

# YopD and LcrH Regulate Expression of *Yersinia enterocolitica* YopQ by a Posttranscriptional Mechanism and Bind to *yopQ* RNA

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**Pathogenic yersiniae secrete 14 Yop proteins via the type III pathway. Synthesis of YopQ occurs when the type III machinery is activated by a low-calcium signal, but not when the calcium concentration is above 100  $\mu$ M. To characterize the mechanism that regulates the expression of *yopQ*, mutants that permit synthesis of YopQ in the presence of calcium were isolated. Yersiniae bearing deletion mutations in *yopN*, *tyeA*, *sycN*, or *yscB* synthesized and secreted YopQ in both the presence and the absence of calcium. In contrast, yersiniae with a deletion in *yopD* or *lcrH* synthesized YopQ in the presence of calcium but did not secrete the polypeptide. These variants displayed no defect in YopQ secretion under low-calcium conditions, revealing that *yopD* and *lcrH* are required for the regulation of *yopQ* expression. Experiments with transcriptional and translational fusions to the *npt* reporter gene suggest that *yopD* and *lcrH* regulate *yopQ* expression at a posttranscriptional step. YopD and LcrH form a complex in the bacterial cytosol and bind *yopQ* mRNA. Models that can account for posttranscriptional regulatory mechanisms of *yop* expression are discussed.**

Three pathogenic *Yersinia* species, *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*, use a type III secretion machinery to transport Yop proteins across the bacterial double membrane envelope (16, 43). The transport of Yop proteins plays an important role during the establishment of bacterial infections, as *Yersinia* spp. lacking type III genes are rapidly phagocytosed and killed by host macrophages (21, 24, 51, 55). The genes that encode the type III secretion machinery and Yop proteins are located on the 70-kb virulence plasmid of yersiniae (17). *Y. enterocolitica yopB*, *yopD*, *yopE*, *yopH*, *yopM*, *yopN*, *yopO*, *yopP*, *yopQ*, *yopR*, *yopT*, *lcrV*, *yscM1*, and *yscM2* encode secretion substrates. *Y. pseudotuberculosis* and *Y. pestis* virulence plasmids encode only *yscM1* (*lcrQ*) but not *yscM2* (58, 61). *yscC*, *yscD*, *yscE*, *yscF*, *yscG*, *yscI*, *yscJ*, *yscK*, *yscL*, *yscN*, *yscO*, *yscP*, *yscQ*, *yscR*, *yscS*, *yscT*, *yscU*, *yscV*, *yscW*, *yscX*, and *yscY* specify components of the type III secretion machinery (1, 2, 7).

During *Yersinia* infection of tissue culture cells, the type III machinery is activated by a series of environmental signals that trigger the transport of specific sets of Yop proteins (42). A temperature shift to 37°C and an environmental glutamate signal lead to the assembly and activation of the type III machinery (31, 42, 71). In the presence of additional signals, i.e., animal serum proteins, *Y. enterocolitica* secretes YopB, YopD, YopR, and LcrV into the extracellular medium (42, 44, 45).

Contact with host cells activates the type III machinery to transport YopE, YopH, YopM, YopN, YopO, YopP, YopT, and YscM1 into the eukaryotic cytosol (9, 10, 28, 36, 41, 49, 50, 52, 59). Our laboratory refers to these transport reactions as type III secretion (YopB, YopD, YopR, and LcrV) and type III targeting (YopE, YopH, YopM, YopN, YopO, YopP, YopT, and YscM1) (41).

Bacterial type III machines assemble into needle complexes, supramolecular structures that are dedicated to protein transport (40). Upon contacting tissue culture cells, the needle complexes of *Yersinia* type III machines insert into the plasma membrane of target cells and serve as protein conduits for the transport of YopE, YopH, YopM, YopN, YopO, YopP, YopT, and YscM1 (32). Needles are presumably also involved in measuring environmental signals, as the needle insertion into host cells could provide the low-calcium signal of the *Yersinia* type III pathway (42).

Some of the views presented here are under debate. Although it seems clear that YopB and YopD are predominantly secreted proteins, portions of the polypeptides are associated with the plasma membrane of eukaryotic cells (41, 65). Wolf-Watz, Cornelis, and colleagues postulated that YopB, YopD, and LcrV form a pore in the host cell plasma membrane through which effector Yops (YopE, YopH, YopM, YopO, YopP, and YopT) are translocated into the cytosol (19, 29, 33, 65). This translocation mechanism is obviously distinct from protein transport through the conduit of type III needles, which do not appear to contain YopB, YopD, and LcrV (32). Another area of debate is the precise location of LcrV. Although LcrV is regularly found in the extracellular medium (45), this polypeptide has also been observed on the surface of yersiniae (53) or transported into the cytosol of tissue culture cells (22).

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The mechanisms by which Yop proteins are recognized by the type III machinery are currently under investigation by several laboratories. Translational fusion of *yop* genes to the 5' end of the *Escherichia coli* neomycin phosphotransferase (*npt*) or *Bordetella pertussis* adenylate cyclase (*cya*) gene results in the type III secretion of hybrid polypeptides (4, 60). As for *yopQ*, truncation of 3' coding sequences is tolerated, and fusion of only the first 15 codons to *npt* is still sufficient to promote type III secretion of hybrid polypeptides (6). Deletion of codons 2 to 15 of *yopQ* abrogated substrate recognition, indicating that the signal encoded in the first 15 codons is indeed necessary for type III transport of hybrid polypeptides (6). Anderson et al. generated nucleotide insertions and deletions of the *yopQ*, *yopN*, and *yopE* secretion signal, immediately following the AUG start codon (4, 6). These frameshift mutations were corrected by reciprocal insertions and deletions at the fusion site between *yopQ* and *npt*, allowing the synthesis of hybrid Npt proteins (4). Some but not all of the frameshifted signals directed mutant proteins into the type III pathway. Anderson et al. suggested that the secretion signal of Yop proteins may be decoded at the level of mRNA translation rather than via signal peptide recognition by the type III secretion machinery (5).

