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YOPH IS DEGRADED INTO FRAGMENTS THAT RETAIN PROTEIN TYROSINE PHOSPHATASE ACTIVITY IN YERSINIA PESTIS

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YOPH IS DEGRADED INTO FRAGMENTS THAT RETAIN PROTEIN TYROSINE PHOSPHATASE ACTIVITY IN *YERSINIA PESTIS*

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ABSTRACT

YOPH IS DEGRADED INTO FRAGMENTS THAT RETAIN PROTEIN TYROSINE PHOSPHATASE ACTIVITY IN *YERSINIA PESTIS*

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The mechanism of virulence of *Yersiniae* involving the protein tyrosine phosphatase YopH was studied. Cells were fractionated into subcellular compartments and the PTPase activity of each fraction was analyzed. Irrespective of species, more than 95% of the total PTPase activity was located within the cytoplasm and culture media. In the Pst^+ strain of *Y. pestis*, diminished extracellular PTPase activity was due to degradation by a Pst plasmid product, presumably the plasminogen activator. The molecular state of YopH PTPase in Pst^+ and Pst^- strains of *Y. pestis* were further analyzed by, immunoblot and FPLC Superose 12 gel filtration. This analysis showed that YopH PTPase exists in one enzymatically active form (44 kDa) in the cytoplasm of a Pst^- strain of *Y. pestis*. More significantly, additional smaller, enzymatically active forms (33 to 37 kDa) of YopH were found to exist in the cytoplasm and extracellular surroundings of the Pst^+ strain of *Y. pestis* .

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INTRODUCTION

Tyrosine phosphorylation is involved in the regulation of cellular processes responsive to environmental stimuli including cell cycle control, signal transduction and oncogenesis. The antagonistic relationship between the protein tyrosine kinases (PTKs) and the protein tyrosine phosphatases (PTPases) is responsible for maintaining a steady state of cellular tyrosine phosphorylation. The involvement of PTKs in cellular processes has already been established. In contrast, the PTPases have only recently been recognized. PTPases are assumed to play an equally important role in regulation and the study of PTPases is currently an expanding area of research.

The PTPase family includes both membrane-bound and cytosolic proteins (Figure 1). The transmembrane PTPases are characterized by an extracellular domain, one membrane-spanning hydrophobic domain, and two PTPase catalytic domains repeated in tandem. The first PTPase domain is enzymatically functional; the second domain is not enzymatic but appears to regulate the substrate specificity of the first (Streuli, 1990). The cytosolic PTPases lack any extracellular or transmembrane domain and have only one PTPase domain. All of the characterized PTPases share a molecular "fingerprint" of conserved residues surrounding the enzyme active site, including a cysteine residue essential for catalysis (Charbonneau, 1989). This "fingerprint" has made possible the identification of multiple PTPases by using low-stringency hybridization techniques.

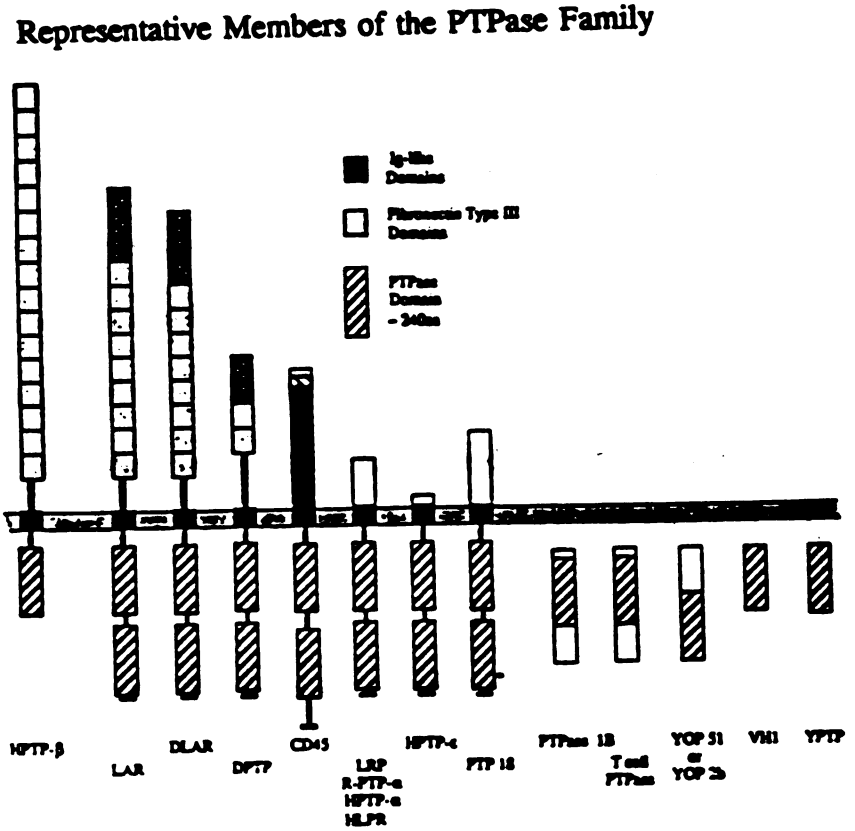

Figure 1. Schematic depiction of the structure of representative members of the transmembrane and cytosolic classes of PTPases (Trowbridge, 1991). YopH (third from right, also designated Yop51 or Yop2b) contains a PTPase domain with an additional N-terminal sequence.

Transmembrane PTPases.

The CD45 (LCA, B220, T200) family of large, heterogeneous glycoproteins (180-220 kDa) is found on all nucleated hematopoietic cells. Tonks et al. (1988a) demonstrated that CD45 has intrinsic PTPase activity. The catalytic C-terminal segmentof CD45 is highly conserved among human, rat and mouse while the external domain of CD45 is very heterogeneous. Exons 4, 5, and 6 can be used selectively to make eight different messages for the external domain. All eight of these combinations have been identified in murine CD45 (Rogers, 1992). Usage of the alternate exons is cell lineage specific with B cells expressing all three alternate exons and T cells predominately expressing none or one exon (Chang, 1989). Recent investigation indicates that exons 7 and 8 are also used selectively in immature ($CD4^-$, $CD8^-$) murine T cells (Chang, 1991). The alternate exons encode segments rich in O-linked glycosylation sites, thereby potentially adding additional heterogeneity to the molecule. The lineage-specific isoform expression of CD45 suggests that the molecule may interact with different ligands on different types of leukocytes.

CD45 is required for signal transduction in response to antigen stimulation in B cells. The state of antigen receptor tyrosine-phosphorylation in resting B cells is maintained by PTPases (Lin, 1992). The ability to transduce second messenger signals was restored to a $mIgM^+$, $CD45^-$ cell line after transfection of CD45 into the cells (Justement, 1991). The small proteins associated with mIgM are responsible for transmitting the signal for activation when the antigen receptor is occupied. These accessory proteins (IgMα, Ig-β and Igγ) are phosphorylated on tyrosine residues in

response to anti-IgM antibody activation (Gold, 1991). Each of these proteins is a substrate for CD45 *in vitro* (Justement, 1991).

