar molecules of a given size in the medium can inhibit YopB-mediated sheep erythrocyte lysis, which allowed an approximate determination of the size of the putative pore: since dextran 4 has an inhibitory effect while raffinose has no significant effect, the inner diameter of the pore would be between 12 and 35 Å. YopB also has a homolog in *Shigella*, namely, IpaB. Interestingly, IpaB, which also contains two possible transmembrane regions, is implicated in the entry of *Shigella* into epithelial cells, but its exact role in this process is not known (223). YopB also shows a moderate level of homology to YopD, and it has a homolog in *P. aeruginosa*, called PopB (379).

Interaction between YopB and YopD. The fact that YopB and YopD are both hydrophobic proteins needed for translocation suggests that they could associate at some stage to fulfill their function. This idea is reinforced by the presence of hypothetical coiled coils in both proteins. In good agreement with this hypothesis, YopB and YopD appear to be associated in the bacterium prior to their secretion (243). In an attempt to localize the domain of YopB that is involved in this interaction with YopD, Neyt and Cornelis (243) analyzed the capacity of a set of truncated YopB proteins to bind to YopD. The outcome of this analysis is that the binding does not occur at one precise site on YopB but, rather, at different sites along the protein. These observations suggest that YopB and YopD could insert together in the eukaryotic membrane and that the putative pore described above could consist of YopB and YopD, but this has not been shown yet. Until now, the pore has been neither purified nor observed on eukaryotic target cells by electron microscopy.

Role of YopQ/YopK. Yop translocation through the putative pore seems to be controlled by the 20.8-kDa YopK/YopQ (162). A *yopK* mutant of *Y. pseudotuberculosis* indeed delivers more YopE and YopH into HeLa cells than does the wild-type strain, whereas a strain overexpressing YopK is impaired in translocation. Overproduction of YopK also leads to a reduction of the YopB-dependent lytic effect on infected HeLa cells and sheep erythrocytes, probably by influencing the size of the pore, as shown by the protective effect of different-sized sugars (161).

Role of LcrV. The *lcrGV sycD yopBD* operon also encodes the LcrV protein, known since the mid-1950s as a protective antigen against plague (53). Unlike YopB and YopD, this Yop exhibits a certain degree of polymorphism; in particular, the region between amino acids 225 and 232 appears to be hypervariable (276). LcrV has been described as a regulatory protein involved in the calcium response, since a mutant with an in-frame deletion mutation in *lcrV* was found to be Ca^{2+} independent and downregulated in transcription of *yop* genes (25, 257, 269, 312, 336). However, recent data from Sarker et al. (294) indicate that LcrV could be a functional element of the translocation apparatus, since an entire deletion of the *lcrV* gene abolishes the secretion of LcrV, YopB, and YopD but has no effect on the secretion of the other Yops. The lack of secretion of YopB and YopD is not due to a lack of transcription or translation or to proteolysis, which indicates that LcrV is specifically involved in the release of YopB and YopD. Recently, a further role of LcrV has been described in the deployment of YopB, which is in turn essential for the vectorial translocation of Yops into eukaryotic cells (246a). In agreement with these observations, LcrV interacts with both YopB and YopD (294), as well as with LcrG (247, 294). On the basis of these results, it was suggested that LcrV constitutes a third component of an organized delivery apparatus (246a, 294).

Conclusion. In conclusion, data available today suggest that the translocation apparatus could act like the perforin from the cytotoxic T lymphocytes, forming a pore through which the effectors are translocated. This putative pore is probably composed of YopB and YopD, which are secreted and possibly assembled with the assistance of LcrV. Inside the bacterium, YopB and YopD are capped with their chaperone SycD/LcrH. LcrG, the first protein encoded by the *lcrGV sycD yopBD* operon, is also involved in translocation, but its exact status needs to be clarified (see below).

Control of Yop Release

The Ca^{2+} paradox and the role of eukaryotic cell contact. We have seen that in vitro, *Yersinia* spp. secrete Yops only in the absence of Ca^{2+}. Since the Ca^{2+} concentrations in the mammalian intracellular compartment are low (micromolar range) but those in the extracellular medium are high (about 2.5 mM), this would suggest that Yops are essentially produced in the intracellular environment. However, this hypothesis contradicts the evidence that *Yersinia* spreads and multiplies extracellularly (140, 141, 209, 210, 309). There is thus a paradox: in vivo, *Yersinia* proliferates under conditions that are supposed to be nonpermissive for Yop production, but it does produce Yops, as evidenced by the specific anti-Yop immune response that develops during an infection (150, 220, 325). The discovery of Yop translocation into eukaryotic cells (285, 321) led to a better understanding of the system and a solution to the Ca^{2+} paradox. Translocation of YopE into target cells occurs in cell culture media containing about 1 mM Ca^{2+}, a medium not permissive for Yop secretion. Since translocation is achieved only by extracellular bacteria adhering at the cell surface (see above), one must assume that eukaryotic cells can

somehow replace Ca^{2+} chelation to trigger Yop secretion. In vivo, contact is thus the real signal inducing Yop secretion.

Proteins involved in control of Yop release by Ca^{2+} chelation: YopN, TyeA, and LcrG. The isolation of Ca^{2+}-blind mutants (383) allowed the identification of three genes involved in the control of Yop release: *yopN* (99), *tyeA* (172), and *lcrG* (295, 311). These mutants are deregulated for Yop secretion in the sense that they secrete Yops even in the presence of Ca^{2+}. LcrG was also described in the previous section. YopN and TyeA are described here and in Table 5.

YopN, also known as LcrE, is a 32.6-kDa protein encoded by the first gene of a locus that also contains *tyeA* and three other ORFs that have been sequenced and characterized (99, 107a, 172, 360). Sequence analysis shows that it is devoid of hydrophobic domains (99) and that the regions spanning amino acids 62 to 108 and 248 to 272 could form coiled-coil structures (172). YopN is massively secreted at 37°C in the absence of Ca^{2+} ions. However, in contrast to the other Yop proteins, YopN can be detected in bacteria grown at 37°C in the presence of Ca^{2+}. In the presence of Ca^{2+}, YopN is accessible to proteases exogenously added to intact bacteria, can be extracted from the bacterial surface with xylene (99, 172), and fractionates with the Triton X-100-insoluble membrane fraction (172). Thus, under low-Ca^{2+} conditions, most of the YopN produced is released in the culture supernatant, while in the presence of Ca^{2+}, the protein is not released but is exposed at the bacterial surface.

TyeA is a 92-amino-acid protein (10.8 kDa) encoded immediately downstream of *yopN* (99, 360). This protein, previously called ORF1 (99, 360), has been named TyeA by Iriarte et al. (172) because it plays a role in translocation of some Yop effectors (see below). TyeA is detected in the bacterial cytosolic fraction and in the Triton X-100-insoluble membrane fraction but not in the culture supernatant irrespective of the presence of Ca^{2+} in the culture medium. Like YopN, TyeA is accessible to proteases exogenously added to intact bacteria and can be removed from the bacterial surface with xylene, indicating that it is loosely associated with the membrane. TyeA has the capacity to bind to the second coiled coil of YopN (172).

LcrG is a 96-amino-acid protein (11.0 kDa) (311) that controls the release of Yops in vitro (295, 311) but is also required for efficient translocation of the Yop effectors (295). LcrG has been shown to be primarily cytosolic, but it has also been detected in the membrane and in the extracellular media (247, 311). Localization of the LcrG protein upon infection of cells by bacteria has not been investigated. LcrG has been shown to bind to LcrV (247, 294). Nilles et al. (246a, 247) presented a model in which LcrG blocks Yop secretion and *LcrV* is required to remove the LcrG-imposed block. The experimental arguments that support this model are that (i) some lcrV mutants are blocked for total Yop secretion (312) and (ii) strains carrying defects in both LcrG and LcrV are calcium blind like single *lcrG* mutants (246a, 312). At this stage, no definitive proof is available. LcrG has also been shown to bind heparan sulfate proteoglycans on the surface of HeLa cells (see below), but the role of this interaction in translocation remains to be investigated (48).