Some Yop proteins, for example YopE, bind to small homodimeric cytoplasmic proteins (Syc, specific Yop chaperone) (68). In the case of YopE, this interaction also leads to the initiation of the polypeptide into the type III pathway (12, 15). Nevertheless, this mechanism may not be universal, as Syc proteins have not yet been described for YopM, YopO, YopP, and YopQ. What is the role of Syc proteins in directing Yops into the type III pathway? Cornelis and colleagues proposed that Syc proteins could act as secretion chaperones or transport pilots (66). In this model, Syc proteins deliver Yops to the type III machinery and may be involved in substrate recognition (69). Karlinsey et al. suggested that Syc proteins may act as modulators of translation of secretion substrates, presumably coupling translation of mRNA with the type III secretion of newly synthesized polypeptides (39). Lloyd et al. proposed the existence of a signal peptide within the first 12 amino acid residues (46). This signal peptide is predominantly composed of alternating polar and hydrophobic residues. Furthermore, a synthetic secretion signal seems functional, as the peptide sequence MSISISISI can direct YopE into the type III pathway (47). In the signal peptide model, Syc proteins may prevent the premature folding or association of secretion substrates (46). Future work will need to distinguish between these models and reveal the mechanism by which Yop proteins are recognized and transported by the type III pathway.

In this report we have focused on the regulation of expression of type III secretion substrates. Synthesis of YopQ occurs when the type III pathway is activated by an environmental calcium signal ( $\leq 80 \mu\text{M}$ ), but not when the calcium concentration is above  $100 \mu\text{M}$  (6). We sought to characterize mutants that permit synthesis of YopQ in the presence of calcium. As reported previously, mutants bearing deletion mutations in *yopN*, *tyeA*, *sycN*, or *yscB* display a calcium-blind phenotype and secrete Yops in the presence and absence of calcium (14, 20, 38, 72). We refer to these strains as class I mutants, as the *Yersinia* variants synthesize and secrete YopQ in the presence and in the absence of calcium. Mutants with a deletion in *yopD*

or *lcrH* synthesize YopQ in the presence of calcium but do not secrete the polypeptide. These class II mutants display no defect in YopQ secretion, revealing that *yopD* and *lcrH* are required for the regulation of *yopQ* expression. Experiments with transcriptional and translational *yopQ* fusions to the *npt* reporter gene suggest that *yopD* and *lcrH* may regulate *yopQ* expression at a posttranscriptional step. YopD and LcrH form a complex in the bacterial cytosol and bind *yopQ* mRNA.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Y. enterocolitica* W22703 (18), MC1 [ $\Delta$ (*yopQ*)] (6), MC4 [ $\Delta$ (*yopB*)] (44), VTL1 [ $\Delta$ (*yopN*)] (41), and VTL2 [ $\Delta$ (*yopD*)] (44) have been described previously. Using allelic exchange, codons 21 to 149 of *lcrH* were replaced with GGA TCC, generating the  $\Delta$ (*lcrH*) strain CT133. The  $\Delta$ (*yopDQ*) strain DA1 was constructed by crossing the *yopQI* mutation into the *yopD* mutant strain VTL2. All other mutations were introduced by allelic exchange using an established strategy (12). *E. coli* strain P90C served as the host for DNA manipulations (48).

**DNA methods and plasmid construction.** Plasmid pDA255, expressing *gst-yopD* under control of *lacI<sup>q</sup>* from the *tac* promoter, has been described previously (41). The *yopB* and *lcrH* open reading frames were PCR amplified using the primers YopB-Kpn (5'-AAGGTACCCAACAAGAGACGACAGACA-3') and YopB-Bam (*yopB*) (41) or LcrH-Kpn (5'-AAGTACCCAACAAGAGACGACAGACA-3') and LcrH-Bam (5'-AAGGATCCTCATGGGTTATCAACGCAC T-3') (*lcrH*). DNA fragments were cut with *KpnI* and *BamHI* and cloned into the corresponding sites of pDA255, generating pNT20 (*gst-yopB*) and pDA326 (*gst-lcrH*). Plasmid pDA325 was generated by PCR amplification with the primers LcrH-Nde (5'-AACATATGCAACAAGAGACGACAGA-3') and LcrH-Bam. The PCR product was cut with *NdeI* and *BamHI* and cloned into the corresponding site of pVL41 (41). An *npt* reporter cassette that allows fusion of *yopQ* sequences has been described previously (pDA183 and pDA243) (6).

To generate pDA330, *npt* sequences were PCR amplified with the primers Npt-Tsf (5'-AAGGTACCTGACTGACTGATCAAGAGACAGGATGAGGA T-3') and Npt-3 (6), and the *yopQ* promoter was amplified with primers YopQ-1 (5'-AAGAATTCAGCCATTATTTTGCTATACCGA-3') and YopQ-TS+1 (5'-AAGGTACCATTATTTTAAAGTACTGAT-3'). Amplified fragments were cut with *EcoRI* and *KpnI* and *KpnI* and *NdeI* and cloned into pDA183. Plasmid pDA340 was generated by PCR amplification of *yopQ* sequences with the primers YopQ-T7 (5'-AAGACGGTTATTAATAGTGAG-3') and Npt-3 using pYopQ<sub>1-15</sub>-Npt as a template. The PCR product was ligated into pCR2.1 (Invitrogen). pDA183 was generated by cloning a *yopQ* promoter and *yopQ* untranslated region (UTR) fusion to the *npt* reporter gene into the pHSG575 derivative pDA15 (12, 64).

pDA243 is a pHSG575 derivative harboring an insert in which the *yopQ* promoter, *yopQ* UTR, and full-length open reading frame are fused to the open reading frame of *npt*. The pHSG575 derivative pDA209 contains a transcriptional fusion of the *yopQ* promoter, *yopQ* UTR, and *yopQ* full-length open reading frame to the open reading frame of *npt*. Translational initiation of *npt* in pDA209 occurs from its own regulatory signals.

**Protein electrophoresis and immunodetection.** Procedures to measure Yop secretion have been reported previously (4). To quantify the concentration of Npt fusions in the presence of calcium in Fig. 2, wild-type and mutant *Yersinia* cultures were induced by temperature shift in the presence of calcium. One milliliter of culture was removed and precipitated with trichloroacetic acid (TCA). Equal amounts of protein, as determined by the cultures' absorbance at 600 nm, were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrotransfer to polyvinylidene difluoride membranes and immunoblotting. The signal was visualized by incubation of the membrane with [<sup>125</sup>I]protein A and quantified on a PhosphorImager. *ysc* genes were expressed as His-tagged fusions in *E. coli*. Recombinant Ysc proteins were purified by affinity chromatography and injected into rabbits to raise the Ysc-specific antisera used (see Table 1).