The T cell PTK $p56^{lck}$ associates with the T cell surface antigens CD4 and CD8 (Veillette, 1988a) and is required for T cell activation (Glaichenhaus, 1991). The activity of $p56^{lck}$ is regulated by tyr phosphorylation at two positions. The phosphorylation of tyr505 negatively regulates the autophosphorylation of tyr394 (Veillette, 1988b; Amrein, 1988). After dephosphorylation of tyr505, tyr394 is autophosphorylated and the kinase is enzymatically active toward exogenous substrates (Marth, 1988). An active CD4/CD8-$p56^{lck}$ complex can phosphorylate CD3 γ, δ, ϵ and ζ chains *in vitro*. (Barber, 1989). CD45 mediates the dephosphorylation of tyr505 (Mustelin, 1989; Ostergaard,1989). T cell lines ($CD4^+$ or $CD8^+$) defective in CD45 expression are dysfunctional in activation-dependent functions (Pingel, 1989; Weaver, 1991).

CD45 is also involved in the interaction between B cells and T cells. CD22β, a B cell surface protein required for Ag response, is an adhesion molecule. It interacts with CD75 on other B cells and with the zero alternate exon form of CD45 on T_h cells (Stamenkovic, 1991).

The leucocyte common antigen related protein (LAR) is also a PTPase. LAR mRNA is found in varying amounts in many cell types including epithelial and endothelial cells (Streuli, 1992). LAR is a large protein with an intracellular segment homologous to CD45. The extracellular segment is comprised of three immunoglobulin-like domains followed by 8 fibronectin type III domains (Streuli, 1988). This pattern of structural motifs resembles the NCAM family of cell adhesion molecules. LAR is synthesized as a 190 kDa protein. During transport to the cytoplasmic membrane,

hydrolysis occurs several residues NH_2-terminal to the membrane-spanning domain producing two LAR products. The 145 kDa E-subunit (external domain) and the 85 kDa P-subunit (PTPase domain) remain non-covalently associated with each other at the cell surface (Streuli, 1992; Yu, 1992). When LAR-expressing cells are grown at saturation density the E-subunit is shed from the cell surface (Streuli, 1992). Release of the E-subunit may regulate the activity of the P-subunit.

Other transmembrane PTPases have been identified through hybridization experiments. HPTPβ is noteworthy as the only membrane-bound PTPase described to have one PTPase domain (Krueger, 1990). Additional transmembrane PTPases are listed in Figure 1.

Cytosolic PTPases.

Protein tyrosine phosphatase 1B (PTP1B) was isolated from both the soluble and particulate fractions of human placenta (Tonks, 1988b). From the cDNA for PTP1B a 50 kDa protein of 435 amino acids was predicted. PTP1B has been described to be functional in two forms, the full length protein (Frangioni, 1992) and a form truncated from the C-terminus (321 amino acids) (Tonks, 1988b). Two structural domains were apparent from the protein sequence: a catalytic PTPase domain and a very hydrophobic tail. Polyclonal anti-PTP1B antibodies localized PTP1B in vivo to the cytoplasmic face of the endoplasmic reticulum (Frangioni, 1992). The 30 amino acid C-terminal hydrophobic tail was identified as the determining factor in the localization of PTP1B, suggesting that the previously reported 321 amino acid form of PTP1B was an artifact

of purification, and not found *in vivo*. The function of PTP1B at the external face of the ER could be to regulate ER-bound proteins by tyrosine dephosphorylation.

T cell PTPase (TCPTP) was isolated from a T cell cDNA library (Cool, 1989). Subsequently TCPTP mRNA has been found in other tissues. TCPTP is very similar to PTP1B. The catalytic domain of TCPTP is 74% homologous to the catalytic domain of PTP1B. The 11 kDa C-terminal region is required for localization of the molecule to the cellular particulate fraction (Cool, 1990). Deletion of the C-terminal region results in a constitutively active PTPase. Over-expression of the truncated form in baby hamster kidney (BHK) cells led to multinucleation of the cells. Unlike most multinucleated cells, the nuclei of the BHK cells transfected with the truncated TCPTP did not divide synchronously (Cool, 1992). These data imply a function for TCPTP in cytokinesis and cell cycle signaling.

Yersinia outer membrane protein H (YopH) is a plasmid-encoded protein common to the human pathogenic *Yersiniae* species and the only PTPase proven to be a virulence factor. The 45 kDa protein has three structural domains. The first 48 amino acids form a conformation required for the protein to be exported out of the cell. Amino acids 48-127 are homologous to Lcr Q, a recently described protein involved in regulation of Yop transcription. The remaining 341 amino acids comprise the PTPase domain (Rimpilainen, 1992). YopH acts synergistically with plasmid-encoded YopE to inhibit phagocytosis of *Yersinia* (Rosqvist; 1988; 1990; 1991). Infection of macrophages *in vitro* by $YopH^+$ bacteria resulted in the dephosphorylation of two phosphotyrosine-containing host proteins that were unaffected by infection with a $YopH^-$ strain (Bliska, 1991). Generally, bacteria do not use tyrosine phosphorylation to modulate cellular processes.

Therefore, it has been proposed that YopH is of eukaryotic origin. The structure of YopH agrees with such a hypothesis (Guan, 1990). The disruption of activation signals in macrophages is an example of the discord an unregulated exogenous PTPase could inflict on the metabolism of a cell.

Additional cytosolic PTPases are listed in Figure 1. Two worthy of an additional note are cdc25 and VH1. cdc25 plays a key role in the cell cycle transition from G_2 to M (Kumagai, 1991). VH1 is a Vaccinia viral protein that may prove to be a virulence factor (Guan, 1991).

Yersinia Virulence Determinants.

The genus *Yersinia* includes three human pathogenic species; *pseudotuberculosis*, *enterocolitica*, and *pestis*. *Y. pseudotuberculosis* and *Y. enterocolitica* typically cause gastrointestinal illnesses. *Y. pestis* is the causative agent of bubonic plague, a disease which has been responsible for nearly 200 million deaths throughout history.

The virulence of these species has been attributed to a 70 kb low Ca^{2+} response (Lcr) plasmid which under conditions of low Ca^{+2} concentration (<2.5 mM) and high temperature (37°C) restricts vegetative growth of the organism and induces production of large quantities of plasmid proteins including the virulence factors: Virulence antigen (V Ag) and Yops (Cornelis, 1991; Straley, 1991). The increased transcription of Lcr plasmid products is mediated by a positive loop that is responsive to an upward temperature shift to above 34°C and a negative loop responsive to an extracellular Ca^{2+} concentration above 2.5 mM. The proteins of these regulatory loops are encoded at the Ca^{2+}-dependence region, a 20kb section of the Lcr plasmid (Straley, 1991).