Contact control. We have seen that secretion and subsequent injection of Yops is an oriented phenomenon in the sense that Yops are essentially directed into the eukaryotic cell and not into the culture medium. Thus, the three proteins that are involved in the in vitro control of Yop release by Ca^{2+} (YopN, LcrG, and TyeA) could also be involved in the contact-induced control of Yop release (41, 98, 172, 259, 285, 295). As expected, $yopN_{45}$ mutants producing a 45-amino-acid trun-

TABLE 5. Control of Yop release

Protein	Size (kDa)	Structural features[a]	Role(s)	Localization		Relevant similarities	References
				Minus Ca^{2+}	Plus Ca^{2+}		
YopN	32.6	Coiled-coil structures (aa 62–108 and aa 248–272)	Element of the putative plug closing the secretion channel; associated with TyeA	Secreted, cytosolic	Cytosolic, surface associated	MxiC (*Shigella* spp.); InvE (*Salmonella* spp.); PopN (*P. aeruginosa*); HrpI (*E. amylovora*); CopN (*C. psittaci*)	6, 38, 99, 117, 165, 172, 379
TyeA	10.8		Element of the putative plug closing the secretion channel; binds to the second coiled-coil domain of YopN; binds to YopD; required for translocation of YopE and YopH but not YopM, YopO, YopP, YopT	Surface associated, cytosolic	Surface associated, cytosolic	Pcr1 (*P. aeruginosa*); SsaL (*Salmonella* spp.)	99, 154, 170, 172, 360, 379
LcrG	11.0	Forms dimers; heparin-binding domain	Involved in control of secretion; required for efficient delivery of all Yop effectors; binds to heparan proteoglycans	Mainly cytosolic	Mainly cytosolic	PcrG (*P. aeruginosa*)	48, 246a, 247, 294, 295, 311, 312, 379

[a] aa, amino acids.

cated YopN secrete more Yops into the eukaryotic cell medium than do wild-type *Yersinia* strains. It has been suggested that YopN could function as a sensor and a stop valve controlling Yop secretion. After contact with the eukaryotic cell, the YopN sensor would interact with a ligand on the target cell surface, be removed, and allow Yop secretion and delivery to the target cell (285). However, YopN has never been shown to interact either with Ca^{2+} or with a cell receptor. The fact that *lcrG* and *tyeA* mutants are also deregulated for Yop secretion in the presence of Ca^{2+} or depolarized in the presence of eukaryotic cells (172, 295, 311) suggests that control of the delivery of the effectors requires not simply YopN but, rather, a complex system comprising at least these three proteins.

Interpenetration of the control and translocation systems. *Yersinia* producing $YopN_{45}$ can still deliver Yops into the cytosol of the target cell. However, since secretion is deregulated, the ratio between the amount of Yops delivered into cells and the amount secreted into the culture medium is very low (1:100). This suggests that translocation is independent of the control of Yop release. However, the situation is more complex and there appears to be interpenetration of the two systems. Indeed, a *tyeA* mutant is deregulated for Yop secretion but impaired in translocation of YopE and YopH but, surprisingly, not of YopM, YopO/YpkA, YopP/YopJ, and YopT (170, 172). Moreover, a $yopN_{\Delta 248-272}$ mutant, producing a truncated YopN protein with the binding domain for TyeA deleted, has the same phenotype as a *tyeA* mutant. This suggests that when YopN is inserted in the control complex, its interaction with TyeA is required for translocation of YopE and YopH but not the other effectors (170, 172). *tyeA* has a dominant phenotype over *yopN* since a double mutant, the $yopN_{45}$ *tyeA* mutant, does not translocate YopE (172), which indicates that TyeA is not required simply for removal of the YopN stop plug. Finally, as discussed above, LcrG is required for efficient translocation of all the known Yop effectors into macrophages (295), but it is also involved in the control of Yop release, since the *lcrG* mutants are Ca^{2+} blind (295, 311).

It is still difficult to establish a model that integrates the dual function of YopN, TyeA, and LcrG. The fact that TyeA is required for translocation of YopE and YopH but not YopM, YopO/YpkA, YopP/YopJ, or YopT raises the hypothesis that some Yops could be specifically delivered to particular cell types. TyeA could be a bacterial ligand with some specificity for given cell types. Although appealing, this hypothesis is purely speculative. One could also speculate that TyeA is required for translocation of Yops having a Syc chaperone and not for the others, but this is contradicted by the fact that YopT has a chaperone and does not require TyeA for its translocation (170).

Heparin interferes with translocation of YopE into HeLa cells. If the control of Yop release is induced by contact with eukaryotic cells, one can speculate that YopN, LcrG, and TyeA form a recognition complex at the bacterial surface that interacts with a receptor on the surface of eukaryotic cells. Information about this hypothetical receptor is still scarce, but the first element appeared recently.

Proteoglycans, i.e., surface proteins to which glycosaminoglycans are attached, are found on practically all types of eukaryotic cells, and they have been shown to be receptors for a variety of microorganisms via their glycosaminoglycans. Boyd et al. (48) tested the possibility that proteoglycans are responsible for the binding of a *Yersinia* sensor to the eukaryotic cell. They observed that LcrG binds HeLa cells by interacting with heparan sulfate proteoglycans (48). LcrG, which has heparin-binding motifs, also binds directly to heparin-agarose beads. Addition of exogenous heparin decreased the level of YopE translocation into HeLa cells. Translocation of YopE was also decreased by treatment of HeLa cells with heparitinase. Thus, heparan sulfate proteoglycans play a role in the delivery of Yops into HeLa cells. However, the addition of heparin was unable to completely abolish translocation. This suggests that perhaps the requirement for heparan sulfate-LcrG interaction can be partially compensated for by another bacterium-eukaryotic cell interaction. Thus, the heparan sulfate-LcrG interaction can be viewed as maximizing the efficiency of LcrG in the translocation process but may not be absolutely essential.

Homologs of YopN, TyeA, and LcrG in other bacteria. Homologs to YopN, LcrG, and TyeA have been identified in other type III secretion systems. YopN is similar to InvE from *Salmonella typhimurium* (117), MxiC from *Shigella flexneri* (6), HrpI of *Erwinia amylovora* (38), PopN of *Pseudomonas aeruginosa* (379), and CopN of *Chlamydia* (165). Homologs to TyeA and LcrG have been identified in the type III secretion system of *P. aeruginosa* (379). TyeA also shows some similarity (25% identity over 97 amino acids) to the C terminus of SsaL, a component of the type III secretion apparatus of *S. typhimurium* pathogenicity island 2 (154). The existence of homologs for all three proteins reinforces the view that they are important pieces of the type III secretion-translocation system.

Yop Effectors and Their Targets

YopE. YopE, originally described as Yop25 in *Y. enterocolitica* (229) and as YOP5 in *Y. pseudotuberculosis* (97), is a 23-kDa protein, containing an N-terminal secretion domain of 15 amino acids and a translocation domain of 50 amino acids (320). Bacterial mutants defective in *yopE* are less virulent in mice after oral infection, intraperitoneal infection, and intravenous injection than are wild-type strains (282, 330). As seen previously, YopE contributes to the ability of *Yersinia* to resist phagocytosis (282). Infection of epithelial cells with *Yersinia* leads to disruption of the microfilament structure of the host cell, due to the action of YopE (283). Within minutes after infection, the host cell rounds up and detaches from the extracellular matrix, a phenomenon referred to as cytotoxicity (282). In HeLa cells infected with *Y. pseudotuberculosis*, the YopE protein is enriched in the perinuclear region (285). As the infection of HeLa cells progresses, the microfilament structure of the cells changes from ordered filaments to a disordered granular appearance, leading to a complete disruption of the actin microfilaments (283). However, the actual enzyme activity and the target of YopE remain to be identified, since YopE does not act directly on actin (283). Since GTPases regulate the polymerization of actin in eukaryotic cells (216), it can be hypothesized that YopE acts on the cytoskeleton through interaction with GTPases.

YopE is homologous to the N-terminal noncatalytic region of the *P. aeruginosa* ADP-ribosyltransferase ExoS (195) (see below), but YopE is not known to act as an ADP-ribosyltransferase.

YopH. YopH, originally described as Yop51 (226) and Yop2b (46), is probably the best characterized Yop. It is a PTPase of 51.0 kDa, composed of several domains, which include two N-terminal domains, of 17 and 71 amino acids, which are important for secretion and translocation, respectively (259, 320); a sequence of 39 amino acids that shows unexpected homology to the regulatory protein YscM/LcrQ (see below) (275, 327); a central proline-rich sequence that binds host cell Src homology 3 (SH3) domains (31); and finally a C-terminal 262-amino-acid domain that is homologous to the catalytic domains of eukaryotic PTPases (32, 132) (Fig. 8). Phosphorylation is one of the mechanisms through which both

