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LcrQ Coordinates with the YopD-LcrH Complex To Repress *lcrF* Expression and Control Type III Secretion by *Yersinia pseudotuberculosis*

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ABSTRACT Human-pathogenic *Yersinia* species employ a plasmid-encoded type III secretion system (T3SS) to negate immune cell function during infection. A critical element in this process is the coordinated regulation of T3SS gene expression, which involves both transcriptional and posttranscriptional mechanisms. LcrQ is one of the earliest identified negative regulators of *Yersinia* T3SS, but its regulatory mechanism is still unclear. In a previous study, we showed that LcrQ antagonizes the activation role played by the master transcriptional regulator LcrF. In this study, we confirm that LcrQ directly interacts with LcrH, the chaperone of YopD, to facilitate the negative regulatory role of the YopD-LcrH complex in repressing *lcrF* expression at the posttranscriptional level. Negative regulation is strictly dependent on the YopD-LcrH complex, more so than on LcrQ. The YopD-LcrH complex helps to retain cytoplasmic levels of LcrQ to facilitate the negative regulatory effect. Interestingly, RNase E and its associated protein RhlB participate in this negative regulatory loop through a direct interaction with LcrH and LcrQ. Hence, we present a negative regulatory loop that physically connects LcrQ to the posttranscriptional regulation of LcrF, and this mechanism incorporates RNase E involved in mRNA decay.

IMPORTANCE All three human-pathogenic *Yesinia* species, *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*, employ a plasmid-encoded T3SS to target immunomodulatory effectors into host immune cells. Several plasmid-encoded regulators influence T3SS control, including the master transcriptional activator LcrF, the posttranscriptional repressor YopD, and the unassigned negative regulatory factor LcrQ. Since LcrQ lacks any obvious DNA or RNA binding domains, its regulatory mechanism might be special. In this study, we screened for proteins that directly engaged with LcrQ. We found that LcrQ cooperates with LcrH of the YopD-LcrH complex to aid in the posttranscriptional repression of *lcrF* expression. This negative-control loop also involved the mRNA decay factor RNase E and its associated RhlB protein, which were recruited to the regulatory complex by both LcrQ and LcrH. Hence, we identify interacting components of LcrQ that shed new light on a mechanism inhibiting T3SS production and biogenesis.

KEYWORDS T3SS, regulation, RNase E, RhlB, chaperone

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All three human-pathogenic *Yesinia* species—*Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*—employ a type III secretion system (T3SS) to deliver immunomodulatory effector proteins into host immune cells (1–4). This has the purpose to hijack cellular signaling involved in host immune responsiveness that enables bacteria to

establish an infection niche (1–3). All structural proteins (termed Ysc for *Yersinia* secretion), as well as the major secreted effectors (termed Yops for *Yersinia* outer proteins), are encoded on a 70-kb conserved virulence plasmid named pYV or pCD. Additionally, a recent report also indicates a subset of immunomodulatory effector proteins are encoded on the *Yersinia* chromosome (5).

Composed of several highly conserved substructures, both T3SS biogenesis and subsequent substrate secretion follow well-orchestrated pathways that are tightly controlled (6–9). In *Yersinia*, *ysc* and *yop* gene expression is stringently controlled at both transcriptional and posttranscriptional levels (7, 10–16). A low Ca^{2+} signal *in vitro* or close eukaryotic cell contact *in vivo* are both stimulators of Ysc-Yop T3SS biogenesis and activity (17, 18). LcrF, the only characterized activator encoded on the pYV plasmid, is an AraC family transcriptional regulator that directly binds to several promoters of T3SS-related genes to activate their transcription (16, 19, 20). Additionally, several pYV-encoded proteins counter this by repressing the T3SS system (21, 22). One such protein is dual-functional YopD, a translocon pore former located at the top of the T3SS needle that also acts as a negative regulator by binding to AU-rich sequences in the 5′ untranslated region (5′ UTR) of *yop* mRNA to regulate its stability and the translation processes (23–25). Crucially, a further role of YopD is to impact the effectiveness of the translational regulator CsrA, which, in turn, enhances LcrF production (26). Central to the multiple functions of YopD is the need for presecretory stabilization through a binary interaction with the cognate type III secretion (T3S) chaperone, LcrH (22, 23, 25, 27, 28).