Proteins Lcr B, C, D, F, V, and K are involved in the positive loop of the low calcium response (Figure 2), however a specific role for each has not been discerned (Straley, 1991). In response to increased temperature the transcription activator Lcr F binds Yop promotors causing induction of Yop gene transcription. The expression of Lcr F is autoregulated in response to temperature (Cornelis, 1986). Lcr F has been sequenced and found to be homologous to the DNA-binding protein AraC in *Escherichia coli* (Cornelis, 1989). Lcr V enhances transcription of itself and the Yops (Bergman, 1991). Lcr K is needed for Yop secretion from the cell (Rosqvist,1990). At temperatures below 34°C, Ca^{2+} has no effect on growth, and V Ag and Yops are produced at a basal rate.

Lcr E, H, Q and R mediate the negative effects of calcium (Fig. 2) (Straley, 1991). Lcr H is a repressor for Ca^{2+} and nucleotide regulated genes (Bergman, 1991). At Ca^{2+} concentrations above 2.5 mM the repressor is made in high quantities and Yop transcription is repressed. Lcr Q is involved in the early stages of Ca^{2+} response (Rimpilainen, 1992). The function of the other proteins is not known.

Products of the Lcr plasmid include the V Ag and the Yops. V Ag is required for virulence (Brubaker,1991). Its function outside of the cell is unknown, but it is suspected to be involved in host immunosuppression (Brubaker, 1991). The protein is secreted from the bacteria by a chaperon-mediated system and does not accumulate in the outer membrane (Straley, 1981). There are thirteen identified Yops. Possible functions of Yops B, C, F, G, I and J are unknown. YopE is cytotoxic (Rosqvist, 1990). Intracellular microinjection of YopE into HeLa cells caused the disruption of the cellular actin microfilament structure (Rosqvist, 1991). YopD is involved in the translocation of

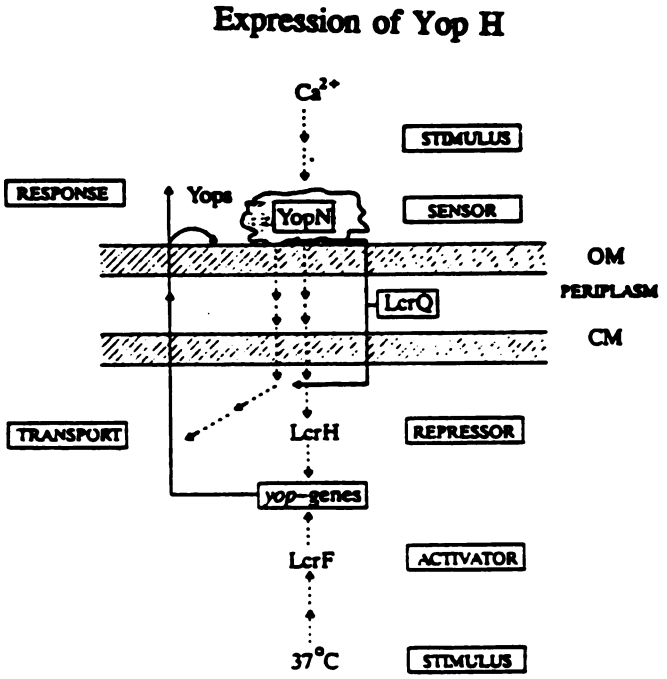

Figure 2. Proposed model of Yop transcription regulation by low calcium concentration and elevated temperature. At calcium concentrations above 2.5 mM the *yop* gene repressor Lcr H is produced and Yop transcription is inhibited. YopN has been suggested as the external calcium sensor. Lcr Q acts between YopN and Lcr H. At temperatures exceeding 34°C the activator Lcr F is produced and *yop* gene transcription is enhanced. OM-outer membrane, CM-cytoplasmic membrane (diagram taken from Rimpilainen, 1992).

YopE across the plasma membrane of the target cell during infection (Rosqvist, 1991). YopE works synergistically with YopH to inhibit phagocytosis. YopH has PTPase activity and has been associated with the dephosphorylation of specific phosphotyrosine residues of macrophages (Guan, 1990; Bliska, 1991). Yops K and L are required for successful growth in newly infected tissues (Brubaker, 1991). YopM is homologous to the platelet surface protein GPIbα, a protein that binds thrombin and initiates the clot formation cascade and other inflammatory responses (Reisner, 1992). YopN has been implicated as a front line sensor of environmental Ca^{2+} concentration in the negative loop of Lcr plasmid gene transcription (Forsberg, 1991).

Characterization of the Lcr plasmid gene products has increased the understanding of *Yersinia* virulence, but since the plasmid is common to all three species, it does not explain why *Y. pestis* causes a more serious disease. *Y. pestis* harbors two additional plasmids not found in *Y. pseudotuberculosis* or *Y. enterocolitica*. The first is a 100 kb exotoxicity (Tox) plasmid which encodes a capsular antigen and a rodent-specific exotoxin (Brubaker, 1991). The second is a 10 kb Pesticinogeny (Pst) plasmid which confers upon *Y. pestis* the ability to make the bacteriocin, pesticin, the immunity protein to pesticin, and an outer membrane-bound protease, the plasminogen activator (Sodeine, 1988a).

The Tox plasmid was considered to be functionally inactive until recently when it was recognized to encode a rodent-specific toxin and the capsular protein (Fraction 1) (Brubaker, 1991). The exotoxin is highly lethal to mice and rats but has no effect on other hosts. Its mode of action is unknown. The capsule antigen is a protein and

polysaccharide complex (Glosnicka, 1980). One function of the capsule during disease is to aid in the prevention of bacterial phagocytosis after infection.

The Pst plasmid substantially contributes to the increased virulence of *Y. pestis*. The bacteriocin, pesticin, aids in colonization by hydrolyzing the cell wall peptidoglycan of species closely related to *Y. pestis* (Ferber, 1979). The plasminogen activator protease plays a fundamental role in the heightened virulence of *Y. pestis*. The protein is synthesized as a 38 kDa molecule. Upon insertion into the outer membrane auto-hydrolysis occurs forming a 35 kDa protein (Sodeinde, 1988a). The plasminogen activator promotes the conversion of plasminogen to plasmin leading to the break up of clots isolating the bacterium and is responsible for the dissemination of the bacterium from the initial site of infection (Beesley, 1967). The cleavage products of plasminogen produced by the serine protease urokinase and the plasminogen activator are indistinguishable from each other by SDS-PAGE, suggesting that the plasminogen activator may also be a member of the trypsin family of serine-proteases (Sodeinde, 1992).