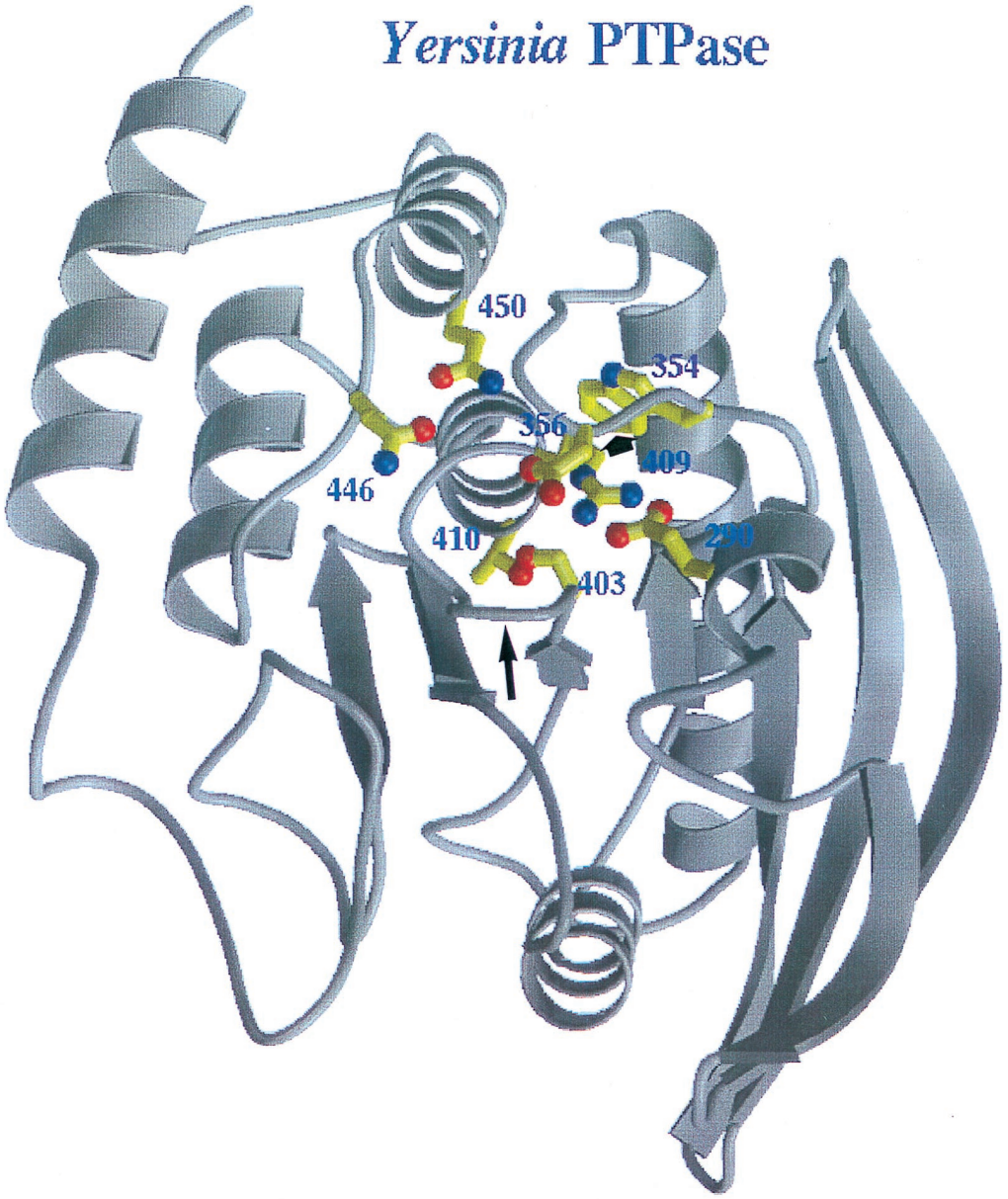

FIG. 9. Catalytic domain of YopH. A ribbon diagram of the (C403S) *Yersinia* PTPase is shown. The sulfate anion has been left out of the active site for clarity. A few critical conserved residues are depicted with stick bonds in yellow and are labeled. The P-loop is depicted by a long arrow, and the flexible loop is indicated by a short arrow. The general acid on the flexible loop, aspartic acid at position 356, can be seen in the "closed" conformation over the active site. Serine 403 is seen in place of the catalytic cysteine, although the two residues have very similar conformations. Diagram courtesy of J. Dixon (University of Michigan Medical School).

bacteria and eukaryotic cells modulate protein activity in response to environmental stimuli (216). Phosphorylation of eukaryotic proteins commonly occurs on serine or threonine; only 0.01% of the total phosphoamino acids within a eukaryotic cell exist as phosphotyrosines. The protein tyrosine phosphorylation process forms part of the signal transduction pathways that control many cellular functions, including fundamental processes such as phagocytosis, mitogenesis, and cell division (216).

The YopH tyrosine phosphatase activity is optimal around pH 5.0, and the protein is active in vitro against synthetic proteins and eukaryotic proteins, such as the insulin receptor, that are phosphorylated on tyrosine (132). The catalytic region of PTPases contains a highly conserved cysteine residue (132). Conversion of this conserved cysteine at position 403 of YopH to alanine (C403A) abolishes the PTPase activity of the recombinant enzyme expressed in *E. coli* (132). A study of the kinetic properties of the purified recombinant *Yersinia* PTPase has shown that this enzyme is by far the most active PTPase known, with a k_{cat} value 25-fold higher than that of the mammalian PTP1 enzyme (385, 386). The PTPase reaction proceeds in two steps. First, the phosphate is transferred from tyrosine to a functional group on the enzyme, and then the phosphoenzyme intermediate is hydrolyzed (133). For YopH, the invariant cysteine 403 residue is directly involved in the formation of a covalent phosphoenzyme intermediate (132).

The three-dimensional structure of the catalytic domain of YopH has been resolved by Su et al. (340) and Stuckey et al.

(339). The YopH PTPase domain consists of an eight-stranded β-sheet surrounded by seven α-helices (Fig. 9). The most prominent characteristic of the structure is a strand-loop-helix motif, representing the phosphate recognition loop (P-loop), that is composed of amino acids 403 to 410 (CGRAGVGRT) in which cysteine 403 is positioned at the center of the protein. The P-loop provides the framework of hydrogen bonds that initially stabilize the negatively charged thiolate of the catalytic cysteine 403 residue. During the catalysis reaction, a substrate-induced conformational change takes place. By analyzing crystals of YopH complexed with the phosphate analog tungsten, a competitive inhibitor of YopH, it was shown that an adjacent loop of amino acids (residues 350 to 360) moves 7 Å into the active site, thereby placing an invariant aspartic acid into the active site, where it participates in phosphotyrosine hydrolysis (339, 387) (Fig. 9). It is remarkable that the structure of the P-loop and the invariant cysteine (position 403) and arginine (position 356) residues is conserved among species from bacteria to mammals. Moreover, the mechanism of dephosphorylation is also conserved from bacteria to mammals (387).

Several studies have demonstrated that YopH is an important virulence determinant of *Yersinia* (42, 330). Insertional inactivation of *yopH* has no measurable effect on bacterial gene expression, growth, or cell viability outside the host. However, *yopH* mutants derived from *Y. pestis* or *Y. pseudotuberculosis* are significantly reduced in virulence. As explained above, *Y. pseudotuberculosis yopH* mutants are less able to resist phagocytosis in vitro by macrophages than are their parental strains (281).

Immunofluorescence studies have demonstrated that after infection of HeLa cells, YopH is localized largely in the cytoplasm of the cells, although a small fraction is colocalized with the plasma membrane (259). During infection of cultured human epithelial cells and macrophages, two host proteins of 55 and 120 kDa are dephosphorylated by wild-type *Y. pseudotuberculosis* within 15 min (34, 36), whereas these proteins are not dephosphorylated in cells infected with the C403A mutant. For identification of the YopH substrates and their localization within the host cell, mutant *Yersinia* strains producing YopH C403A (258) or C403S (35) were used, because these catalytically inactive PTPases form stable complexes with their substrate. After infection of HeLa cells with *Yersinia*, YopH (C403A) interacts with tyrosine-phosphorylated forms of FAK and $p130^{CAS}$ and colocalizes with these proteins in focal adhesions. In contrast, the active YopH leads to inhibition of bacterial uptake, dephosphorylation of $p130^{CAS}$ and FAK, and disruption of peripheral focal complexes (35, 258, 281). Focal adhesions are sites where integrin receptors serve as a transmembrane bridge between extracellular matrix proteins and intracellular signaling proteins. FAK is involved in the early steps of the integrin-mediated signaling cascade and is therefore believed to function as a transmitter and amplifier (216). The kinase substrate $p130^{CAS}$ interacts with FAK via an interaction between its proline-rich sequence and a C-terminal SH3 domain of $p130^{CAS}$ (35, 258). In macrophages, the inactive YopH specifically recognizes and interacts with tyrosine-phosphorylated $p130^{CAS}$ (88).

As described above, YopH inhibits phagocytosis by PMNs and macrophages, mediated by complement receptors (291) or Fc receptors (87), respectively. Interestingly, these receptors also mediate phosphorylation of FAK or $p130^{CAS}$ upon stimulation (135, 260). Dephosphorylation of $p130^{CAS}$ by YopH could prevent the early steps required for the formation of focal adhesions. Integrin-mediated adhesion of phagocytes to endothelia or extracellular matrix proteins plays an important role during inflammation. Interference with this process due to YopH-mediated dephosphorylation of $p130^{CAS}$ might therefore have an important implication for leukocyte function during *Yersinia* infection.