Additionally, LcrQ, also known as YscM in *Y. enterocolitica*, has long been known to block Yop secretion when accumulated in the bacterial cytoplasm (17, 21, 29). However, the mechanism underlying this blockage has remained elusive. LcrQ shares 42% identity to the first 128 residues of the T3SS effector YopH (21). This explains why both LcrQ and YopH share a T3S chaperone, SycH (30–32). The derepression of *yop* expression is relieved once SycH interacts with LcrQ/YscM (33). This interaction also facilitates the type III secretion of LcrQ to the outside environment, which further elevates Yop synthesis and secretion (31). In fact, fusion of glutathione *S*-transferase (GST) tag to LcrQ protein, disruption of the T3SS apparatus, or deletion of SycH, all of which prevent LcrQ secretion, lead to decreased expression and secretion of Yops (31, 33). Hence, retention in the bacterial cytoplasm is coupled with the negative regulatory role of LcrQ.

LcrQ lacks any obvious DNA or RNA binding motifs (21, 23, 31). This is consistent with the inability to detect a specific association between LcrQ/YscM and *yop* mRNA (23, 24). These data suggested a novel mechanism of LcrQ-mediated T3SS inhibition. An initial model posits that YopD association with a small subpopulation of 30S ribosomal particles enables LcrQ/YscM to block *yop* mRNA translation (27). However, it remains unclear how this mechanism would actually result in the specific inhibition of *yop* mRNA translation.

Our previous study showed that LcrQ shared regulatory targets with the master regulator LcrF and the relative levels of these two proteins controlled T3SS synthesis (34). We failed to observe a direct protein-protein interaction between LcrF and LcrQ (34), questioning, at the time, how these two regulators might counterbalance each other to regulate T3SS. With a view to understand this process, the present study reports on an interaction between intracellular LcrQ and the T3S chaperone LcrH. We characterized this interaction in the context of repressing LcrF levels by the YopD-LcrH complex during bacterial growth under T3SS restrictive conditions. We also demonstrate that YopD abrogates the secretion of LcrQ. These observations provide a molecular basis for how LcrQ exerts a negative regulatory role on *Yersinia* T3SS.