In addition to initiating the clot dissolution cascade, the plasminogen activator may enhance the ability of *Y. pestis* to evade host defense mechanisms by hydrolyzing some of the Lcr plasmid Yop proteins. The Yop proteins of the non-pesticinogenic *Yersinia* species accumulate in the outer membrane and are extremely antigenic. In the pesticinogenic *Y. pestis*, some Yop proteins are hydrolyzed by the plasminogen activator presumably during translocation through the outer membrane (Sodeinde, 1988b). Yops M and N appear to avoid degradation and YopE is degraded into a functional product (Reisner, 1992; Mehigh, 1989). Yops H, K and L are hydrolyzed but are still required

for virulence, suggesting that they too may be processed into forms retaining fundamental activities. It is possible that *Y. pestis* specifically trims the Yops into functional smaller pieces that are less antigenic thereby delaying an immune response from the host. Such modification is supported by the observation that passive anti-*Y. pestis* Yops antibodies confer immunity to the enteropathogenic *Y. pseudotuberculosis* but are not protective against *Y. pestis* (Brubaker, 1991). The continual turnover of membrane proteins mediated by the membrane-bound plasminogen activator would contribute to the increased virulence of *Y. pestis* and explain why *Y. pestis* requires Yops for virulence even though the proteins are hydrolyzed after synthesis.

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CHAPTER 2

YopH is Degraded into Fragments that Retain Protein Tyrosine Phosphatase Activity in *Yersinia Pestis*

FOOTNOTES

Abbreviations: PTPase, protein tyrosine phosphatase; FPLC, fast protein liquid chromatography.

ABSTRACT

The mechanism of virulence of *Yersinia pestis* involving the protein tyrosine phosphatase, YopH, was studied by the analysis of the subcellular distribution of phosphatase activity and by the examination of the proteolytic processing of YopH phosphatase. The intracellular distribution of YopH protein tyrosine phosphatase (PTPase) activity was compared in the human-pathogenic *Yersinia* species; *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. pestis* D34 (Pst^-) and *Y. pestis* D27 (Pst^+). Cells were fractionated into subcellular compartments and the PTPase activity of each fraction was analyzed. Irrespective of species, more than 95% of the total PTPase activity was located within the cytoplasm and culture media. Less than 5% was found in the inner membrane, periplasm, and outer membrane compartments combined. This distribution supports the concept that YopH PTPase activity is transported rapidly to the extracellular environment without accumulating significantly in the inner or outer membrane, or in the periplasm. In the Pst^+ strain of *Y. pestis*, diminished extracellular PTPase activity was due to degradation by a Pst plasmid product, presumably the plasminogen activator. However, significant amounts of PTPase activity were detectable in both the cytoplasm and extracellular fraction of the Pst^+ strain of *Y. pestis*. Examination of the subcellular distribution of PTPase specific activity indicated a relative enrichment of PTPase activity in the outer membrane of each *Yersiniae* studied. The molecular state of YopH PTPase in Pst^+ and Pst^- strains of *Y. pestis* was further analyzed by immunoblot with anti-YopH and by FPLC Superose 12 gel filtration. This analysis showed that YopH PTPase exists in one enzymatically active form (44 kDa) in the cytoplasm of a *Y. pestis* strain cured of the Pst plasmid (Pst^-). More significantly, additional smaller, enzymatically active

forms (33 to 37 kDa) of YopH were found to exist in the cytoplasm and extracellular surroundings of *Y. pestis* (Pst^+). These results show that small but significant amounts of the processed YopH PTPase were released from Pst^+ cells and that several of the processed YopH forms retained PTPase activity. The virulence of *Y. pestis* (Pst^+) may thus depend on this released PTPase.

INTRODUCTION

The genus *Yersinia* includes three human pathogenic species; *pseudotuberculosis*, *enterocolitica*, and *pestis* (reviewed in reference 3). *Y. pseudotuberculosis* and *Y. enterocolitica* typically cause gastrointestinal illnesses. *Y. pestis* is the causative agent of the bubonic plague, a disease which has been responsible for nearly 200 million deaths throughout history.

The virulence of these species has been attributed to a 70 kb Low Ca^{2+} response (Lcr) plasmid which, under conditions of low Ca^{+2} concentration (<2.5 mM) and high temperature (37°C), down regulates vegetative growth of the organism and induces production of large quantities of plasmid proteins (5,20). These proteins include the *Yersinia* outer membrane proteins (Yops), a group of at least thirteen virulence factors that are not membrane bound, but instead appear to be transient in the outer membrane as they are released into the extracellular surroundings (7,9). Yops K, L, M and E aid in the initial bacterial growth within newly infected tissue and are required for full virulence (3). YopE is cytotoxic and acts synergistically with YopH, another essential factor, to inhibit phagocytosis (13,14,15). Recently, YopH was identified as a member of the protein tyrosine phosphatase (PTPase) family (6). This is a particularly interesting observation because bacteria typically do not phosphorylate tyrosine residues. However, the state of tyrosine phosphorylation in eukaryotic cells is involved in the regulation of many essential functions and physiologic responses.

Characterization of the Lcr plasmid gene products has increased the understanding of *Yersinia* virulence, but since the plasmid is common to all three species it does not explain why *Y. pestis* causes a more serious disease. Understanding of the increased

virulence was further complicated when it was discovered that Yops do not accumulate in the outer membrane of *Y. pestis*, as they do in *Y. pseudotuberculosis* and *Y. enterocolitica* (16,19). Although no net increase in Yops is seen, evidence of Yop usage *in vivo* exists; including a mutation rendering the cells YopE$^-$ or YopH$^-$ causes the bacterium to become avirulent (8).

Y. pestis harbors two additional plasmids not found in *Y. pseudotuberculosis* or *Y. enterocolitica*. The first is a 100 kb exotoxicity (Tox) plasmid which encodes a capsular antigen and a rodent-specific exotoxin (3). The second is a 10 kb Pesticinogeny (Pst) plasmid which confers upon *Y. pestis* the ability to make the bacteriocin pesticin, the immunity protein to pesticin, and an outer membrane-bound protease, the plasminogen activator (17). Investigation into the Pst plasmid led to the discovery that the plasminogen activator was digesting some of the Yops of *Y. pestis* after they were synthesized in the bacterial cytoplasm (18).

The advantages of Yop degradation in *Y. pestis* are largely unknown. However, YopE provides a model to explain why *Y. pestis* requires Yops for virulence, even though the proteins are hydrolyzed after synthesis. In *Y. pestis*, YopE exists as a stable degradation product that retains an essential function (16). It is possible that *Y. pestis* trims the Yops into functional smaller pieces that are less antigenic thereby delaying an immune response from the host. Such modification would contribute to the increased virulence of *Y. pestis*.