YopM. YopM, originally described as Yop48 in *Y. enterocolitica* (74, 239) and as Yop2a in *Y. pseudotuberculosis* (97), was first sequenced in *Y. pestis* (208). It is a strongly acidic protein with an isoelectric point of 4.06 and a mass of 41.6 kDa (41, 208). It is hydrophobic at both the N- and C-terminal ends and contains 12 leucine-rich repeated motifs (LRRs) (193) (Fig. 8). There is some interstrain variability in the size of YopM (40). In particular, Boland et al. (40) noted that YopM from a *Y. enterocolitica* O:8 strain isolated from a patient with a severe infection is 56.9 kDa instead of 41.6 kDa. This increased size results from the duplication of part of the gene that probably occurred because of the repetitive nature of this gene (40). Therefore, unlike the other Yop effector proteins that are well conserved among different *Yersinia* species, YopM is somewhat heterogeneous.

According to the 50% lethal dose test, both *Y. pestis* and *Y. enterocolitica yopM* mutants have a strongly reduced virulence in mice (207, 239). Furthermore, bacterial counts in the liver and spleen of *Y. enterocolitica*-infected animals showed that the *yopM* mutant had a reduced ability to multiply in the host (239).

YopM is significantly homologous to IpaH of *Shigella* (148) and y4fR of *Rhizobium* (104); however, no function is known for either of these proteins. Due to the presence of LRRs, YopM shows a moderate similarity to a great number of proteins containing LRRs, including the α-chain of the platelet membrane glycoprotein 1b (GP1bα). GP1bα binds thrombin and von Willebrand factor (208), and therefore the ability of YopM to bind thrombin was studied. In vitro studies showed that purified YopM has thrombin-binding activity and competitively inhibits thrombin-induced platelet activation in vitro, suggesting that YopM is an extracellular effector (207, 274). However, this role remains to be confirmed, since no thrombin-binding site has been identified so far in YopM and since the domains of GP1bα that are known to be involved in the interaction with thrombin are located outside the region with homology to YopM (77, 125). Furthermore, the similarity to GP1bα is less than 24% and is essentially due to the LRR repeats. Today, the data bases contain many proteins, in particular proteoglycans, containing LRR motifs that are more similar to YopM than is GP1bα.

Recently, by using the Yop-Cya approach, Boland et al. (41) demonstrated that YopM is delivered inside eukaryotic cells. The secretion signal of YopM is contained within the first 40 N-terminal residues, and the translocation domain is contained within the first 100 residues. However, the directionality of intracellular Yop delivery is not as high as that of the other delivered Yops. Therefore, although YopM is likely to react with an as yet unknown intracellular target, an extracellular role cannot be excluded.

YpkA/YopO. YpkA/YopO had been described first in *Y. enterocolitica* as Yop84 (74), and later, when a letter code was adopted, it was renamed YopO (239). When the gene encoding this Yop in *Y. pseudotuberculosis* was sequenced and found to be homologous to the eukaryotic serine/threonine kinases, it was named YpkA, for "*Yersinia* protein kinase A." The protein is thus named YopO in *Y. enterocolitica* and YpkA in *Y. pseudotuberculosis* and in *Y. pestis* (257a, 330). YopO/YpkA shows 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 (155).

Galyov et al. (110) described the ability of the 81.7-kDa

YpkA protein to catalyze autophosphorylation of a serine residue in vitro. Removal of a major part of the catalytic domain (from amino acids 207 to 388) does not totally abolish phosphorylation. However, a disruption of the ORF downstream from the catalytic domain results in a kinase null mutant, indicating that at least part of the kinase function is located outside the catalytic domain. The effect of this mutation on virulence was studied by challenging mice orally. No lethal infection could be observed with the mutant strain, in contrast to a challenge with the wild-type strain. Nevertheless, the mutant strain was able to colonize the Peyer's patches to an extent similar to that shown by the wild type at the initial stage of the infection. Furthermore, no colonization of the spleen was observed and the colonization of the Peyer's patches decreased in the later stages of the infection (111).

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 the cells, the YpkA protein is targeted to the inner surface of the plasma membrane (137). No target protein of YpkA/YopO has been identified yet.

YopJ/YopP. The 32.5-kDa YopJ/YopP was first detected as YopJ in *Y. pestis* (330) and as Yop30 (74) and later YopP (69a) in *Y. enterocolitica*. YopP contains 288 residues (232), and for a while it was thought that YopJ contains 264 residues (111); however, recent data suggest that YopJ also contains 288 residues (251). YopJ/YopP, together with YopO/YpkA, is encoded by a single operon (74, 111). It is considered a "minor" Yop in the sense that in vitro, it is secreted in smaller amounts than most of the other Yops, e.g., YopE. A *yopJ* mutant strain appeared to be fully virulent in an intravenous mouse model (111, 330, 332). However, in vitro, both YopP and YopJ induce apoptosis in murine macrophages (232, 237). Induction of apoptosis requires type III secretion and a translocation step (Fig. 4). The apoptotic process is cell type dependent, since *Yersinia* is not able to induce apoptosis in epithelial cells (232, 237, 290) or in fibroblasts (237). By using the mechanism of apoptosis during the infection process, *Yersinia* might eliminate macrophages without inducing an inflammatory response and thereby might favor extracellular proliferation in lymphoid tissues (see above).

Interestingly, YopP and YopJ have a high level of similarity to AvrRxv from *Xanthomonas campestris* (372), AvrA from *Salmonella* (144), and y410 from *Rhizobium* (104). No function is known for AvrA and y410. However, AvrRxv is one of many plant pathogen avirulence proteins that mediate the hypersensitive response, a process that is likely to result from the activation of a programmed cell death pathway (234, 372). However, no cytotoxic effect has been described for AvrA so far. 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 Yop effector that has been described and characterized recently (170). It 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. YopT shows some similarity to the C-terminal end of p76, an immunoglobulin-binding protein present in the serum-resistant strains of *Haemophilus somnus* (67), but the relevance of this similarity is not known.

Conclusion. In conclusion, the presence of at least six Yop effectors (Table 6) could reflect the interaction of *Yersinia* with one or more cell types at different stages of activation or development, thereby inducing different cell responses. The outcome of this multifactorial process is the ability of *Yersinia*

TABLE 6. Yop effectors

Protein	Size (kDa)	Action on the cell	Enzymatic activity	Relevant similarities	Localization in the target cell	Sufficient signals for[a]:		Reference(s)
						Secretion (aa or codons)	Translocation (aa)	
YopE	22.9	Disruption of the cytoskeleton; rounding up of cells; detachment from extracellular matrix	Cytotoxicity	ExoS (*P. aeruginosa*); SptP/StpA (*Salmonella*)	Perinuclear region	11	50	10, 14, 184, 282, 283, 300, 321, 378
YopH	51.0	Disruption of peripheral focal complexes	PTPase	Eukaryotic phosphatases; SptP/StpA (*Salmonella*)	Cytoplasm	17	71	14, 31, 32, 46, 132, 184, 258, 321, 340
YopM	41.6	Unknown	Unknown	y4fR (*Rhizobium*); IpaH (*Shigella*); numerous proteoglycans; GP1bα	Unknown	40	100	41, 104, 148, 207, 208
YpkA/YopO	81.7	Rounding up of cells	Ser/Thr kinase	Ser/Thr kinases; COT oncogene	Inner surface of plasma membrane	77	77	74, 110, 137, 324
YopP/YopJ	32.5	Apoptosis of macrophages; inhibition of TNF-α release	Cytotoxicity	AvrRxv (*X. campestris*); AvrA (*Salmonella*)	Cytoplasm	99	99	41, 144, 232, 237, 251, 324
YopT	35.5	Disruption of actin filaments	Cytotoxicity	p76 (*H. somnus*)	Unknown	124	124	170

[a] aa, amino acids.

to obstruct a cellular immune response. Remarkably, all the effector Yops have homologs in a taxonomically diverse group of pathogens including animal and plant pathogens. Interestingly, *Salmonella* secretes the SptP/StpA protein, whose C terminus exhibits homology to YopH while the N terminus exhibits sequence similarity to YopE and ExoS (14, 184). This implies that these shared genes were already present before the divergence of these pathogens into different species, that they result from a successful coevolution, or that they were recruited by horizontal transfer. The last hypothesis is favored by the fact that the mechanism of type III secretion is also highly conserved and widespread among bacterial species that are taxonomically distant.

