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# MINIREVIEW

# Yops of *Yersinia* spp. Pathogenic for Humans

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## Yops

The *Yersinia* Yops are a set of virulence and virulence accessory factors synthesized and secreted by the three species of *Yersinia* pathogenic for humans in response to certain environmental cues (17). The genes encoding the Yops are found on a 70- to 75-kb plasmid that is highly conserved and essential for *Yersinia* virulence (17). These proteins will be discussed here with emphasis on the new paradigms they present for mechanisms of pathogenesis and environmental modulation of virulence gene regulation.

## PATHOGENESIS OF YERSINIAE

*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* all grow in the lymphoid tissue of humans and may cause invasive, systemic disease. They vary in their levels of invasiveness for animals, with the order generally being *Y. pestis* > *Y. pseudotuberculosis* > *Y. enterocolitica*. One exceptional serotype of *Y. enterocolitica* (O:8) matches or exceeds the virulence of *Y. pseudotuberculosis* in mice. *Y. pestis* is ordinarily transmitted by the bite of a flea, while the enteropathogenic *Y. pseudotuberculosis* and *Y. enterocolitica* are transmitted by an oral-fecal route. These yersiniae cycle between ambient environmental temperatures and 37°C in mammals. It is perhaps not surprising, therefore, that many *Yersinia* virulence factors are thermally regulated in their expression and/or activity.

To fully appreciate the role of Yops in *Yersinia* pathogenesis, it is important to consider several other virulence proteins that facilitate or modify Yop activity. At least two Yops function within eucaryotic cells. Effective bacterial adherence to the host cell is necessary for expression and functional deployment of these proteins (6, 69). Three *Yersinia* proteins, invasin (40), YadA (8), and an adhesin possibly related to the pH 6 antigen (2), provide productive bacterial attachment to the eucaryotic cell surface (4, 6, 69). Invasin promotes bacterial attachment to, and entry into, eucaryotic cells by binding with high affinity to multiple β1 integrins (38). β1 integrins comprise a widely expressed family of receptors that promote attachment of eucaryotic cells to extracellular matrix proteins such as fibronectin and collagen and function in cell-to-cell interactions and signal transduction (36). Invasin is chromosomally expressed in *Y. pseudotuberculosis* and *Y. enterocolitica* (40, 57). No functional invasin has been demonstrated in *Y. pestis*, although *inv*-homologous sequences are present (58). Invasin is maximally expressed at 28°C (39), which suggests that it may function early in infection, when the enteropathogenic yersiniae penetrate intestinal lymphoid follicles (Peyer's patches) (76).

YadA is a virulence plasmid-encoded fibrillar adhesin (43, 44) that is maximally expressed by *Y. pseudotuberculosis* and *Y. enterocolitica* at 37°C (8, 42). *Y. pestis* lacks this protein because of a frameshift mutation (71). YadA mediates yersinial attachment to host cells (33) and other targets, including fibronectin and collagen (19, 75, 89). YadA also promotes entry of *Y. pseudotuberculosis* into epithelial cells (6, 37, 41). This cellular attachment and entry process appears to be mediated by β1 integrins, and collagen or fibronectin expressed on the surface of eucaryotic cells could serve to link YadA and this host cell receptor (6, 41). The YadA entry pathway is inhibited by the antiphagocytic activities of YopE and YopH (see below and reference 6). The biological activity of YadA seems to favor ileal colonization especially to locally damaged areas, and, in concert with Yop functions, to prevent surface-attached bacteria from entering eucaryotic cells.

The pH 6 antigen (2) is a chromosomally encoded surface protein of *Y. pseudotuberculosis* and *Y. pestis* that is expressed maximally at 37°C and acidic pH (2, 49, 50). pH 6 antigen is likely responsible for thermoinduced binding of plasmid-cured *Y. pseudotuberculosis* invasin mutants to epithelial cells (4, 37) and may be the only Yop-productive adherence mechanism present in *Y. pestis*. Accordingly, pH 6 antigen is an essential virulence determinant of *Y. pestis* (50). Presumably, this determinant is important in systemic disease if the cellular inflammatory response, tissue necrosis, and products of bacterial metabolism create locally acidic conditions. Moreover, since the pH 6 antigen is expressed in the phagolysosome of macrophages, it may also play an intracellular role (50).