To address the question of the role of YopH post-translational modification in *Y. pestis* virulence, we asked if YopH could exist as a stable cleavage fragment with enzymatic activity. We compared the intracellular location of YopH PTPase activity in

Y. pseudotuberculosis, *Y. enterocolitica*, *Y. pestis* D34 (Pst^-) and *Y. pestis* D27 (Pst^+). In addition, biochemical analysis indicated that YopH existed in multiple catalytically functional forms in *Y. pestis*.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study were *Y. pseudotuberculosis* PB1, *Y. enterocolitica* WA, and *Y. pestis* KIM. Their relevant characteristics are described in Table I.

Media and Culture Conditions. Bacteria were grown as previously described (20). Stocks preserved in glycerol at -20°C were transferred to tryptose blood agar base (Difco Laboratories, Detroit, MI) and incubated at 26°C. Zahorchak modified Higuchi's medium (24) was inoculated from slants and the bacteria allowed to complete six doublings at 26°C before being diluted to an optical density at 620nm of 0.1 to 0.2 units. The diluted culture was left at 26°C until growth had restarted, then shifted to 37°C to induce the Lcr^+ bacteria to cease vegetative growth and increase Yop synthesis.

Subcellular Fractionation of the *Yersiniae* Species. Bacteria were fractionated using the method of Osborn and Munson (11) modified for *Yersinia* (20). Briefly, the cells were forced into spheroplasts by treatment with lysozyme and EDTA, sonicated, and centrifuged. The cytoplasmic contents were found in the supernatant and the inner and outer membranes in the pellet. The two membranes were separated by centrifugation through an isopycnic sucrose gradient.

FPLC Separation of Cytoplasm and Culture Media Components. Cells were pelleted and the culture media collected. Sonication was used to lyse the harvested cells. The culture media was precipitated with ammonium sulfate and dialyzed over night in 0.05M Tris-HCl, pH 8.0. The cytoplasm and culture media preparations were centrifuged at 18,000 rpm for 30 min at 4°C. The final supernatant fractions were filtered through a low protein-binding membrane. The total protein was measured by the

Table I. The characteristics of the bacterial strains used in this study.

Strain	Designation	Lcr plasmid	Pst plasmid
Y. pseudotuberculosis PB1	B15	+	-
	B16	-	-
Y. enterocolitica WA	D21	+	-
	D22	-	-
Y. pestis KIM	D27	+	+
	D28	-	+
	D34	+	-
	D35	-	-

Bradford protein-dye binding assay (2). Samples were diluted to 0.3 mg/ml in elution buffer (2.7 mM KCl, 1.1 mM KH_2PO_4, 1.4 M NaCl, 8.1 mM Na_2HPO_4, 10 mM DTT) and 60 μg of protein added to an FPLC Superose 12 sizing column (Pharmacia, Piscataway, NJ). Fractions were collected into BSA (final concentration 0.5 mg/ml) to stabilize the dilute protein solution. Storage was at -20°C.

PTPase Reaction Substrate Preparation. Radiolabeling of the synthetic peptide Raytide (Oncogene Science, Manhasset, NY) at its single tyrosine residue was performed as follows. Raytide (10 μg) was incubated overnight at 37°C with 2.0 units $pp60^{c\text{-}src}$ tyrosine kinase (Oncogene Science), γ-^{32}P-ATP (NEN/Dupont, Wilmington DE), 15 mM ATP and 30 mM $MgCl_2$, in kinase assay buffer (50 mM HEPES, pH 7.5; 0.1 mM EDTA; 0.015% Triton X-100). The Raytide was recovered by addition of trichloroacetic acid (TCA) (22) (0.1ml of 5 mg/ml bovine serum albumin (BSA) and 0.5 ml of cold 10% TCA/20 mM NaH_2PO_4) to the kinase reaction mixture. Precipitation occurred over 30 min on ice. The protein pellet was washed three times with 10% TCA/20 mM NaH_2PO_4 before being dissolved in 0.1 ml 0.2M Tris-HCl, pH 8.0.

PTPase Assay. The tyrosine phosphatase activity of the samples was evaluated by a modification of methods described previously (22). The assay reaction components were 5 μl of 10X phosphatase assay buffer (250 mM HEPES, pH7.5; 50 mM EDTA; 100 mM DTT), 5μl of sample, 0.7-1.0 X10^5 cpm of ^{32}P-Raytide and ddH_2O for a final volume of 50μl. Reactions were incubated at 37°C and stopped by the addition of 750 μl of acidic charcoal mixture (0.9 M HCl, 90 mM $Na_4P_2O_7$; 20 mM NaH_2PO_4; 4% w/v Norit A (Sigma Chemical Co., St. Louis, MO)). The proteins were absorbed by the acidic charcoal, leaving the released $^{32}PO_4$ free. The charcoal was pelleted and 0.4 ml

of the supernatant added to 8 ml of scintillation cocktail and the amount of released $^{32}PO_4$ measured.

Immunoblot. Proteins were precipitated on ice for 15 minutes with 10%TCA/20 mM NaH_2PO_4 and washed three times in cold absolute ethanol before being dissolved in SDS-PAGE sample buffer containing DTT. A 4-15% gradient acrylamide gel was used to separate the proteins by molecular weight before they were transferred to a nitrocellulose membrane and probed first with a polyclonal rabbit anti-YopH antibody (the generous gift of Dr. Hans Wolf-Watz, Umea, Sweden) and secondly with a goat anti-rabbit IgG-horseradish peroxidase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN). Peroxidase activity was detected with the enhanced chemiluminescence (ECL) Western blot detection reagents from Amersham (Arlington Heights, IL).

RESULTS

Localization of PTPase activity in the human-pathogenic *Yersiniae*. It has been reported that Yops are associated with the outer membrane of Yersinia. However, since Yops do not have a hydrophobic domain for membrane insertion the nature of their interaction with the outer membrane is unclear (3). Subcellular fractionation allowed each compartment of the cell to be evaluated individually for the presence of active YopH PTPase. The PTPase activity distribution among the subcellular fractions of *Y. enterocolitica* WA, *Y. pseudotuberculosis* PB1, *Y. pestis* D34 (Pst^-) and *Y. pestis* D27 (Pst^+) was compared, as shown in Figure 1. PTPase activity was monitored by the dephosphorylation of ^{32}P-Raytide, a reported substrate for YopH (6). Each compartment was evaluated against the same compartment of an identical strain cured of the Lcr plasmid (Lcr^-). None of the Lcr^- samples had any detectable PTPase activity (data not shown). A comparison of individual subcellular divisions across the Pst^- strains showed a low percentage (<5%) of the total enzyme activity in the inner membrane, periplasm, and outer membrane compartments combined. At least 95% of the total PTPase activity was localized to the cytoplasm of the bacteria or the surrounding culture media. This distribution reflects the transient nature in the membrane compartments of a protein that is rich in the cytoplasm and accumulates extracellularly. The Pst^+ strain, *Y. pestis* D27, had <5% of the total PTPase activity in the membrane compartments plus the culture medium, leaving the cytoplasm as the only compartment rich in catalytically active YopH.