Functional Conservation among Different Bacterial Species

We have seen that several other bacterial species possess a type III virulence system. Are these 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 the ADP-ribosyltransferase ExoS from *P. aeruginosa* (453 residues total) has 54% similarity to the entire YopE (see above), and the protein encoded by the gene next to *exoS* (ORF1) is very similar to SycE/YerA (368). These observations prompted Frithz-Lindsten et al. (105) to introduce the two genes from *P. aeruginosa*, transcribed from the p_{lac} promoter, into *Y. pseudotuberculosis*. Since they observed that the recombinant *Y. pseudotuberculosis* could secrete ExoS, they next investigated whether ExoS would be delivered by a recombinant *Y. pseudotuberculosis* into HeLa cells, just like YopE. They introduced the *exoS* gene and ORF1 into a noncytotoxic double *yopE yopH* mutant of *Y. pseudotuberculosis* and used the mutant to infect 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 2,000-fold-reduced ADP-ribosyltransferase activity, they still observed cytotoxicity, which indicated that ExoS is a bifunctional protein endowed with a YopE-like cytotoxic activity. These experiments demonstrated that the closely related *Yersinia* and *Pseudomonas* type III systems are functionally interchangeable. Given the taxonomic distance between these two species, the observation is important because it strengthens the idea of horizontal spread of these type III systems.

The group of H. Wolf-Watz also observed that *Y. pseudotuberculosis* can secrete IpaB from *Shigella flexneri* and that *Salmonella typhimurium* can secrete YopE (284). The latter recombinant *Salmonella* strain is also cytotoxic for HeLa cells, suggesting that YopE could even be translocated across the cell plasma membrane.

YadA ADHESIN AND YlpA LIPOPROTEIN

Discovery and Description

Y. enterocolitica and *Y. pseudotuberculosis* synthesize a pYV-encoded outer membrane protein called YadA (formerly called Yop1 or P1) (20, 43, 45). YadA is named for "*Yersinia* adhesin A," a name that was given during the 1990 Keystone meeting on *Yersinia*, in Frisco, Colo. The expression of the gene encoding this protein (*yadA*, formerly *yopA*) is under the control of VirF, and it is expressed only at 37°C, like the *yop* and *ysc* genes (43, 187). YadA is thus a Yop regulon protein (see "Regulation of transcription of the virulon genes," below), but unlike the Yops, it is produced in the presence and absence of Ca^{2+} (43, 228, 315). YadA is not expressed by *Y. pestis* even though its pYV plasmid contains the *yadA* gene. The *yadA* gene of *Y. pestis* has a single-base-pair deletion that results in a shift in the reading frame of the gene and an mRNA with a reduced half-life, and so the YadA protein is not produced (268, 286, 316).

yadA encodes a 44.1- to 47.1-kDa protein (the exact size depends on the strain) that is seen on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) when prepared in the presence of high concentrations of urea or upon prolonged boiling in the presence of excess reducing agent (313, 384). Sample preparation by boiling for shorter periods in the presence of SDS results in the appearance on SDS-PAGE of an approximately 200-kDa band that is a polymer of YadA. By electron microscopy, YadA has been seen as a fibrillar extracellular matrix around the bacteria (188). Each individual fibrilla had a diameter of 1.5 to 2.0 Å and a length of 50 to 125 Å. In another study, YadA was seen as tack-like projections protruding from the bacterial surface (384). The protein causes autoagglutination of *Yersinia* and mannose-resistant haemagglutination of guinea pig erythrocytes (20, 188, 198, 221, 313).

YadA can bind a variety of eukaryotic extracellular and cell surface molecules including collagens, fibronectin, and laminins (85, 96, 304, 305, 345). It has been shown to bind to rabbit intestinal tissue (both the brush border membranes and mucus), eye lens capsule basement membranes, and human intestinal submucosa (96, 249, 250, 314). YadA also mediates the binding of *Yersinia* to cultured cells, such as HeLa and HEp-2 (152, 282). Thus, YadA is a major adhesin of *Yersinia* for attachment to eukaryotic cells. Although invasin, an outer membrane protein encoded by the chromosome of *Yersinia*, is the major determinant for internalization of the bacteria into eukaryotic cells via interaction with β_1-integrins (176, 177), YadA too can mediate internalization of the bacteria into eukaryotic cells, and this is due, at least in part, to interaction with β_1-integrins (35, 380).

Role of YadA in Virulence

YadA plays a protective role for *Yersinia*. As discussed above, it contributes to the protection of *Y. enterocolitica* against killing by PMN extracts and against killing by human serum (20, 221, 362). This latter ability is due to the binding of factor H by YadA, which leads to the inactivation of C3b and a subsequent decrease in the deposition of membrane attack complexes on the bacterial surface (66, 262). YadA also mediates the inhibition of the anti-invasive effect of interferon (52).

The role played by YadA in the resistance of *Y. enterocolitica* to phagocytosis and killing by eukaryotic cells is disputed (65, 152, 291, 361), but it has been suggested that YadA would act by binding to eukaryotic cells and, in doing so, allow delivery of the Yops (see above) (291, 321).

There is a major difference between *Y. enterocolitica* and *Y. pseudotuberculosis* in the influence of a *yadA* mutation on virulence in mice. A *Y. enterocolitica yadA* mutant is attenuated for virulence (254, 277, 278, 342). Due to impaired colonisation of the Peyer's patches, *Y. enterocolitica* bacteria are eliminated and smaller numbers of bacteria are found in the mesenteric lymph nodes, spleen, and liver (188, 277). In addition, there is less inflammation and necrosis in the liver (254). The virulence attenuation is not a result of YadA being required for movement of *Yersinia* from the intestinal lumen to the Peyer's patches—the *yadA* mutant strain does this very well (277). It is due to the requirement of YadA for persistence, i.e., survival and multiplication, in the Peyer's patches, and perhaps for dissem-

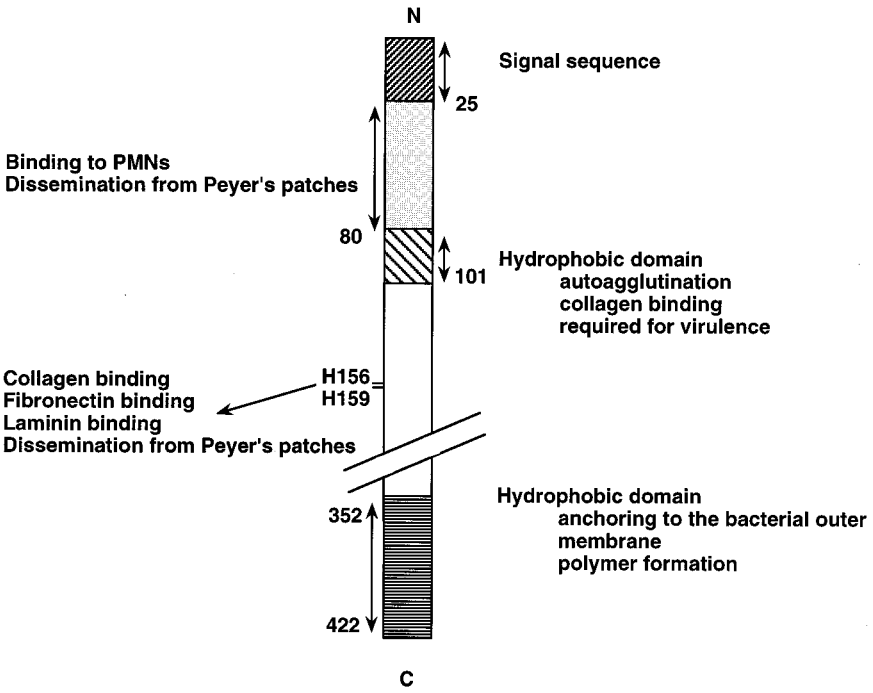

FIG. 10. Representation of the YadA protein of *Y. enterocolitica* O:8 showing the mapped domains and their functions (277, 278, 314, 316, 342).

ination of the bacteria from the Peyer's patches to other sites in the body (254, 277). In contrast, a *Y. pseudotuberculosis yadA* mutant is just as virulent as the wild type (45, 139, 286) and can colonize the Peyer's patches just as efficiently. It was previously reported that an *inv yadA* double mutant was hypervirulent compared to the wild-type strain or the *yadA* or *inv* single mutants (286). However, more recent results suggest that this *inv yadA* double mutant was not isogenic to the parental wild-type strain. A newly constructed *inv yadA* double-mutant strain maintained the same virulence as the parental strain, demonstrating that neither invasin nor YadA plays an important role during *Y. pseudotuberculosis* infection (139). The severe virulence of *Y. pestis* is thus probably not due to the *inv yadA* mutant phenotype, as originally suggested, but could be due to additional virulence factors, which are lacking in *Y. enterocolitica* and *Y. pseudotuberculosis* (139). The differences between the *Yersinia* species with regard to the role of YadA in virulence probably result from the importance of the interplay of additional adhesion factors.