*Y. pestis* harbors two additional virulence plasmids that are not found in the enteropathogenic yersiniae. Each encodes a property that modifies or supplements Yop function, the fraction 1 and plasminogen activator protease (Pla) proteins. The antiphagocytic activities of YopE and YopH may be augmented by the presence of the fraction 1 protein capsule. Fraction 1 may help protect the bacterium from the onslaught of inflammatory polymorphonuclear leukocytes at the site of fleabite inoculation (15). The Pla protease is maximally active at 37°C and is localized to the outer membrane of *Y. pestis* (51, 52, 81, 87). Pla attacks several of the Yops, and rapid turnover results in low, steady-state amounts of the Yops on the bacterial surface in vitro (72, 73, 82). The Yops vary in their susceptibilities to Pla activity, and undegraded forms of YopM and YopN are secreted into the bacterial growth medium (66, 83). Because they lack Pla activity, the enteropathogenic yersiniae accumulate large

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amounts of Yops on their surface and shed these proteins into the medium (55, 62, 86).

## Yops: REGULATION AND SECRETION

The genes encoding the 10 or 11 known Yops are contained in both mono- and polycistronic operons scattered throughout the virulence plasmid (17, 84). The polycistronic operons also encode proteins involved in Yop regulation (e.g., *lcrGVHyopBD*) (3, 59). Additional polycistronic operons encode other regulatory proteins and ones involved in Yop secretion (22, 30, 54, 67, 84, 90). All *yop* operons examined so far and several of the regulatory operons belong to a regulon called the *yop* regulon, in which thermal induction is mediated by the plasmid-encoded LcrF protein and moderated by the chromosomally encoded YmoA protein (16–18, 34, 45, 91). At 37°C, the *yop* operons, most of the regulatory operons, and *ysc* operons specifying the Yop secretion apparatus are subject to a second level of regulation. Their transcription is downregulated ca. three- to two-fold in response to the presence of millimolar concentrations of $Ca^{2+}$ and nucleotides such as ATP and GTP (11). This regulation and its associated effect on growth of yersiniae in vitro have been termed the low-$Ca^{2+}$ response (LCR). The effect of the regulation on Yop secretion is especially strong and effectively shuts off the release of Yops (17). YopN, also known as LcrE, plays a major role in regulation by $Ca^{2+}$ (see below). Regulation by ATP occurs by a genetically distinct mechanism which so far has not been found to involve Yops (64).

The Yops lack classical bacterial N-terminal signal sequences and are exported without processing by a virulence plasmid-encoded secretion system (53, 55). A protein conformation present in the N-terminal domain appears to target Yops for release (53). This system is proving to be a complex mechanism which requires multiple gene products of several operons, including the large *ysc* locus, the multicistronic *lcrB* locus, and *lcrDR* (21, 30, 54, 60, 61). Homologs of LcrD with regulatory and secretory functions have been reported for other bacteria. One group of homologous sequences is associated with flagellar synthesis (e.g., in *Caulobacter crescentus* [65, 74], *Escherichia coli* [cited in reference 24], *Bacillus subtilis* [14], and *Campylobacter jejuni* [56]). Another group is associated with virulence in *Salmonella typhimurium* (24), *Shigella flexneri* (1), and several bacteria pathogenic for plants, e.g., *Pseudomonas solanacearum* (28) and *Xanthomonas campestris* (20).