The sites of YopH PTPase enrichment were identified by a comparison of PTPase specific activity found in each of the subcellular fractions (Figure 2). Examination of the

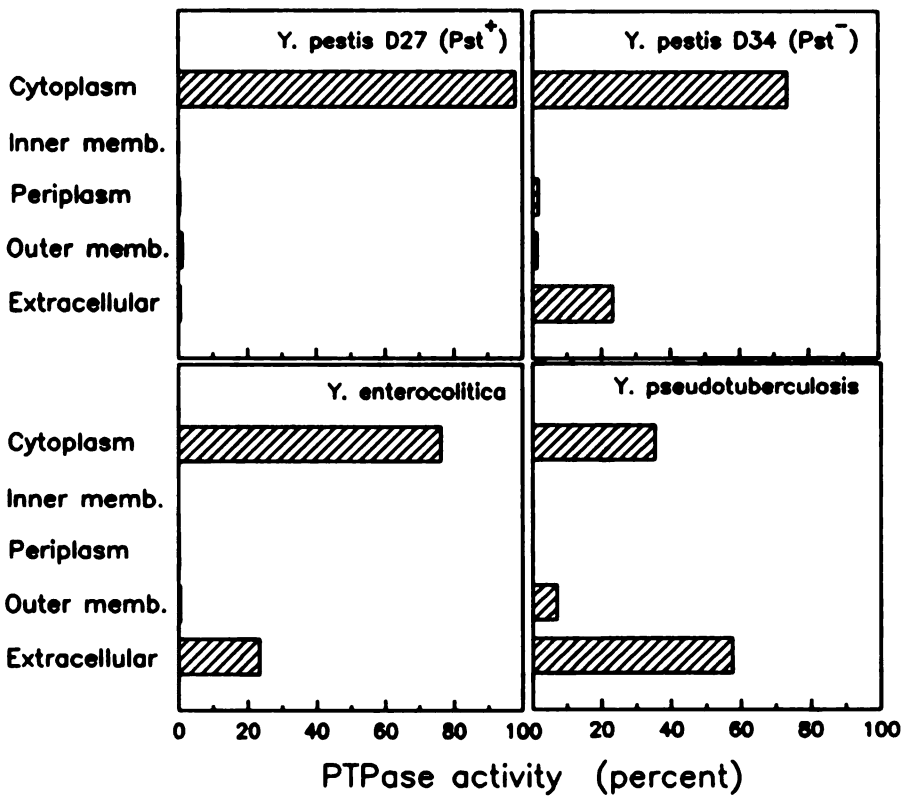

Figure 1. Percent distribution of YopH PTPase activity in subcellular fractions of *Yersiniae*. Lcr$^+$ species of *Yersinia* were grown in conditions maximal for Yop synthesis. Cellular fractions were assayed for PTPase activity (U=μmol $^{32}PO_4$ released per min).

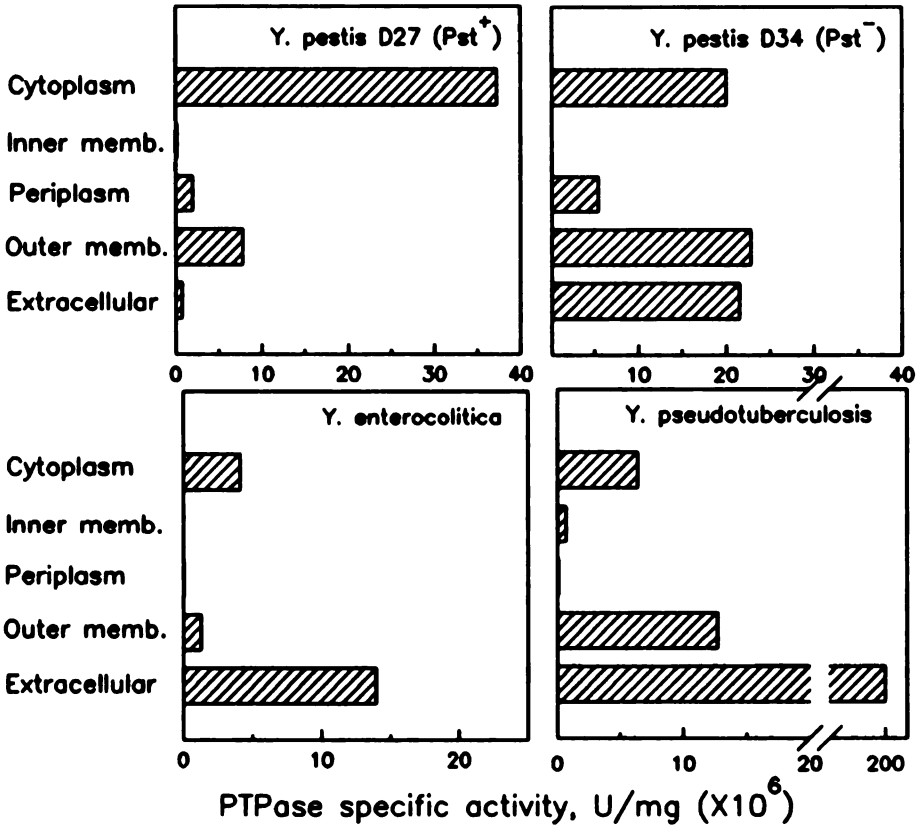

Figure 2. Distribution of YopH PTPase specific activity in subcellular fractions of *Yersiniae*. Cells were grown as described in Figure 1 and assayed for PTPase specific activity (U per mg protein X 10^6).

subcellular distribution of PTPase specific activity indicated a relative enrichment of PTPase activity in the outer membrane of each *Yersiniae* studied. In addition, our data revealed that the PTPase activity in the Pst^+ *Y. pestis* strain also accumulated in the outer membrane and culture supernatant, although to a much lesser extent than the Pst^- strains.

YopH exists in multiple forms in *Y. pestis* D27 (Pst^+). We then wished to determine whether the Pst plasmid-encoded protease, plasminogen activator, degrades YopH after synthesis in *Y. pestis* (18). Multiple forms of YopH were found in the cytoplasm of both D34 (Pst^-) and D27 (Pst^+) strains of *Y. pestis* after TCA precipitation and immunoblot analysis using polyclonal anti-YopH antibody. Proteins at several molecular weights reacted with the anti-YopH antibody (Figure 3A). The predominant immunoreactive form in both Pst^- and Pst^+ strains had an apparent M_r 44 kDa in agreement with previous reports. Substantial amounts of degradation products centered around 31 kDa were observed in the Pst^+ strain. The Pst^- strain also exhibited an immunoreactive form at about 32 kDa. Samples of Pst^+ cytoplasm stored at -20°C showed further degradation (Figure 3B) demonstrating that the degradation process continues after fractionation. The Pst^- strain however exhibited little loss of the 44 kDa form upon storage. In accordance with the immunoblot data, the PTPase activity in the Pst^+ cytoplasm declined upon storage (data not shown).