Structure-Function Analysis of YadA from *Y. enterocolitica*

The YadA proteins from various strains of *Y. pseudotuberculosis* and *Y. enterocolitica* differ as a result of substitutions and deletions/insertions within the coding DNA (316). Studies on the structure-function relationships of YadA have focused primarily on *Y. enterocolitica* serotype O:8 (Fig. 10). The N terminus of each YadA comprises a typical signal sequence (316). YadA is thus not exported by the Ysc secretion machinery but is instead probably exported by the Sec system. A *Y. enterocolitica* mutant lacking amino acids 29 to 81, a region that varies among the *Yersinia* species, does not adhere to PMNs and so does not cause inhibition of the oxidative burst (278). This mutant is attenuated in virulence and is comparable to a complete *yadA* mutant strain. This suggests that the effect on PMNs mediated by YadA is important for the virulence of *Y. enterocolitica*. Residues 83 to 101 compose one of the conserved hydrophobic domains of YadA. Deletion of these amino acids results in a YadA protein that cannot promote autoagglutination, does not bind collagen and has impaired ability to bind to basement membranes (342). This domain is also involved in the binding of YadA to human intestinal submucosa via collagen and laminin (314). Most importantly, this mutant is avirulent, reinforcing the importance of YadA binding to eukaryotic cells for virulence of *Y. enterocolitica*. The conserved histidines 156 and 159 are necessary for collagen binding, are important for binding to fibronectin and laminin, and are needed for binding to HEp-2 cells (277). A *Y. enterocolitica* strain carrying a *yadA* gene with mutations in these two histidine residues colonizes the Peyer's patches but does not disseminate to the mesenteric lymph nodes, spleen, or liver. These results indicate that YadA plays a role in the dissemination of *Y. enterocolitica* from the Peyer's patches to other sites in the body. Finally, the conserved hydrophobic C-terminal amino acids of YadA have been shown to be involved in surface exposure of the protein and polymer formation (342).

Conclusion for YadA

To summarize, YadA is a *Yersinia* adhesin that binds eukaryotic cells by a number of molecules. In *Y. enterocolitica*, the adhesive ability of YadA is essential for virulence and could be required for the translocation of the Yop proteins into eukaryotic cells and so for the effects of these Yop proteins on the target cells. A number of domains of YadA have been mapped as being important for the various functions of the protein and for virulence.

YlpA

YlpA is a pYV-encoded lipoprotein that is produced at 37°C in the absence of Ca^{2+} (64). Its expression is dependent on VirF, making it a member of the Yop regulon. Like YadA,

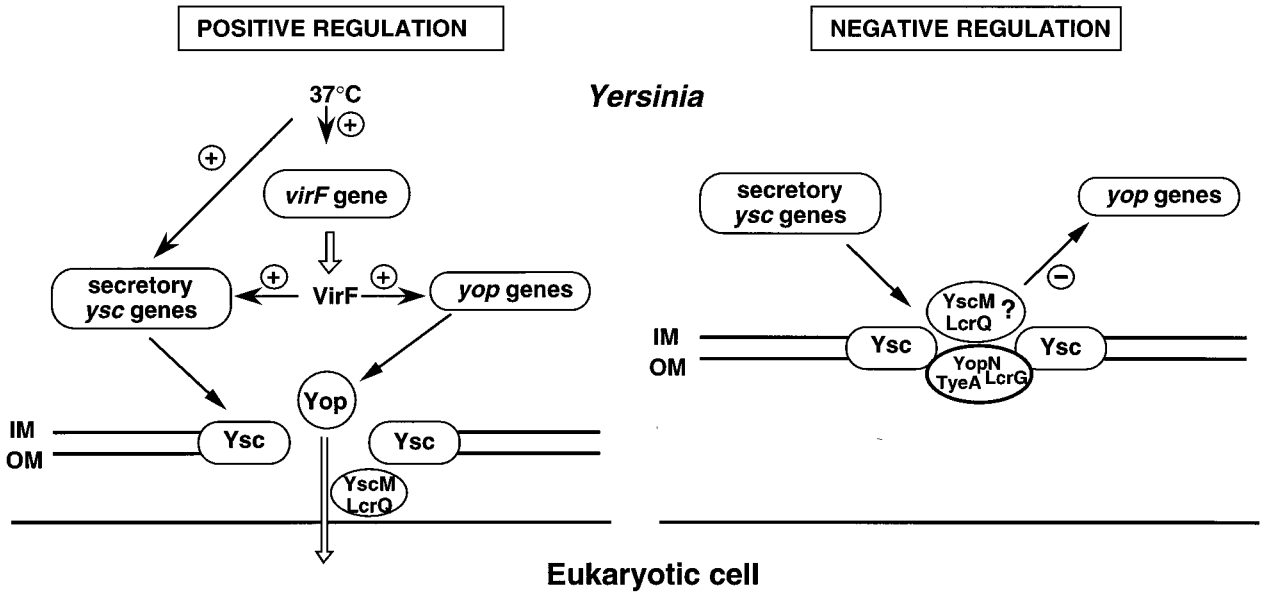

FIG. 11. Schematic representation of the two circuits regulating *yop* gene transcription. IM, inner membrane; OM, outer membrane.

YlpA has a typical signal sequence and is localized to the outer membrane independent of the Ysc secretion machinery. YlpA is highly homologous to the TraT proteins of a number of bacterial plasmids (up to 88% identity at the amino acid level). TraT proteins are involved in serum resistance (235), surface exclusion during conjugation (1), and inhibition of phagocytosis by macrophages (2), but the involvement of YlpA in these processes is unknown. A mutation in YlpA does not affect virulence in the mouse model (64).

GENETICS

Regulation of Transcription of the Virulon Genes

Effect of temperature and Ca^{2+} on in vitro transcription. As discussed above, Yop secretion in vitro occurs only at 37°C in the absence of Ca^{2+}. This secretion correlates with growth arrest, a phenomenon known for a long time as Ca^{2+} dependency (50). By contrast, the production of YadA is independent of the Ca^{2+} concentration but is still thermoregulated (43, 187). This was the first indication that temperature and Ca^{2+} influence two different regulatory networks. The first permits full expression of all the pYV-encoded virulence functions when the environment is ideal and the temperature reaches 37°C, while the second prevents the production of the Yops and YlpA only in the presence of 2.5 mM Ca^{2+} ions (for reviews, see references 70 and 335). Neither of these two regulatory networks is perfectly understood so far, but it is quite clear that they are independent of each other. They are summarized in Fig. 11.

The transcriptional activator VirF/LcrF. In 1986, Yother et al. mutagenized the virulence plasmid pCD1 of *Y. pestis* with Tn*5* and identified a locus which positively controls the transcription of two loci (called *trtA* and *trtB* at that time) in response to temperature. They named this regulatory locus *lcrF* (382). In 1987, Cornelis et al. (74) described *virF*, the *Y. enterocolitica* homolog of *lcrF*, and showed that a mutation in this locus, which is located between *virB* and *virC* (Fig. 3), drastically reduces the transcription of a *yopH-lacZ* operon fusion. The *virF* gene was isolated and sequenced by the same group 2 years later (72).

VirF/LcrF is a 30.9-kDa protein that belongs to the AraC family of regulators (72). This family includes regulators of degradative pathways in *E. coli* and *Pseudomonas putida* as well as regulators involved in the control of virulence of *Shigella* spp., enterotoxigenic *E. coli*, *P. aeruginosa*, and the phytopathogen *Ralstonia solanacearum* (80, 114, 164, 180; for a review, see reference 109). Transcription of many pYV genes, including all the *yop* genes, *sycE*, *ylpA*, *yadA*, and the *virC* operon, is dependent on VirF/LcrF (64, 72, 228, 315, 366). These genes and operons constitute the *virF*/*lcrF* regulon. By contrast, VirF/LcrF seems to be dispensable or less important for transcription of the *virA* and *virB* operons, which encode the Ysc secretion apparatus (199), and of some other genes such as *sycH* (365). All these genes, dependent on or independent of VirF/LcrF, are silent at low temperature but strongly expressed at 37°C. They constitute the *yop* stimulon. Note that the *yop* stimulon is larger than the *virF*/*lcrF* regulon.

DNase I footprinting experiments carried out by Wattiau and Cornelis (367) on four promoters (*yopE*, *yopH*, *virC*, and *lcrGVsycDyopBD*) showed that VirF binds to a 40-bp region localized immediately upstream from the RNA polymerase binding site. These VirF-binding sequences are located in an AT-rich region and appear either isolated or repeated in opposite orientation. This site contains the 13-bp consensus sequence TTTTaGYcTtTat (in which nucleotides conserved in $\geq$60% of the sequences are in capital letters and Y indicates C or T) (367).