**Purification of GST fusion proteins.** *Yersinia* expressing glutathione S-transferase (GST)-YopB, GST-YopD, or GST-LcrH were grown in tryptic soy broth (TSB) at 37°C for 3 h in the presence of 5 mM CaCl<sub>2</sub> or 5 mM EGTA. Thirty minutes after beginning incubation at 37°C, the expression of GST fusions was induced by the addition of 1 mM IPTG (isopropylthiogalactopyranoside). Cells were harvested by centrifugation, suspended in 10 mM HEPES (pH 7.5)–100 mM potassium acetate–2 mM magnesium chloride–1 mM dithiothreitol (DTT) and lysed in the presence of 50  $\mu\text{M}$  phenylmethylsulfonyl fluoride at 10,000 lb/in<sup>2</sup>

in a French pressure cell. The lysate was cleared by centrifugation at 15,000 × g for 15 min and applied to affinity chromatography. Glutathione-Sepharose, 1.5-ml bed volume, was equilibrated with 50 mM Tris-HCl-150 mM NaCl, pH 7.5, and charged with lysate. The column was washed with 30 ml of 50 mM Tris-HCl-150 mM NaCl-10% glycerol, pH 7.5, and bound proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 8.0.

**Reverse transcriptase PCR.** *Yersinia* cultures were grown at 37°C in the presence or absence of calcium ions for 2 h. Total RNA was isolated as described previously (6), and 5 µg of RNA was treated with 2 U of DNase I for 30 min at 37°C, followed by phenol extraction and ethanol precipitation. Each sample was divided into three tubes, and cDNA was prepared. The oligonucleotides Npt-3 (6) or Cat-2 (5'-AAGGATCCAAATTACGCCCGCCCTG-3') were annealed to RNA by heating to 37°C for 5 min, followed by a 5-min ice incubation in reverse transcriptase buffer. Avian myeloblastosis virus (AMV) reverse transcriptase (Promega) was added together with 0.5 mM deoxynucleoside triphosphates and incubated at 42°C for 60 min. Reactions were phenol extracted, and the DNA was ethanol precipitated and suspended in 40 µl of H<sub>2</sub>O. The cDNA was used as the template for PCR amplification with three sets of primers: YopQ+1/Npt-3, Npt-1/Npt-3 (4, 6), and Cat-1 (5'-AAGGTACCGAGAAAAAATCAC TGGATATA-3')/Cat-2. PCR products were separated by electrophoresis on agarose gels.

**RNA electrophoretic mobility shift assay.** *yopD*, *lcrH*, and a transcriptional *yopD-lcrH* fusion were cloned into pET16 (Novagen). Recombinant plasmids pKR2 (*yopD*), pKR4 (*lcrH*), and pKR6 (*yopD-lcrH*) were transformed into *E. coli* BL21(DE3) (63). His<sub>6</sub> affinity-tagged proteins were expressed by T7 polymerase induction of *E. coli* cultures with IPTG (63). *E. coli* cells (10<sup>12</sup> cells) were lysed in a French press at 18,000 lb/in<sup>2</sup> (total extract), and insoluble material was removed at 115,000 × g. The supernatant (load) was applied to Ni-nitrilotriacetic acid (NTA) preequilibrated with 0.05 M Tris-HCl-0.15 M NaCl, pH 7.5. The column was washed with the same buffer containing 0.02 to 0.07 M imidazole and eluted with 0.5 M imidazole.

An RNA probe (-45 through +45 relative to the AUG of *yopQ*) was synthesized in vitro using T7 RNA polymerase and [ $\alpha$ -<sup>32</sup>P]UTP. RNA was heated at 95°C for 2 min and cooled to 4°C for 10 min. Purified protein and 10 fmol of *yopQ* RNA were incubated in 25 mM HEPES-150 mM KCl-10 mM MgCl<sub>2</sub>-1 mM DTT-1% glycerol-40 U of RNasin (Promega), pH 7.5, on ice for 10 min prior to separation by electrophoresis on a 4% polyacrylamide gel. Competition experiments used unlabeled *yopQ* mRNA and *E. coli* tRNA.

## RESULTS

**Genes required for *yopQ* expression.** When the type III machinery is induced by the chelation of calcium ions, yersiniae synthesize and secrete YopQ into the extracellular medium (6). To identify the genes that are required for the regulation of *yopQ* expression, we generated nonpolar mutations in the *Y. enterocolitica* W22703 virulence plasmid using an allelic exchange strategy (12). The *Yersinia* virulence plasmid carries 22 *ysc* genes (*yscA*, *yscC*, *yscD*, *yscE*, *yscF*, *yscG*, *yscI*, *yscJ*, *yscK*, *yscL*, *yscN*, *yscO*, *yscP*, *yscQ*, *yscR*, *yscS*, *yscT*, *yscU*, *yscV*, *yscW*, *yscX*, and *yscY*) that specify secretion machinery components and 10 regulatory genes (*lcrG*, *lcrV*, *lcrH*, *ycN*, *ycH*, *yopD*, *yopN*, *yscB*, *yscM1*, and *yscM2*) that control the activity of the type III pathway (17). *Yersinia* strains harboring virulence plasmids with null mutations in any one of those genes were analyzed for YopQ synthesis and secretion. Three mutant phenotypes were detected: loss of calcium regulation of YopQ synthesis and secretion (class I); loss of calcium regulation of YopQ synthesis but not of type III secretion (class II); and loss of YopQ synthesis under low-calcium conditions (class III) (Fig. 1A).