Multiple forms of YopH have PTPase activity. To determine whether the multiple protein forms reactive with anti-YopH antibody were catalytically active, the components of bacterial cytoplasm and extracellular preparations from *Y. pestis* D34 (Pst^-) and D27 (Pst^+) were separated by size using FPLC-Superose 12 chromatography

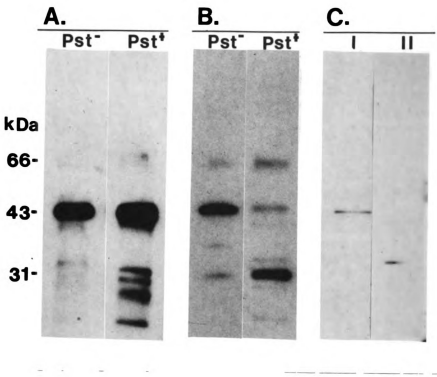

Figure 3. Anti-YopH immunoblot of Pst$^-$ and Pst$^+$ *Yersinia pestis* cytoplasmic proteins. Equivalent amounts of cytoplasmic proteins from Pst$^-$ and Pst$^+$ *Y. pestis* strains grown under conditions stimulatory for Yop synthesis were TCA precipitated, solvated in SDS-PAGE sample buffer and separated on a 4-15% gradient acrylamide gel. Cytoplasmic proteins were electroeluted onto a nitrocellulose membrane and probed with an anti-YopH polyclonal antibody. Panel A) Cytoplasm from *Y. pestis* D34 (lane Pst$^-$) and *Y. pestis* D27 (lane Pst$^+$); B) Partially degraded samples of cytoplasm from *Y. pestis* D34 (Pst$^-$) and *Y. pestis* D27 (Pst$^+$); C) Anti-YopH immunoblot of PTPase-active FPLC fractions (from Figure 4; pool I and II) from *Y. pestis* D34 and *Y. pestis* D27 cytoplasm respectively. The position of the M_r standards is indicated on the left side in kDa.

and the PTPase activity of each fraction monitored (Figure 4). In the cytoplasm of *Y. pestis* D34 (Pst$^-$) the PTPase activity resided in a prominent peak eluting at an apparent M_r of 44 kDa (Figure 4A, I). Additionally, a small but significant amount of activity was consistently found as a shoulder at about 60 kDa which could not be resolved because of the prominence of the 44 kDa form . The presence of this peak under the native conditions of FPLC but not the denaturing conditions of SDS-PAGE resolution followed by Western blot analysis, suggests that some YopH is associated with an unidentified, small protein in the bacterial cytoplasm. In agreement with such a hypothesis, only one 44 kDa form of YopH was observed in the extracellular fraction of the Pst$^-$ strain (Figure 4B).

In contrast, the cytoplasm of *Y. pestis* D27 contained at least four forms of active PTPase (Figure 4C). The largest form eluted at a volume corresponding to approximately 60 kDa and was identical in size to the form found in the Pst$^-$ strain. The majority of PTPase activity appeared among a series of peaks eluting at positions with M_r of 44 kDa to 33 kDa. Most of the activity was found in the smaller forms (37 and 33 kDa). Evaluation of D27 (Pst$^+$) culture supernatant by FPLC analysis showed that this strain had much less total extracellular PTPase activity than the Pst$^-$ strain. Moreover, the exported active PTPase existed in multiple forms eluting at a volumes corresponding to 44 kDa and 37 kDa (Figure 4D).

Cytoplasmic protein fractions with PTPase activity were pooled and analyzed by immunoblot detection to confirm that the peaks from the FPLC analysis corresponded to the proteins identified with the anti-YopH antibody (Figure 3C). Immunoblot analysis of the pooled, TCA precipitated FPLC fractions of *Y. pestis* D34 (Pst$^-$) cytoplasm

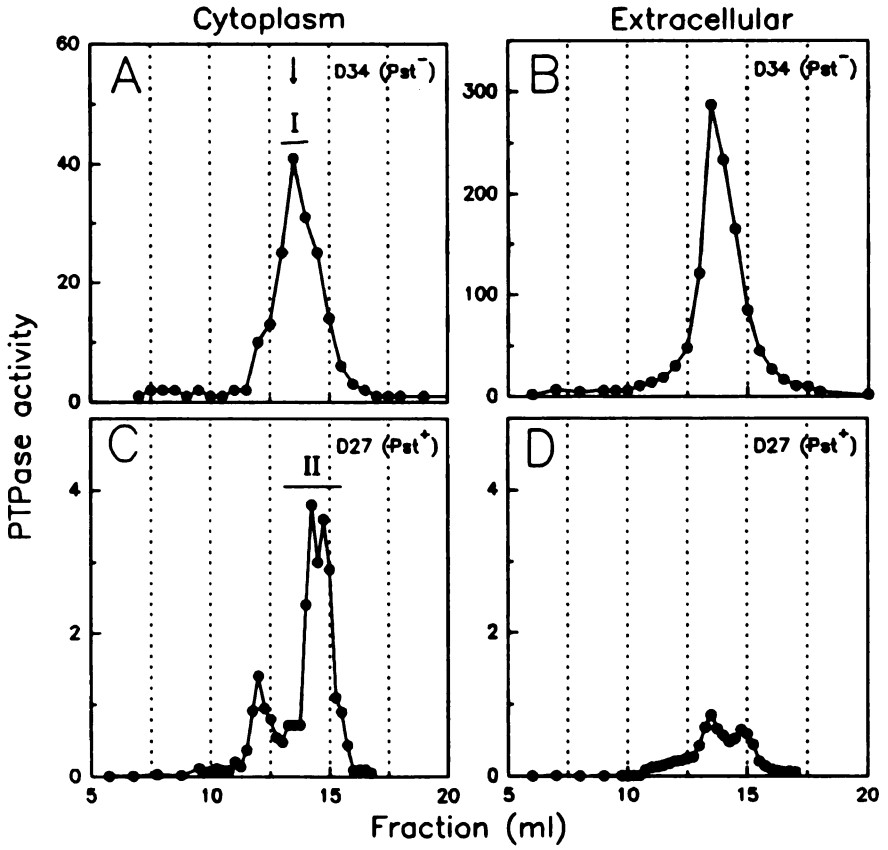

Figure 4. PTPase activity of FPLC separated cytoplasmic and extracellular proteins of Pst⁻ and Pst⁺ *Yersinia pestis*. Components of cytoplasm and extracellular media from cultures grown in conditions maximizing Yop production were separated using a FPLC-Superose 12 sizing column. Eluate fractions were analyzed for PTPase activity (μmol $^{32}PO_4$ released per min X 10^9). Panel A) cytoplasm of *Y. pestis* D34; B) extracellular medium of *Y. pestis* D34; C) cytoplasm of *Y. pestis* D27; D) extracellular medium of *Y. pestis* D27. The arrow denotes the fraction in which ovalbumin (44 kDa) was eluted.

revealed that the main peak of PTPase activity (from Figure 4A, I) corresponded to the major 44 kDa band (Figure 3C, I). The minor amount of YopH existing in the 60 kDa YopH-protein complex was too small to be detected by immunoblot. Analysis of D27 (Pst^+) FPLC fractions revealed only one immuno-reactive band at 37 kDa (Figure 3C, II), corresponding to one of the major peaks of PTPase activity. The low protein levels in the others sizes of YopH present in the cytoplasm of the Pst^+ strain were undetectable by Western blot.