In *Y. enterocolitica*, the *virF* gene itself is strongly thermoregulated. This thermoinduction still occurs in *E. coli* containing an isolated *virF* gene transcribed from its own promoter (72). Expression of the *virF* gene must thus be thermoregulated by a chromosomal gene rather than by a pYV gene. The fact that *virF* is itself thermoregulated can explain why the Yops are produced only at 37°C. However, it does not prove that temperature control occurs only through the regulation of *virF*. In fact, when *virF* is transcribed at low temperature from a *tac* promoter, the *yop* and *yadA* genes are only poorly transcribed. In contrast, at 37°C, the response to isopropyl-β-D-thiogalactopyranoside (IPTG) mimics the normal response to thermal induction (199). In conclusion, expression of the *yop* stimulon

is first controlled by temperature but expression of some of its genes is reinforced by the action of VirF, whose synthesis is also temperature controlled.

In *Y. pestis*, transcription of *lcrF*::*lacZ* transcriptional fusions is independent of temperature (160). However, comparison of the amount of LcrF protein produced per unit of message at low and high temperature indicates that the efficiency of translation of the *lcrF* mRNA increases with temperature (159). To account for this observation, Hoe and Goguen (159) presented a model in which a secondary structure of mRNA could sequester the *lcrF* Shine-Dalgarno sequence and so regulate LcrF synthesis.

With regard to these two models, one might think that there are two separate modes of regulation in *Y. enterocolitica* and in *Y. pestis*. However, VirF and LcrF are so similar in their sequence and in their function that it is difficult to imagine that they could be regulated differently. Data from the two groups could be reconciled in a model in which both transcription and translation of *virF*/*lcrF* are temperature dependent.

Role of the histone-like protein YmoA and chromatin structure. To identify the chromosomal regulator of the *yop* stimulon, Cornelis et al. (71) carried out transposon mutagenesis of a *Y. enterocolitica* strain which carried *lacZ* fused to *yopH*. Two chromosomal mutants strongly transcribed *yopH*, *yopE*, and *yadA* at 28°C but did not secrete the Yops at this temperature (71). Transcription of the regulatory gene *virF* was itself increased at 28°C, which could account for the increased transcription of the genes of the regulon. Although expression of these genes was deregulated at low temperature in the mutants, there was still an increase of transcription upon transfer to 37°C. Hence, the thermal response was not abolished but, rather, "modulated." The phenotype is thus not that of a classical repressor-minus mutant, but the mutations nevertheless specifically affected a component of the temperature response. In both mutants, the transposon was inserted in the same small gene that Cornelis et al. (71) called *ymoA* for "*Yersinia* modulator." The *ymoA* gene encodes an 8.1-kDa protein extremely rich in positively and negatively charged residues. Although there is no sequence similarity between YmoA and the histone-like proteins HU, IHF, and H-NS (H1), it is very likely that YmoA is a histone-like protein (71). This idea has been reinforced by the fact that the level of supercoiling was higher in *ymoA* mutants than in the wild-type strain, as shown by chloroquine agarose gel electrophoresis of plasmid DNA (71). To determine whether the chromatin structure influences expression of *yop* genes, Lambert de Rouvroit et al. (199) measured the expression of a *yopH-cat* operon fusion in the *ymoA* mutant. They showed that the *yopH* promoter becomes independent of VirF in the *ymoA* mutant but still remains thermoinducible. This result suggested that chromatin structure and temperature strongly affect the *yopH* promoter itself. 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. (279) confirmed that temperature alters DNA supercoiling and DNA bending by VirF in *Y. enterocolitica* and hypothesized that temperature dislodges a repressor, perhaps YmoA, bound on promoter regions of VirF-sensitive genes and of some other thermoregulated genes.

The role of chromatin structure in thermoregulation of virulence gene transcription is now well established. It is reviewed and discussed in detail by Dorman and Ni Bhriain (81). All the properties of the *ymoA* mutants are strikingly reminiscent of the *virR* mutants of *Shigella flexneri* lacking H-NS (123). YmoA is, however not the counterpart of H-NS from *E. coli* or *Shigella*. A homolog of YmoA was discovered in *E. coli* as a regulator of hemolysin production (246). The gene encoding this regulator, called *hha*, can complement the *ymoA* mutations of *Y. enterocolitica*, provided that it is expressed at an adequate level (230). YmoA and Hha are thus the first representatives of a new class of histone-like proteins regulating the expression of topologically sensitive promoters. YmoA can thus be listed along with the histone-like proteins H-NS, IHF, FIS, HU, and LRP. It is striking that the searches for thermoregulators in *Yersinia* spp., in *Shigella* spp., and in uropathogenic *E. coli* converged on histone-like proteins (156).

Feedback control of Yop synthesis by the secretion apparatus. The first molecular studies on the transcription of pYV genes in *Y. pestis* and *Y. enterocolitica* were done in the mid-1980s by using mini-Mu d*lac* insertion mutants. This strategy permits researchers to monitor the expression of various genes under different conditions simply by measuring the β-galactosidase activity of promoters fused to *lacZ*. In 1984, Goguen et al. showed that Ca^{2+} ions do not affect the transcription of *lcrA-lacZ*, *lcrB-lacZ*, and *lcrC-lacZ* gene fusions in *Y. pestis* (120). This was confirmed for their homologs in *Y. enterocolitica* (*virA*, *virB*, and *virC*) by Cornelis et al. (73). While Ca^{2+} had little effect on transcription of these loci, which are now known to encode the Ysc secretion apparatus, it had a very clear effect on *yop* expression: expression of *yop-lacZ* operon fusions was dramatically decreased by the presence of 2.5 mM Ca^{2+} in the medium (73, 74, 239, 330). Later, Northern blotting experiments confirmed that Ca^{2+} abolishes the transcription of *yopE* and *yopH* (46, 100). Thus, the presence of Ca^{2+} ions blocks not only the secretion of Yops but also their synthesis. It is difficult to imagine how an ion like Ca^{2+} could penetrate into the bacterial cytosol to block transcription. It is more plausible that the abundance of Ca^{2+} could influence Yop secretion from the bacterial surface. Cornelis et al. (74) thus suggested that Ca^{2+} would essentially stop secretion and that a feedback inhibition mechanism would block transcription of *yop* genes when secretion is compromised. This hypothesis is reinforced by the fact that mutations in the *virA*, *virB*, and *virC* loci, which encode the Ysc secretion apparatus, severely downregulate expression of the *yop* genes (9, 72, 264, 265, 375). It is also consistent with the fact that transcription of the *ysc* genes themselves is not strongly influenced by Ca^{2+} (see above).

As described above, *yopN*/*lcrE*, *lcrG*, and *tyeA* mutants have a Ca^{2+}-blind phenotype: they express and secrete Yops in the presence, as well as in the absence, of Ca^{2+} in the media (99, 172, 311). The fact that these mutants were derepressed for Yop expression even under repressive conditions (presence of Ca^{2+} ions) leads to the idea that feedback regulation is of the negative type.

As we have seen previously, this regulation by Ca^{2+} permits control of Yop secretion in vitro while contact with eukaryotic cells is probably the signal triggering Yop secretion in vivo. In 1996, Pettersson et al. (261) studied the transcriptional activity of a *Yersinia yopE-luxAB* fusion in the presence of HeLa cells. They observed that transcription of *yopE* was induced in the bacteria associated with HeLa cells whereas no signal was observed in the bacteria attached to the glass coverslip. These observations clearly demonstrate that the bacteria initiated *yop* transcription only after contact with the target cell had been established.

LcrQ/YscM. By analogy with the secreted anti-sigma factor involved in the regulation of flagellum synthesis (49, 169, 197), Rimpiläinen et al. (275) suggested that feedback inhibition could be mediated by a negative regulator that is normally expelled via the Yop secretion machinery. They suggested that in *Y. pseudotuberculosis*, 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 the absence of secretion (presence of Ca^{2+} or mutation in the genes coding for the secretion machinery), an *lcrQ* mutant indeed synthesizes more Yops than the wild type does. In the presence of Ca^{2+}, this mutant secretes YopD and LcrV. Recently, the same group showed that LcrQ is rapidly secreted when bacteria are shifted from a medium containing 2.5 mM Ca^{2+} (nonpermissive conditions for Yop secretion) to a medium containing a Ca^{2+} chelator (permissive for Yop secretion), which fits quite well with the "secreted negative regulator" hypothesis (261). In *Y. enterocolitica*, the situation appeared to be slightly different: YscM, the counterpart of LcrQ, is also a secreted protein (327), but a *yscM* mutant does not show any sign of derepression of Yop synthesis in the absence of Yop secretion, although overproduction of YscM blocks Yop synthesis (8). The reason for this discrepancy was recently elucidated by the discovery of a gene related to *yscM* (now called *yscM1*) on the pYV virulence plasmid of *Y. enterocolitica*, which was called *yscM2*. A *yscM1 yscM2* double mutant of *Y. enterocolitica* shows the same phenotype as the *lcrQ* mutant of *Y. pseudotuberculosis*. Thus, two different YscM proteins in *Y. enterocolitica* behave like LcrQ in *Y. pseudotuberculosis* (327).