RESULTS

Cytoplasmic-located LcrQ downregulates the promoter activities of *yop* genes.
To confirm the negative regulatory role of LcrQ protein, we first detected the mRNA
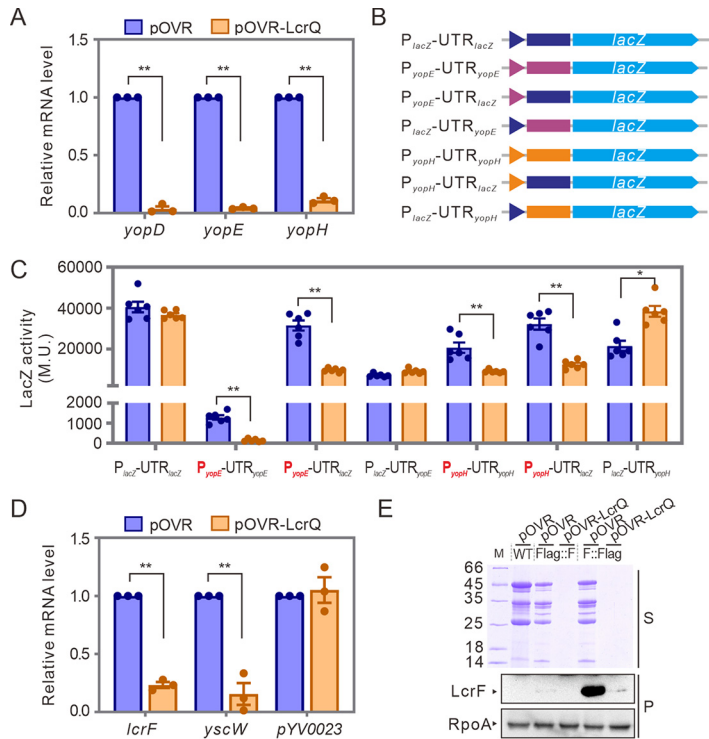
FIG 1 LcrQ inhibits promoter activities of *yop* genes by repressing expression of the master regulator LcrF. (A) Relative mRNA levels of *yopD*, *yopE*, and *yopH* in LcrQ-overexpressed strain. The mRNA levels in YpIII strain carrying the pOVR plasmid were normalized to 1, respectively. (B) Schematic of *lacZ* fusion constructs. Promoters and 5′ UTR from different genes are colored differently. (C) Effects of overexpressed LcrQ on activity of *lacZ* fusion constructs shown in panel B. The LacZ activity is indicated by Miller unit (M.U.) from β-galactosidase activity assay. (D) Effects of LcrQ overexpression on mRNA level of *lcrF*. The *yscW*, which is cotranscribed with *lcrF*, was also tested. The *pYV0023* gene was used as a control. (E) Repression of LcrQ to expression of LcrF protein. The Flag tag was fused to either the N terminus (Flag::F) or C terminus (F::Flag) of LcrF protein in the coding region. Expression level of LcrF protein was detected by anti-Flag antibody. RpoA was detected as a loading control. *, $P < 0.05$; **, $P < 0.01$.

levels of *yopD*, *yopE*, and *yopH* genes in YpIII parental strain overexpressing *lcrQ*. Elevated cytoplasmic LcrQ abrogated mRNA levels of these genes under T3SS-induced conditions (Fig. 1A), which corroborated other reports (34, 35). We next aimed to identify the regulatory element targeted by LcrQ. For this purpose, we used a transcriptional fusion assay. We constructed a series of chimeric clones composed of the promoter alone, the 5′ UTR alone, or both promoter and 5′ UTR of *yopE* and *yopH* genes in front of the promoterless *lacZ* reporter (Fig. 1B). Where the endogenous regulatory element was lacking, it was substituted by the equivalent element from the regulatory sequences of the *lac* operon (Fig. 1B). As shown in Fig. 1C, LcrQ did not repress the β-galactosidase activities of clones carrying the *lac* promoter fused with 5′ UTR of *yopE* or *yopH* genes but significantly repressed the clones carrying promoters of *yopE* or *yopH* genes. Although we could not exclude the possibility that LcrQ may regulate expression of *yopE* and *yopH* through other regions (such as the coding region or 3′ UTR), our data suggest that LcrQ can downregulate Yops expression by repressing the promoter activities of *yop* genes.