The products of class I genes (*yopN*, *tyeA*, *yscN*, and *yscB*) are thought to act by occluding the secretion channel (13, 72). Synthesis and secretion of YopQ in these mutants occur even in the presence of calcium. Class II genes (*yopD* and *lcrH*) appear to specify regulatory proteins that prevent the expression of *yopQ* in the presence of calcium (see below). Class III

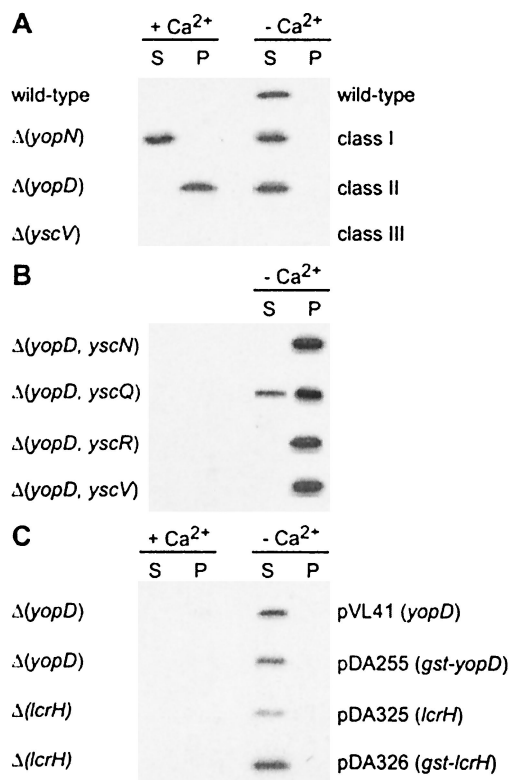


FIG. 1. Synthesis and type III secretion of YopQ in wild-type and mutant yersiniae. (A) *Y. enterocolitica* W22703 (wild type) and isogenic mutant strains VTL1 [ $\Delta$ (*yopN*)], VTL2 [ $\Delta$ (*yopD*)], and KUM1 [ $\Delta$ (*yscV*)] were grown in the presence and absence of calcium (low calcium is an inducing condition for type III secretion). Cultures were centrifuged, and proteins secreted into the culture medium (S, supernatant) were separated from the cell sediment (P, pellet). YopQ secretion was measured by immunoblotting with specific antiserum. (B) *Y. enterocolitica* strains W22703 (wild type), VTL2 [ $\Delta$ (*yopD*)], and CT133 [ $\Delta$ (*lcrH*)] were grown at 37°C in the presence and absence of calcium. Culture supernatants (S) and bacterial extracts (P) were separated on SDS-PAGE and analyzed by immunoblotting with specific antiserum ( $\alpha$ -YopQ). *Yersinia* strains were transformed with plasmids encoding wild-type *yopD* (pVL41) (44), *gst-yopD* (pDA255) (44), *lcrH* (pDA325), or *gst-lcrH* (pDA326). (C) YopQ secretion in *Y. enterocolitica* strains carrying knockout mutations in both the *yopD* and *ysc* genes.

genes encode components of the secretion machinery (*yscA*, *yscC*, *yscD*, *yscE*, *yscF*, *yscG*, *yscI*, *yscJ*, *yscK*, *yscL*, *yscN*, *yscO*, *yscP*, *yscQ*, *yscR*, *yscS*, *yscT*, *yscU*, *yscV*, *yscW*, *yscX*, and *yscY*), and knockout mutations in these genes abrogate the expression of *yopQ* (6). YscM1 (LcrQ) and YscM2, factors that are thought to control *yop* transcription, were not considered for the regulation of *yopQ* translation (61).

**Genes required for YopQ secretion.** *E. coli* expressing the secretion genes of the plant pathogen *Erwinia chrysanthemi* export YopQ into the extracellular medium (3). The type III machinery of *E. chrysanthemi* is encoded by 11 *hrc* genes, all of which display homology to *Yersinia ysc* genes (*yscC*, *yscD*, *yscJ*, *yscL*, *yscN*, *yscQ*, *yscR*, *yscS*, *yscT*, *yscU*, and *yscV*) (30). These 11 *ysc* genes are conserved among type III machines of other gram-negative bacteria, and 9 genes, *yscD*, *yscL*, *yscN*, *yscQ*, *yscR*, *yscS*, *yscT*, *yscU*, and *yscV*, are also present in flagellar type III systems (34). To assess whether *yscD*, *yscL*, *yscN*, *yscQ*, *yscR*, *yscS*, *yscT*, *yscU*, and *yscV* are required for the secretion

TABLE 1. *Yersinia* type III machinery components and their role in YopQ secretion

Type III protein	Ysc solubility <sup>a</sup> (%)	YopQ secretion by $\Delta(\text{ysc}, \text{yopD})$ mutant <sup>b</sup> (% of control)
YscD	0	70
YscL	100	0
YscN	50	0
YscQ	100	20
YscR	0	0
YscS	0	0
YscT	NT	0
YscU	0	0
YscV	0	0
Control	NA	100

<sup>a</sup> *Yersiniae* were lysed by French press, and crude extracts were centrifuged (100,000  $\times$  g). Supernatant and sediment were separated and analyzed by immunoblotting with specific antisera. The amount of immuno reactive Ysc in the supernatant is shown. NT, not tested; NA, not applicable.

<sup>b</sup> YopQ secretion in low-calcium-induced *Yersinia* cultures was measured by immunoblotting. *Y. enterocolitica* VTL2 (ysc wild type)  $\Delta(\text{yopD})$  secreted 100% YopQ, whereas  $\Delta(\text{ysc}, \text{yopD})$  mutants displayed the secretion defects recorded.

of YopQ, the *yopD* knockout allele was combined with various *ysc* mutations, generating mutant strains capable of synthesizing YopQ (Fig. 1B).

$\Delta(\text{yscL}/\text{yopD})$ ,  $\Delta(\text{yscN}/\text{yopD})$ ,  $\Delta(\text{yscR}/\text{yopD})$ ,  $\Delta(\text{yscS}/\text{yopD})$ ,  $\Delta(\text{yscT}/\text{yopD})$ ,  $\Delta(\text{yscU}/\text{yopD})$ , and  $\Delta(\text{yscV}/\text{yopD})$  strains were completely defective in the type III secretion of YopQ, and mutants  $\Delta(\text{yscQ}/\text{yopD})$  and  $\Delta(\text{yscD}/\text{yopD})$  displayed significant defects in YopQ secretion (Table 1). Thus, all nine genes, *yscD*, *yscL*, *yscN*, *yscQ*, *yscR*, *yscS*, *yscT*, *yscU*, and *yscV*, are required for the efficient secretion of YopQ. To determine the subcellular locations of YscD, YscL, YscN, YscQ, YscR, YscS, and YscU, crude *Yersinia* extracts were subjected to ultracentrifugation at 100,000  $\times$  g, sedimenting bacterial membranes. YscD, YscR, YscS, YscU, and YscV were found in the sediment of centrifuged samples, whereas YscL, YscQ, and some YscN remained soluble in the cytoplasmic supernatant. Together these results suggest that the *Yersinia* type III machine is composed of cytoplasmic and membrane components, with YscN representing a mobile machinery protein.