## V ANTIGEN (LcrV)

The V antigen, also known as LcrV, is a 37- to 41-kDa protein that is subject to the same regulation as are the Yops. It escapes degradation by Pla and is secreted into the medium by the *ysc*-specified mechanism (78). V antigen is distinguished historically from the Yops (discovery in 1956 [13] versus 1981 for the Yops [62, 86]) and was found to have a regulatory role in the LCR in addition to its putative antihost function (3, 63). For these reasons it is called LcrV. However, some of the Yops are now known to be involved in LCR regulation (see below), and the distinction between LcrV and Yops may be mainly historical. LcrV is a protective antigen in all three yersiniae pathogenic for humans (11, 12) but does not accumulate on the surface of the enteropathogenic yersiniae or *Y. pestis* even in the absence of Pla (55, 78). Accordingly, it is believed to have an antihost function which can be neutralized by antibody. The regulatory role of LcrV is not well understood. LcrV is necessary for maximal expression of Yops and for a puzzling growth requirement associated with strong expression of the LCR in vitro (63, 80).

### YopE

YopE is a ca. 23-kDa cytotoxin that induces rounding and detachment of cultured cells from the extracellular matrix (69). Cytotoxicity appears to occur only after attachment of bacteria to the surface of the eucaryotic target cell (26, 62, 69). YopE present in culture supernatants or in sonicates of bacterial cultures is not active when added exogenously to eucaryotic cells (26, 62, 70). No functional expression of YopE from intracellular yersiniae has been detected (69). These observations suggest that YopE is introduced into the host cell cytoplasm by surface-attached yersiniae (26, 62, 69, 70). There it causes complete destruction of the actin microfilaments without significantly affecting intermediate filaments or microtubules (70). The mechanism of disruption is likely indirect, as YopE has no effect on actin microfilament polymerization or stability in vitro. The original view (26) for the role of cytotoxicity in *Yersinia* pathogenesis still holds (69): YopE may locally poison phagocytes. The full consequences of this activity are unknown, but YopE does have a demonstrable antiphagocytic effect in vitro on the uptake of yersiniae into macrophages (69) and epithelial cells (6).

### YopD

The ca. 33-kDa YopD protein is encoded by the last cistron of the *lcrGVHyopBD* operon (3, 59) and is necessary for the entry of YopE into a target cell (70). The predicted YopD sequence, unlike those of YopG, YopH, and YopQ, contains a centrally located potential transmembrane domain and a carboxy-terminal putative amphipathic helix (31). These are viewed as potentially having an active role in conducting YopE through the eucaryotic membrane (31, 70).

### YopB

The cistron upstream of *yopD* in *lcrGVHyopBD* encodes the ca. 42-kDa YopB protein (3, 59). This Yop protein also is predicted to have a hydrophobic character, with two centrally located putative transmembrane regions (31). Interestingly, its predicted sequence has significant similarity to those for the contact hemolysin IpaB of *Shigella flexneri* and the RTX family of hemolysins and toxins (31).

### YopH

The ca. 51-kDa YopH protein is a protein tyrosine phosphatase (29). A region of 262 amino acids in the YopH C terminus contains homology to eucaryotic protein tyrosine phosphatases and comprises the active domain of the enzyme (29). $YopH^-$ insertion mutants of *Y. pestis* and *Y. pseudotuberculosis* are significantly reduced in virulence (9, 85), and *Y. pseudotuberculosis* $YopH^-$ insertion mutants are less able than wild-type *Y. pseudotuberculosis* to resist phagocytosis by macrophages in vitro (68). Studies with *Y. pseudotuberculosis* strains that express catalytically inactive derivatives of this enzyme have shown that protein tyrosine phosphatase activity is the essential virulence function of YopH (7). The antiphagocytic activity of YopH in both macrophages and epithelial cells is dependent on protein tyrosine phosphatase activity (6, 35). YopH-specific dephos-

phorylation of eucaryotic proteins has been seen in macrophages and epithelial cells infected with *Y. pseudotuberculosis* in vitro (5, 7). The primary function of YopH may be to subvert the signal transduction processes of cells of the immune system such as phagocytes and lymphocytes during bacterial infection (5, 7, 29). YopH, either in purified form or when present in secreted Yop fractions, is inactive when added exogenously to eucaryotic cells (4). Like YopE, YopH presumably must be directed into the host cell cytoplasm by effectively attached bacteria. It is not known whether YopD plays a role in this process. Although no tyrosine phosphorylated proteins have yet been found in yersiniae, the possibility that YopH functions within the bacteria has not been ruled out. The amino-terminal 128 residues of YopH are homologous to LcrQ, a protein involved in negative regulation of the LCR (67). This domain of YopH could play a subtle role in the LCR, but a YopH$^-$ deletion mutant of *Y. pseudotuberculosis* appears to have normal LCR regulation (9).