DISCUSSION

The lack of virulence of Yop$^-$ mutants and the degradation of Yop proteins by the *Y. pestis*-specific protease plasminogen activator presents a largely unexplained dilemma in our understanding of the role of Yops in *Y. pestis* virulence. The sequence of YopH suggests that the protein is of eukaryotic origin (6). This implies that the PTPase molecule gene was trapped from a eukaryotic host and is now a potent virulence factor. Continued study may reveal more examples of key eukaryotic regulatory enzymes that are modified and used successfully as virulence factors by eukaryotic pathogens.

Yops are synthesized in the cytoplasm and exported to the extracellular surroundings. On the basis of percentage of total PTPase activity our data confirmed that most of the YopH PTPase activity resides in the cytoplasm and in the extracellular media, with much smaller amounts found in the periplasm and the outer membrane. The PTPase activity found extracellularly in the Pst$^+$ cultures, although much lower than in the Pst$^-$ cultures, was present in quantities sufficient for enzymatic analysis. Examination of the specific activity of these fractions indicated that enrichment of YopH occurs in the periplasm and, in particular, in the outer membrane and culture supernatant in the Pst$^-$ strains. The Pst$^+$ strain also shows an accumulation of YopH specific activity in the periplasm and outer membrane, however the specific activity of the extracellular PTPase was comparatively low. These data support the concept that YopH PTPase activity of *Y. pestis* (Pst$^+$) is not significantly diminished in the cytoplasm, but is lost upon translocation through the outer membrane into the extracellular compartment.

The plasminogen activator protease is an outer membrane protein. The cleavage products of plasminogen produced by the serine protease urokinase and the plasminogen

activator are indistinguishable from each other by SDS-PAGE, suggesting that the plasminogen activator may also be a member of the trypsin family of serine-proteases (20), a group capable of cleavage on the C-terminal side of arginine and lysine residues. About one out of every ten amino acids of YopH is an arginine or lysine, allowing many possible sites for plasminogen activator-mediated hydrolysis. The significance of cleavage to virulence remains to be determined since we have shown that many cleavage products retain catalytic activity.

To investigate whether degradation products of YopH could account for the PTPase activity found in the extracellular surroundings of *Y. pestis* D27 (Pst^+), the YopH PTPase activity of plasminogen activator positive (Pst^+) and negative (Pst^-) *Y. pestis* cells was evaluated after FPLC separation of the samples. This allowed comparison of the molecular forms of the PTPase found in the cytoplasm and extracellular media of both *Y. pestis* strains. In Pst^- cells YopH activity appeared as one large peak by Superose 12 chromatography. We concluded that this peak represents the intact, enzymatically active form of YopH. However, the fact that this catalytically active molecular form was observed both under native conditions (FPLC) but not denaturing conditions (SDS-PAGE) suggests that this is not a YopH precursor protein which is processed to yield the abundant 44 kDa form, but instead represents a small amount of YopH associated with an additional cytoplasmic protein. Enzymatically active YopH was also present in the cytoplasm of Pst^+ cells but the PTPase activity was present in several forms. The first form was identical in apparent molecular weight to the first shoulder of the Pst^- YopH PTPase activity peak. The other forms exhibited a range of sizes from 44 kDa peak to 33 kDa. The activity at lower apparent molecular weights

than 44 kDa are attributable to YopH degradation products. We believe these are degradation products rather than unique PTPases because there is no PTPase activity in an Lcr^-, Pst^+ strain of *Y. pestis* (data not shown); curing of the plasminogen activator containing plasmid results in one predominant form of PTPase activity (Fig. 4) and proteins of several sizes were observed to interact with an anti-YopH antibody (Fig. 3).

The isolation of intact YopH in *Y. pestis* D27 (Lcr^+, Pst^+) was complicated by the fact that continuing degradation occurred during the isolation procedure and during storage. Very fresh preparations contained more YopH PTPase activity and more of the 44 kDa form than samples which had been frozen and stored. Preparations from strain D34 (Pst^-) did not loose activity after storage, nor did the activity shift to a form other than the prominent 44 kDa form. This suggests that the plasminogen activator is functional within the cytoplasmic preparation and hydrolyzing the 44 kDa form of YopH into smaller proteins, some of which remain active.

It is possible that the cleavage of YopH could contribute to the virulence of *Y. pestis*. Smaller fragments may be less antigenic while still enzymatically active. The accumulation of unprocessed Yops in the outer membrane could lead to the development of anti-Yop antibodies. In the Pst^+ species, very little YopH accumulates in the extracellular surroundings and much of what is exported is processed into smaller forms. The continual turnover of the Yop and the smaller size of the protein may delay the onset of an effective immune response. This point is illustrated by the observation that anti-Yop antibodies provide passive protection from the enteropathogenic species, *Y. pseudotuberculosis* but not *Y. pestis* (3). Further, it is possible that proteolytic trimming removes a regulatory domain resulting in a constitutively active protein that the host is

unable to down regulate after internalization. Residues at the termini of two other non-transmembrane PTPases, TCPTP and PTP1B, have been proposed to be regulatory domains, with removal resulting in a constitutively active enzyme (4,23). The addition of an exogenous, unregulated PTPase into a macrophage could disrupt the activation signal cascade, thereby delaying an appropriate immune response and enhancing the virulence of *Y. pestis*. Infection with *Y. pseudotuberculosis* expressing YopH has been demonstrated to inhibit phagocytosis and is associated with dephosphorylation of cellular proteins (1,13).

A detailed investigation of a Yop protein such as YopH with a defined enzymatic activity will help clarify the nature of the enhanced pathogenesis of Yersinia pestis. Here we report the subcellular distribution of YopH PTPase, a required virulence factor. In addition, catalytically functional YopH of Pst^+ *Y. pestis* was found to exist not only in the native 44 kDa form but also as smaller forms not previously described.

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