**LcrH and YopD regulate *yopQ* expression.** Class II mutant strains,  $\Delta(\text{yopD})$  and  $\Delta(\text{lcrH})$ , fail to prevent *yopQ* expression in the presence of calcium. To determine whether the mutant phenotype can be complemented in *trans*, *Y. enterocolitica* VTL2 [ $\Delta(\text{yopD})$ ] and CT133 [ $\Delta(\text{lcrH})$ ] were transformed with plasmids containing wild-type alleles. When introduced into the corresponding mutant strain, plasmid-encoded *yopD* or *lcrH* prevented *yopQ* expression in the presence of calcium (Fig. 1C). Previous work showed that YopD is exported by the type III pathway (27). LcrH (SycD) binds to YopD and YopB in the bacterial cytoplasm and functions as a chaperone for the secretion of these polypeptides (67). Fusion of YopD to the C terminus of GST abolishes type III secretion of the hybrid protein, causing GST-YopD to reside in the bacterial cytoplasm (44).

We asked whether the defect of the  $\Delta(\text{yopD})$  strain in *yopQ* regulation would be complemented by GST-YopD. Plasmid-encoded GST-YopD but not GST-LcrH restored the calcium regulation of *yopQ* expression in the  $\Delta(\text{yopD})$  strain (Fig. 1C and data not shown). Similarly, the regulatory defect of the  $\Delta(\text{lcrH})$  strain was complemented by GST-LcrH but not by GST-YopD (Fig. 1C and data not shown). Thus, LcrH and YopD each perform an essential function in regulating the expression of *yopQ* in the cytoplasm of *Y. enterocolitica*.

**YopD and LcrH regulate *yopQ* expression by a posttranscriptional mechanism.** To examine the regulatory defect of *yopD* and *lcrH* mutant *yersiniae*, *yopQ* sequences were fused to the 5' end of the *npt* open reading frame (57). Bacteria were grown at 37°C in the presence of calcium, and the concentration of Npt protein in bacterial extracts was measured by immunoblotting (Fig. 2). The data are presented as the ratio of Npt expression between mutant and wild-type cells. Fusion of the *yopQ* promoter to *npt* revealed that the *yopD* and *lcrH* mutant strains caused only a modest increase in transcription of the reporter gene (1.5- to 2-fold). However, fusion of *yopQ* translational initiation signals, i.e., the promoter and 5' untranslated leader of *yopQ* to *npt*, detected a defect in posttranscriptional regulation, as  $\Delta(\text{yopD})$  and  $\Delta(\text{lcrH})$  *yersiniae* increased expression by five- to sixfold. Fusion of the entire *yopQ*

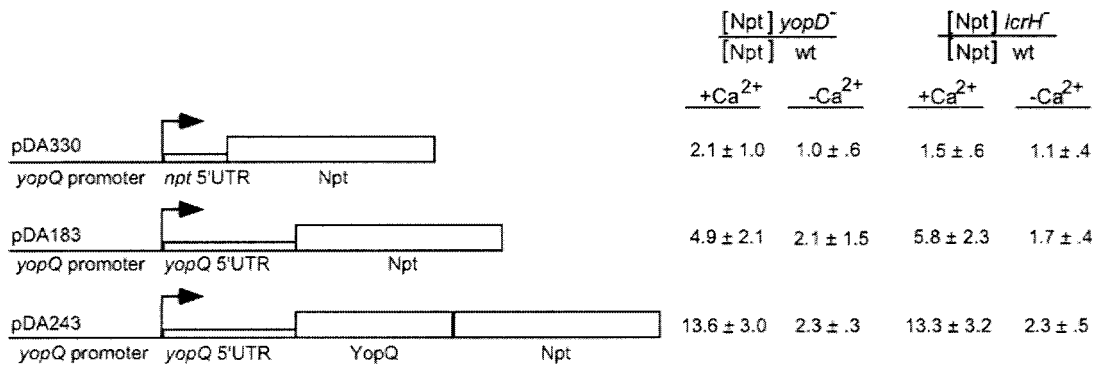


FIG. 2. *lcrH* and *yopD* regulate *yopQ* expression at a posttranscriptional step. Expression of *yopQ* was measured by fusing the reading frame of *npt* to either the *yopQ* promoter (pDA330), the promoter and 5' UTR (pDA183), or the 3' end of the *yopQ* reading frame specifying a translational YopQ-Npt fusion (pDA243). Plasmids were transformed into *Y. enterocolitica* W22703 (wild type) or the isogenic  $\Delta(\text{lcrH})$  (CT133) and  $\Delta(\text{yopD})$  (VTL2) mutant strains. *Yersinia* strains were grown in the presence or absence of calcium, and bacterial extracts were prepared by TCA precipitation. The concentration of Npt reporter was determined by immunoblotting and is reported as the ratio between mutant and wild-type cells. Data were averaged from more than three independent experiments. The standard deviation ( $\pm$ ) is indicated.