### YpkA

Recently, *Y. pseudotuberculosis* was found to secrete an 81.7-kDa protein kinase named YpkA (25). This autophosphorylating enzyme was shown to have significant homology to eucaryotic Ser/Thr protein kinases and to be essential for virulence of yersiniae in mice (25). The *ypkA* gene is carried by the virulence plasmid, and proteins similar in size to YpkA among the Yops of *Y. pestis* and *Y. enterocolitica* have been documented. Hence, YpkA probably represents another *Yersinia* Yop showing remarkable similarity to a eucaryotic enzyme (25).

### YopM

The ca. 42-kDa YopM protein binds human α-thrombin but not prothrombin and can compete successfully with human platelets for thrombin (46, 66). YopM is relatively resistant to degradation by Pla in *Y. pestis* (47, 83) and may act extracellularly to sequester thrombin during the inflammatory response. Sequestration of thrombin as it is produced locally in small amounts from more abundant prothrombin could provide an anti-inflammatory effect (66). Accordingly, YopM$^-$ insertion mutants of *Y. pestis* and *Y. enterocolitica* have decreased virulence in mice (46, 59).

### YopJ

The ca. 31-kDa YopJ protein is not essential for the virulence of *Y. pestis* in mice challenged intravenously (85, 88). Hence, its role may lie in the initial stages of invasiveness that are bypassed in this animal model.

## YopN/LcrE AND OTHER SECRETED Lcr REGULATORY PROTEINS

The ca. 33-kDa YopN protein is identical to LcrE (22) and is important for negative regulation of Yop and LcrV expression at 37°C in the presence of $Ca^{2+}$ (22, 92). Several features of YopN/LcrE suggest an important role for this protein in the LCR regulation hierarchy. YopN/LcrE is localized to the bacterial surface (22) and escapes degradation by Pla in *Y. pestis* (83). Mutants that are unable to express YopN/LcrE on the bacterial surface are unable to downregulate Yop expression at 37°C in the presence of $Ca^{2+}$. Growth temperature has the major regulatory effect on YopN/LcrE expression: transcriptional and translational downregulation at 37°C in response to $Ca^{2+}$ is modest (approximately 3-fold compared with 5- to 2-fold for other Yops) (22, 90). However, the deduced amino acid sequence of YopN/LcrE does not contain a motif indicative of a $Ca^{2+}$-binding protein (90). Because of this, it has been suggested that YopN/LcrE does not bind $Ca^{2+}$ directly. In vivo, it might interact with some macromolecule which causes the same putative signal-transducing conformational change that $Ca^{2+}$ may induce in vitro (22). Additional proteins released during Yop secretion participate in the pathway of negative regulation by $Ca^{2+}$. These include LcrG (79) and LcrQ (67). Genetic data indicate that the 12.4-kDa LcrQ protein may act after YopN/LcrE in this pathway (67).

## OVERVIEW AND SPECULATION

The Yops appear to fall into two broad classes: those, like YopM and YopE, that have purely antihost functions and ones like YopN/LcrE, YopD, LcrG, and perhaps LcrQ that have predominantly regulatory and/or Yop-targeting functions. LcrV and perhaps YopH are bifunctional.