The hypothesis that LcrQ and YscM are secreted negative regulators is essentially based on two observations: (i) an *lcrQ* single mutant and a *yscM1 yscM2* double mutant display a reduction of feedback inhibition when Yop secretion is prevented, and (ii) overproduction of any one of these proteins shuts off Yop synthesis. However, some observations must be made. First, *lcrQ* and *yscM1 yscM2* mutants secrete YopD and LcrV in the presence of Ca^{2+}. How would the lack of a negative regulator remove the external stop-valve YopN to open the secretion channel, and, if the secretion channel is open, why would the other Yops not be secreted? Second, Stainier et al. (327) observed that overexpression of these proteins in a simplified system consisting only of a *yopH-cat* reporter gene and *virF* had no effect on *yopH* transcription, while a negative effect was observed in the presence of a pYV plasmid. Taken together, these various observations suggest that LcrQ/YscM is not a transcriptional repressor and that one or more pYV-encoded proteins are required to act with this protein in the feedback inhibition mechanism.

Recently, Williams and Straley (373) suggested that in *Y. pestis*, YopD acts with LcrQ in the feedback inhibition mechanism. This idea is supported by the fact that a *yopD* mutant secretes some YopM and LcrV in the presence of Ca^{2+}, despite the presence of LcrQ in the bacterial cell. Overexpression of LcrQ in this mutant does not have the same strong repressive effect as it does in the wild-type strain. These results suggests that LcrQ requires YopD to function as a negative regulator.

Lack of interference with flagellum assembly. It has been known for a long time that *Yersinia* is motile (369). The genes coding for the three flagellins and for proteins implicated in the building of the flagellum in *Y. enterocolitica* were described recently (89, 185). Production of flagella in *Y. enterocolitica* occurs below 30°C and requires sigma factor 28 of the RNA polymerase (185, 186). The similarity between some Ysc proteins and proteins involved in flagellar assembly suggests that the Yop secretion system might have evolved from the secretion system operating in flagellum biogenesis. Therefore, one could imagine that these two systems are regulated in the same way. However, it has been shown that *yop* genes are expressed only at 37°C and expression is independent of sigma factor 28 (174). By making use of two different sigma factors, *Yersinia* could thus avoid the simultaneous expression of the two systems, which could functionally interfere with each other.

Role of RpoS on pYV-encoded virulence factor expression. Virulence genes of enteropathogenic bacteria are often regulated by growth phase and environmental signals. Iriarte et al. (173) wondered whether the alternative sigma factor RpoS is required for *Yersinia* virulence. They constructed a nonpolar *rpoS* mutant of *Y. enterocolitica* and analyzed the secretion of Yop proteins and the colonization of mouse tissues. The *rpoS* mutant secreted all the Yops as well as the wild-type strain did. In contrast to what has been observed with *Salmonella* (245, 374), there was no difference between the abilities of an *rpoS* mutant strain and its wild-type parent strain to colonize the Peyer's patches and spleen of mice after intragastric inoculation (173). Badger and Miller (18) similarly showed that a *Y. enterocolitica rpoS* mutant is not affected in virulence for mice.

Organization of the pYV Plasmid

Ancillary functions: replication and stabilization. The pYV plasmid is nonconjugative and incompatible with the sex factor F as a result of the presence of an incompatibility *incD* determinant which is part of the partition system (19, 28). This partition system, like that of F, consists of two proteins, called SpyA and SpyB, and a site called *spyC* (171, 355). The replicon is of the RepFIIA type (archetype R100), like that of many large antibiotic resistance and virulence plasmids (354). The replication machinery consists of an origin of replication (*oriR*) and two genes, *repA* and *repB*, encoding proteins of 33.5 and 9.5 kDa, respectively (354). RepA is the replicase, while RepB acts as a regulator (354). In the pYV plasmid of *Y. enterocolitica* O:9, the replication and partition regions are separated by about 20 kb, which contains the *yadA* gene and vestigial transposons, one of which is Tn*2502* (see below).

The genetic maps of the pYV plasmids from the various *Yersinia* species are quite similar except for the presence of the *ars* transposon and some reshuffling that occurred during evolution. The most striking differences between *Y. enterocolitica* and *Y. pseudotuberculosis* are a large inversion of almost half the plasmid (28) and, within the inverted region, the inversion of the region containing the partition system *spy* and the *yopE* and *sycE* genes (Fig. 3).

Operon encoding arsenic resistance in *Y. enterocolitica*. The pYV plasmid of the low-virulence strains of *Y. enterocolitica* (O:1,2,3, O:1,2, O:3, O:9, and O:5,27) contains a class II transposon, Tn*2502*, which confers arsenite and arsenate resistance (244). This resistance involves four genes: three are the homologs to the *arsRBC* genes present on the *E. coli* chromosome, but the fourth, *arsH*, has no known homolog. ArsR is an arsenite-inducible transcriptional repressor, ArsB forms a transmembrane channel, and ArsC catalyzes the reduction of arsenate to arsenite, which is in turn expelled by the ArsB transport system. One can only speculate on the role of the 26.4-kDa ArsH protein. The most likely hypothesis is that it acts as a regulator. The *ars* operon is not present on the pYV plasmid of the more virulent "American" strains (serotypes O:4, O:8, O:13a,13b, O:18, O:20, and O:21) of *Y. enterocolitica* or on the pYV plasmid of *Y. pseudotuberculosis* or *Y. pestis*. The presence of this operon thus constitutes the first significant difference between the virulence plasmids from different *Yersinia* species (244). It is also the first example of a virulence plasmid carrying resistance genes (244). The fact that the *ars* genes are present in all the serotypes of low-virulence *Y. enterocolitica* suggests that the low-virulence strains, which are distributed worldwide, constitute a single clone that probably emerged quite recently. At present, pigs represent the major

reservoir of pathogenic strains of *Y. enterocolitica*, and pork meat is recognized as the major source of human contamination (204, 343). Neyt et al. (244) speculated that the *ars* transposon might have favored the establishment of a strain of *Yersinia* in pigs. Arsenic compounds were largely used before World War II as therapeutic agents to protect pigs from diarrhea caused by *Serpulina hyodysenteriae*.

CONCLUSIONS AND FUTURE PERSPECTIVES

The Yop story can be compared to the fairy tale "the beauty and the beast," in the sense that it started as a nightmare with the somehow repulsive "Ca^{2+} dependency" phenomenon to end up as the innovative concept that we describe in this review. However, unlike a fairy tale, the Yop story is far from being complete. Many intriguing questions remain in every aspect of the system. Let us first consider the control of Yop synthesis and release. In spite of enormous efforts invested, it is fair to say that we still do not understand the feedback inhibition control. We have a candidate for a secreted repressor (LcrQ/YscM), but its mode of action is not understood. Contact control is one of the most appealing aspects of the system, but two major questions remain: (i) what is the degree of "directionality," and (ii) 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 an initial clue but certainly not the definite answer. Although several pieces of the control system have been identified and characterized at the bacterial surface, the actual bacterial ligand has not yet been identified. Is it YopN, the YopN-associated TyeA, or LcrG whose exact localization remains elusive? Another question concerning control is whether de novo protein synthesis is required for Yop translocation or is every bacterium equipped with a lethal dose of Yops before contact? Now, let us consider the translocation apparatus that is deployed at the interface between the bacterium and the eukaryotic cell. Although this is not firmly established, the existence of a pore is very likely, as suggested by the contact hemolysis shown by Håkansson et al. (138), but this pore remains 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 a pilus underneath YopB and YopD? What is the function of LcrG in translocation? Electron microscopy techniques such as those that provided wonderful images of the assembling flagellum (248) and of the type III secretion apparatus of *S. typhimurium* (194a) and structural biology will probably provide the conclusive evidence. Understanding the translocation process itself will probably still require years of effort by molecular biologists. 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 to assemble. However, in this area, the hottest question is that of the secretion signal. Does the Ysc apparatus recognize mRNA, and how? What is the exact role of the Syc chaperones? Are they bodyguards, pilots, or both? Finally, cell biology has a lot of answers to give. Why does *Yersinia* 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 and the action of others slower but irreversible (YopP/YopJ)? Alternatively, would some effectors be specifically designed for some cell types and others for other cell types?

Besides answering all these fascinating basic questions, one could also envision developing possible medical applications of the new concepts that came into sight. One can envision that such a sophisticated virulence apparatus could be an appropriate target for "antipathogenicity drugs." Would the spectrum of such drugs be broad enough? One may also consider engineered *Yersinia* cells as vectors to deliver antigens when a cytotoxic T-lymphocyte response is desirable. This second application is probably close to realization, at least in the laboratory.

In conclusion, research on *Yersinia* has been extremely fruitful in terms of concepts. Since the Yop virulon is at the leading edge of "type III secretion," it probably represents the most suitable system to investigate 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 led to new ideas, which we believe will pay off sooner or later.

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