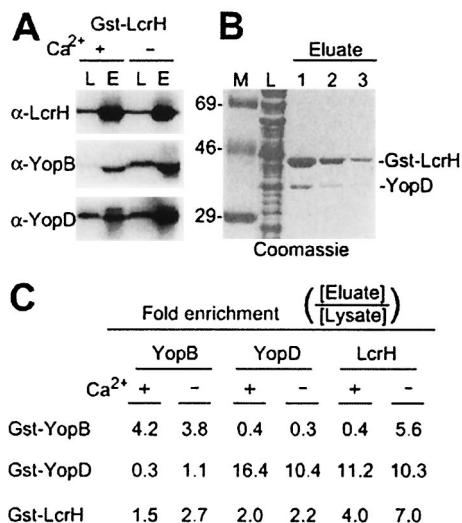


FIG. 3. GST-LcrH binds to YopD or YopB. Plasmid pDA326 was transformed into the  $\Delta(lcrH)$  mutant *Yersinia* strain CT133, and the expression of GST-LcrH was induced by the addition of IPTG. Bacterial lysates were cleared by centrifugation, and GST-LcrH was purified by affinity chromatography on glutathione-Sepharose. (A) Purification was assessed on Coomassie-stained SDS-PAGE and by immunoblotting of load and eluate fractions. (B) When expressed in  $\Delta(yopD)$  cells, subjected to affinity chromatography, and measured on Coomassie-stained SDS-PAGE, GST-YopD bound to LcrH but not to YopB (VTL2 harboring pDA255). Molecular size markers (lane M, in kilodaltons), lysate (L), and eluate fractions (1, 2, and 3) are indicated. (C) Purification of GST-YopB, GST-YopD, and GST-LcrH was measured by subjecting affinity chromatography load (lysate) and eluate fractions to immunoblotting. Chemiluminescent signals were scanned and quantified and are reported as the ratio of signal intensity between eluate and lysate. This experiment was performed in duplicate, with yersiniae grown in the presence and absence of calcium.

gene to *npt* caused an even greater (13- to 14-fold) increase in reporter gene expression. Together these data suggest that YopD and LcrH regulate the expression of *yopQ* at a posttranscriptional step.

**LcrH binds YopB and YopD in the bacterial cytoplasm.** Previous work reported LcrH (SycD) binding to YopB and YopD (66). These studies involved the copurification of over-expressed proteins in *E. coli* or the binding of LcrH to YopD immobilized on a nitrocellulose filter. To measure the binding of LcrH to YopB or YopD in the *Yersinia* cytoplasm, GST fusions to YopB, YopD, and LcrH were expressed from the IPTG-inducible *tac* promoter. Bacterial extracts were subjected to affinity chromatography, and copurification of polypeptides was measured by immunoblotting.

As expected, both YopB and YopD copurified with GST-LcrH. GST-YopB and GST-YopD each copurified with LcrH but not with one another (Fig. 3). Purification of GST-YopB from the cytoplasm of the  $\Delta(lcrH)$  mutant strain was greatly increased when yersiniae were grown in the absence rather than in the presence of calcium. For all other purifications, the growth of yersiniae in the presence or absence of calcium did not significantly alter the purification profiles. Thus, LcrH binds to either YopB or YopD to form cytoplasmic bipartite complexes (66), whereas YopD does not appear to bind to YopB. Consistent with this interpretation of the data is our

observation that  $\Delta(lcrH)$  and  $\Delta(yopD)$  but not  $\Delta(yopB)$  strains are defective in repressing YopQ synthesis (data not shown).

**YopD/LcrH are required for the degradation of *yopQ* mRNA.**

Posttranscriptional control of gene expression can occur as a block in translation (25) and/or as degradation of mRNA (26). Synthesis of a plasmid-encoded transcript encompassing both the *yopQ* and *npt* open reading frames (6) was induced by temperature shift in the  $\Delta(yopQ)$  *Yersinia* strain MC3 (Fig. 4). *npt* was expressed in both the presence and absence of calcium, whereas *yopQ* was expressed and YopQ polypeptide was secreted only in the absence of calcium (Fig. 4) (6). Total RNA

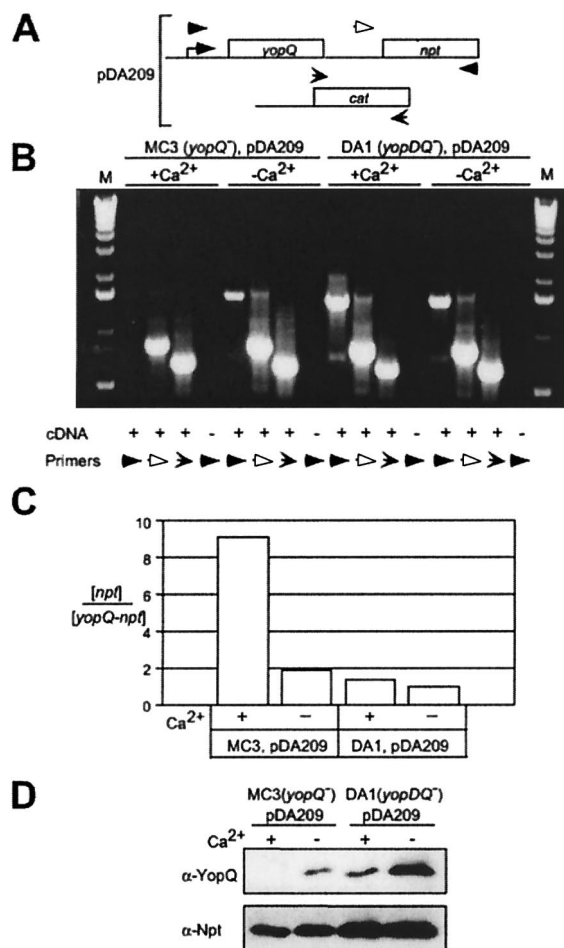


FIG. 4. YopD is required for degradation of *yopQ* mRNA. (A) Plasmid pDA209 carries the *cat* gene and a transcriptional *yopQ-npt* fusion. The drawing displays the annealing positions of oligonucleotide primers (arrowheads). (B) *Y. enterocolitica* strains MC3 [ $\Delta(yopQ)$ ] and DA1 [ $\Delta(yopDQ)$ ] were transformed with pDA209 (6) and grown in either the presence or absence of calcium. RNA was purified and cDNA was synthesized (+ and - indicate the addition or omission of AMV reverse transcriptase, respectively) using oligonucleotides that anneal at the 3' end of *yopQ-npt* or *cat*. cDNA template was used for PCR amplification (arrowheads), and products were analyzed on ethidium bromide-stained agarose gel. The 1-kb DNA ladder was used for size calibration (lane M). (C) The ratio of *npt/yopQ* to *npt* transcripts in various strains and growth conditions was determined by quantifying fluorescent signals. (D) Cell extracts of *Y. enterocolitica* MC3(pDA209) and DA1(pDA209) were analyzed by immunoblotting with  $\alpha$ -YopQ and  $\alpha$ -Npt.

was isolated, and cDNA was synthesized using reverse transcriptase and an oligonucleotide annealing to the 3' end of *npt*. The cDNA was PCR amplified using the same 3' primer as well as 5' primers annealing to either the transcriptional start site of *yopQ* or the ribosome-binding site of *npt*.