So far, despite the fact that many LCR operons have been sequenced, no transmitter or receiver motifs for two-component regulatory systems have been identified in the LCR regulatory system. Instead, yersiniae appear to have evolved a novel mechanism for regulating the synthesis and export of a set of antihost virulence proteins. The mechanism is complex, and many of its components have yet to be fully characterized. For example, the 13-cistron *ysc* locus contains the gene encoding LcrQ as well as other genes with regulatory and secretory functions that remain to be studied (27, 54, 67). Indeed, Yop secretion may be part of a feedback mechanism for transducing environmental signals into effects on LCR gene expression (17). Judging from the homologies that appear in taxonomically distant bacteria, the LCR regulatory mechanism may prove to be a new paradigm for bacterial regulation and secretion.

The role of $Ca^{2+}$ in LCR regulation is not yet understood. Prior to the accumulation of evidence indicating that yersiniae do not enter the cytoplasm of eucaryotic cells, it was proposed that the presence versus the absence of $Ca^{2+}$ signalled the presence of extra- versus intracellular environments, respectively (10). However, it is now apparent that bacterial entry into eucaryotic cells is not required for LCR induction, as yersiniae attached to the surface of host cells express functional Yop activity (4, 69). It is possible that $Ca^{2+}$ is not a physiologically relevant signal and that low-$Ca^{2+}$ conditions may artifactually upregulate the LCR operons in vitro by mimicking the relevant signals encountered at the host cell surface. Alternatively, $Ca^{2+}$ could be relevant if a low-$Ca^{2+}$ environment is present at the host cell surface or if the signal due to $Ca^{2+}$ is nullified by the interaction of the bacterium with a host cell macromolecule. This latter scheme provides a regulatory role for the abundant $Ca^{2+}$ in blood and interstitial spaces likely encountered by the bacterium during systemic infection.

Studies utilizing tissue culture infection models indicate that YopE and YopH are deployed into, and are active against, epithelial cells as well as phagocytic cell types (5–7, 68, 69). Bacterium-host cell interactions mediated by invasin and YadA are known to result in functional expression of YopE and YopH antiphagocytic activity, which effectively blocks penetration of yersiniae into cultured epithelial cells. Because of this, it is unclear how the bacteria effectively

penetrate the intestinal epithelial barrier to initiate infection. One could speculate that the tissue culture infection models inadequately reproduce the in vivo conditions. For example, there may exist additional signalling pathways that downregulate LCR-regulated operons, and one or more of these might operate on the luminal side of an M cell. Alternatively, there may be mechanisms of uptake into the M cells of the Peyer's patch that do not promote Yop delivery. It also is possible that there are other levels of Yop gene regulation that are active within the host environment. Differential Yop expression of this type has been considered ever since the Yops were found to be encoded by multiple operons scattered on the virulence plasmid (e.g., see reference 85). An additional layer of regulation for the YopE cytotoxin is indicated by the *yopE*-specific negative regulatory locus *yerA* that is situated adjacent to the monocistronic *yopE* operon (23).

An important outcome of the intracellular targeting of YopE and YopH is that these proteins might be protected from neutralization by antibody. The prediction can be made that these Yops would not be protective antigens. This is in contrast to Yops such as YopM, YopN, and LcrV, which have extracellularly exposed domains. Direct targeting to the eucaryotic host cell cytoplasm may also protect Yops from degradation by Pla in *Y. pestis*.

The Yop expression system appears to be involved in extracellular survival of yersiniae in the hostile environment of the mammalian reticuloendothelial system. This is accomplished in part by preventing phagocytosis of yersiniae (YopH and YopE), poisoning leukocytes (YopE), subverting signal transduction within cells of the immune system (YopH and presumably YpkA), and sequestration of thrombin (YopM). A likely consequence of this multifactorial process is a delay in the development of a cell-mediated immune response. It may be that yersiniae are exposed to the intracellular environment only during the initial stages of infection, while the primary growth phase during systemic infection occurs extracellularly. Available histopathological evidence supports this concept for enteropathogenic *Yersinia* infection of rodents (32, 48, 77).

The *Yersinia* Yops provide a wealth of insights into new pathogenic mechanisms and present a new paradigm for how bacteria may sense and respond to environmental cues. Future studies should provide a better understanding of *Yersinia* pathogenesis as well as unique insights into the cell biology of the host.

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