In the absence of calcium (when YopQ is expressed), PCR amplification detected similar amounts of full-length *yopQ-npt* transcript as well as 3' *npt* mRNA sequence. The addition of calcium to the culture medium not only blocked type III secretion but also reduced the concentration of the full-length *yopQ-npt* transcript. In contrast, the amount of the 3' *npt* portion of the *yopQ-npt* transcript was not reduced, suggesting that the mRNA sequence encoding *yopQ* had been degraded. As a control, equal amounts of *cat* transcripts were amplified from yersiniae grown in the presence and absence of calcium. If the degradation of transcript requires the presence of YopD/LcrH, a  $\Delta(yopDQ)$  double mutant strain may be defective in reducing the *yopQ* mRNA concentration. This was tested, and equal amounts of *yopQ-npt*, *npt*, and *cat* transcripts were found in the  $\Delta(yopDQ)$  strain grown in either the presence or absence of calcium. Thus, YopQ expression seems to be regulated by a mechanism that requires formation of the YopD-LcrH complex and that may be accompanied by the degradation of *yopQ* mRNA.

**YopD-LcrH complex binds *yopQ* mRNA.** To test whether YopD and LcrH repress YopQ synthesis by binding to mRNA, recombinant proteins were purified and mixed with  $^{32}\text{P}$ -labeled *yopQ* mRNA generated by in vitro transcription with T7 polymerase (Fig. 5). YopD/LcrH bound  $^{32}\text{P}$ -labeled *yopQ* mRNA, as indicated by mobility shifts of the RNA probe during polyacrylamide gel electrophoresis (Fig. 5A and B). RNA binding of YopD and LcrH appears to be specific for *yopQ* transcripts, as mobility shifts were prevented by the addition of excess unlabeled *yopQ* mRNA. Although YopD and LcrH could also bind to *E. coli* tRNA, significantly greater amounts of the tRNA were required to displace YopD and LcrH from *yopQ* mRNA (Fig. 5F). LcrH alone did not bind  $^{32}\text{P}$ -labeled *yopQ* mRNA (Fig. 5C and D). Expression of YopD alone in either *E. coli* or *Y. enterocolitica* caused aggregation and insolubility of this polypeptide in the bacterial cytoplasm (Fig. 5E).

Thus, formation of YopD-LcrH complexes seems to be a prerequisite for binding of these polypeptides to the *yopQ* transcript. Multiple gel-shifted  $^{32}\text{P}$ -labeled *yopQ* species were observed at higher concentrations of YopD and LcrH (Fig. 5B). This could be due to the binding of multiple YopD and LcrH molecules to each transcript. We calculated the concentration for 50% binding to  $^{32}\text{P}$ -labeled *yopQ* mRNA as  $8 \times 10^{-6}$  M YopD and LcrH. As the on and off rates of proteins for mRNA targets are affected by equilibrium changes during gel electrophoresis (11), this measurement must be considered an approximation of the affinity of YopD and LcrH for the *yopQ* transcript.

***yopD* mutations suppress synthesis and secretion defects of *yopQ* signal mutants.** Previous work mapped the *yopQ* secretion signal to codons 1 to 15. The +1 and -2 frameshift mutations of this element did not affect secretion signaling; however, the -1 frameshift mutation severely reduced reporter gene expression when type III machines were induced by low calcium (6). One explanation for this phenotype is that the *yopQ*<sub>-1</sub> mutation may be defective in secretion signaling, preventing initiation of the mutant transcripts into the type III pathway.

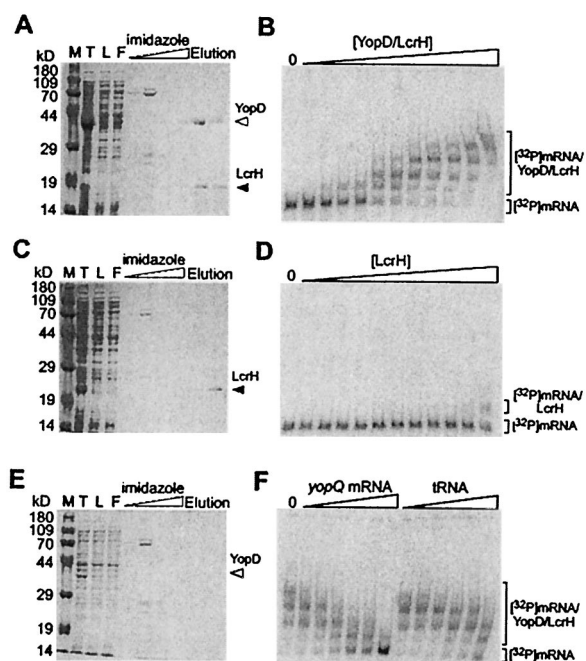


FIG. 5. YopD and LcrH complexes bind *yopQ* mRNA. A transcriptional *yopD-lcrH* fusion (A), *lcrH* (C), or *yopD* (E) was cloned into pET16 (Novagen) and transformed into *E. coli* BL21(DE3). Protein was expressed by IPTG induction of T7 polymerase. Total cell extracts (T) were centrifuged, and the cleared supernatant (L, load) was applied to affinity chromatography on Ni-NTA. Flowthrough (F), column wash samples with increasing stringency (imidazole), and elution with 0.5 M imidazole were analyzed by separating proteins on SDS-15% PAGE and staining with Coomassie blue. The migration of molecular size markers is indicated.  $^{32}\text{P}$ -labeled *yopQ* mRNA was obtained by in vitro transcription of pDA340 with [ $^{32}\text{P}$ ]UTP and gel purification.  $^{32}\text{P}$ -labeled *yopQ* mRNA (10 fmol) was incubated in the presence of increasing amounts of purified YopD and LcrH (0, 0.9, 1.8, 2.7, 3.6, 4.5, 5.4, 6.3, 7.2, 8.1, 9, and 27 ng) (B) or LcrH alone (D) and separated by electrophoresis on a 4% polyacrylamide gel. Specificity of *yopQ* mRNA binding (9 ng of YopD and LcrH to 10 fmol of  $^{32}\text{P}$ -labeled *yopQ* mRNA) was assessed by adding excess unlabeled *yopQ* or *E. coli* tRNA (0, 10, 20, 50, 80, 100, and 500 fmol) (F).

To test whether *yopD* is required for the reduced synthesis of YopQ<sub>-1</sub>-Npt, we examined reporter gene expression in various genetic backgrounds (Fig. 6). Wild-type yersiniae synthesized only small amounts of YopQ<sub>-1</sub>-Npt, and the polypeptide remained in the bacterial cytoplasm. The  $\Delta(yopD)$  mutant strain synthesized 15-fold more YopQ<sub>-1</sub>-Npt, and 58% of the polypeptide was secreted into the extracellular medium, suggesting that the *yopD* knockout mutation suppressed both polypeptide synthesis and secretion defects caused by the *yopQ*<sub>-1</sub> mutation.  $\Delta(yopN)$  mutant yersiniae are known to increase virulence gene expression in the presence of calcium; however, the mutant cells were unable to increase expression or secretion of the YopQ<sub>-1</sub>-Npt fusion. Together these data suggest that YopD and LcrH prevent polypeptide synthesis of defective secretion signals, presumably by sequestration of unused transcripts and initiation into a degradation pathway.

## DISCUSSION

Regulatory control of the *Yersinia* type III pathway was first revealed by the isolation of low-calcium response (*lcr*) mutants

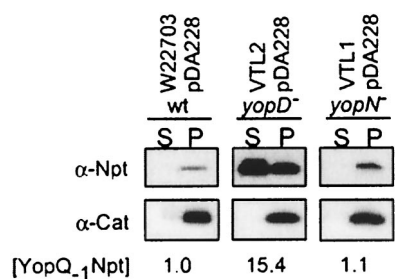


FIG. 6. *yopD* mutation suppresses the signaling defect of the YopQ<sub>-1</sub>-Npt mutation. The secretion signal of *yopQ*, i.e., codons 1 to 15 fused to *npt*, was mutated by deleting a nucleotide immediately following the AUG translational start. The reading frame shift was corrected at the fusion site with the *npt* reporter, generating the YopQ<sub>-1</sub>-Npt fusion (pDA228). Wild-type *Y. enterocolitica* W22703 (wt), the  $\Delta(yopN)$  mutant strain VTL1, and the  $\Delta(yopD)$  mutant VTL2 were transformed with pDA228. Cultures were induced for type III secretion by growing yeast in the absence of calcium. Cultures were centrifuged, and the extracellular medium was separated into supernatant (S) and bacterial pellet (P). The expression and location of YopQ<sub>-1</sub>-Npt and cytoplasmic chloramphenicol acetyltransferase (Cat) was measured by immunoblotting with specific antibody. The secretion of YopQ<sub>-1</sub>-Npt was quantified with an alpha imager: W22703, 0%;  $\Delta(yopD)$  mutant VTL2, 58%; and the  $\Delta(yopN)$  mutant strain VTL1, 0%. The relative concentration of the protein [YopQ<sub>-1</sub>-Npt] is indicated for each strain.

(72). *lcrE* (*yopN*) mutants display a temperature-sensitive growth defect (71), as these strains secrete massive amounts of Yop proteins when grown at 37°C in the presence of calcium (23). Loss-of-function mutations in *syncN*, *tyeA*, or *yscB* cause a similar phenotype (14, 20, 35, 37, 38). The products of the *lcrE* (*yopN*), *syncN*, *tyeA*, and *yscB* genes presumably block the type III secretion pathway in the presence of calcium (16). Mutations in *lcrH* and *yopD* also affect calcium regulation of the *Yersinia* type III pathway. The growth defect of *lcrH* and *yopD* mutants is not as severe as that of *lcrE* (*yopN*), *syncN*, *yscB*, and *tyeA* strains (56, 62, 72). *lcrH* and *yopD* are expressed from the *lcrGVHyopBD* operon (8). Both genes have previously been implicated in regulating expression of the *yop* virulon (8, 70), but the mechanism of regulation was hitherto unknown.

We observed that *yopN*, *syncN*, *yscB*, and *tyeA* mutations abolish the calcium regulation of YopQ synthesis and secretion (class I mutants). In contrast, *yopD* and *lcrH* mutations abolish the regulation of YopQ synthesis without altering the regulation of YopQ secretion (class II mutants). It is suggested here that regulation of *yopQ* expression occurs at the posttranscriptional level and is mediated by *yopD* and *lcrH*. YopD and LcrH bind directly to *yopQ* transcript and could act as a repressor of translation. YopD and LcrH may bind to several different transcripts, as *yopD* and *lcrH* mutations are known to affect the expression of *yopE*, *yopQ*, and *yopM*, among others (8, 70). We speculate that YopD/LcrH may function as a repressor for the translation of transcripts that encode type III secretion substrates. When the type III pathway is inactive, this regulatory mechanism presumably represses the synthesis of Yop proteins and prevents the accumulation of polypeptides that cannot be secreted. Type III export of YopD may deplete the repressor complex from the bacterial cytoplasm, triggering recognition of *yop* transcripts by the secretion machinery. Thus, secretion of YopD can be viewed as a regulatory switch for the activation of the type III pathway.

It is conceivable that YopD and LcrH prevent the recognition of translational initiation signals by ribosomes and regulate translational initiation of *yopQ* mRNA. If so, the binding site of YopD and LcrH could be located within the untranslated leader of *yopQ* (nucleotides -178 through +3 relative to the AUG start codon), as fusion of this region to *npt* is sufficient for *yopD/lcrH*-mediated regulation. Recent experiments suggest the possibility that additional genes are required for *yopD*- and *lcrH*-mediated control of *yop* gene expression. Williams and Straley as well as Lee et al. suggest that *yscM1* and *yscM2* act at the same regulatory step of *yop* gene expression (42, 70). This is a surprising result, as LcrQ (YscM1 and YscM2) is believed to control the transcriptional regulation of *yop* expression (54, 61). Future work will need to unravel the molecular mechanism of *yopD*-, *lcrH*-, *yscM1*-, and *yscM2*-mediated regulation of *yop* expression.

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