LcrQ represses the expression of the master transcriptional regulator LcrF. Since LcrF is the only transcriptional activator of the Ysc-Yop T3SS encoded on the pYV plasmid (16), we next asked if LcrQ could regulate the expression of *lcrF*. We first detected the mRNA levels of *lcrF* in LcrQ-overexpressed and Δ*lcrQ* strains. As expected, the mRNA level of *lcrF* was increased in a Δ*lcrQ* strain under T3SS-inducible conditions (Fig. S1A in the supplemental material), while it was repressed when LcrQ was overexpressed in the YpIII parental strain (Fig. 1D). To confirm this regulatory effect, we
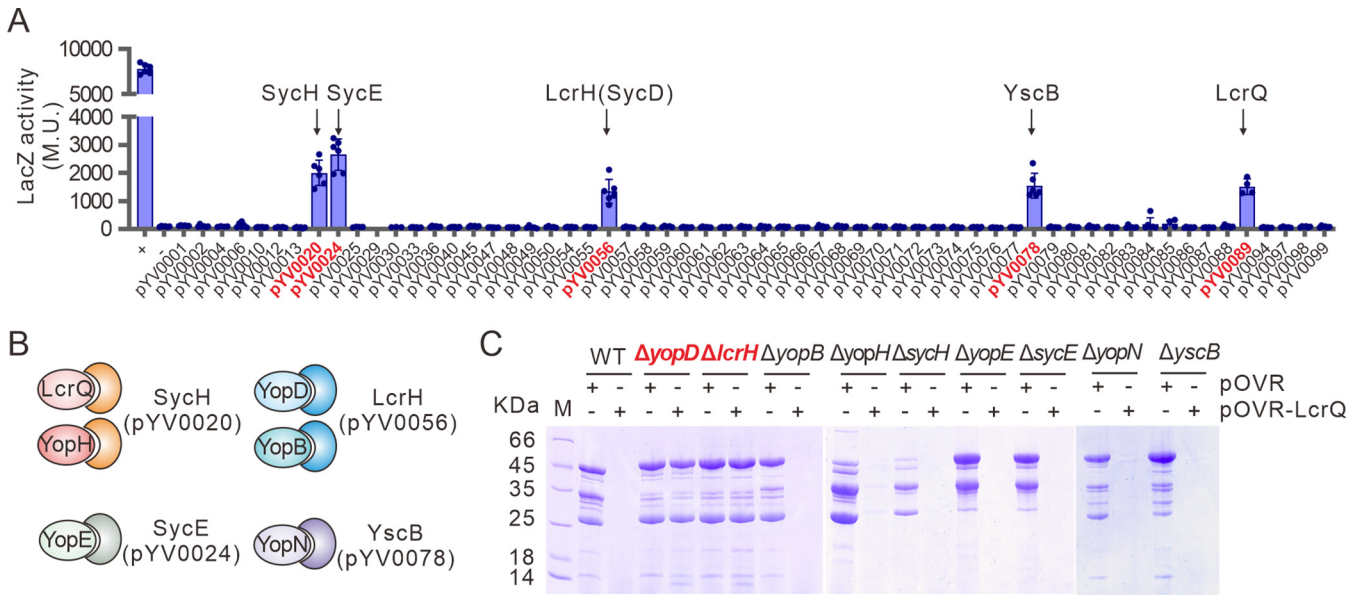
FIG 2 Negative regulatory role of LcrQ to LcrF is dependent on the presence of YopD/LcrH complex. (A) Screening of LcrQ-interacting *Yersinia* T3SS proteins. Bacterial adenylate cyclase two-hybrid system was applied in protein-protein interaction screening. Gene locus numbers of proteins that showed positive interaction with LcrQ are indicated in red. (B) Pairs of Yop effectors and their chaperones. (C) Effects of overexpressed LcrQ on Yops secretion in YpIII WT or mutants lacking a Yop-encoding gene (ΔyopD, ΔyopB, ΔyopH, ΔyopE, or ΔyopN) or their associated chaperone-encoding gene (ΔlcrH, ΔsycH, ΔsycE, or ΔyscB).

examined the LcrF protein levels by Western blotting in this strain overexpressing LcrQ. To facilitate LcrF detection, we inserted a Flag tag-encoding fragment at the 5′ and 3′ termini within the *lcrF* gene in *cis* in the YpIII genome. The transcription of *lcrF* mRNA was only slightly influenced by inserting this Flag tag at either end (Fig. S1C). However, the Flag::LcrF was barely detectable in Western blot assay using anti-Flag antibody (Fig. 1E), probably due to alterations in protein conformation or protein stability induced by the tag. Regardless, overexpression of LcrQ in these strains repressed the expression of recombinant LcrF in both Flag::LcrF and LcrF::Flag strains, which consequently abrogated T3SS production (Fig. 1E). These data taken all together confirmed that LcrQ downregulates the production of LcrF.

The negative regulatory role of LcrQ is dependent on a YopD-LcrH complex. Previous analyses have shown that LcrQ does not contain any DNA or RNA binding motif (21, 23, 31). Our recent study also indicated that LcrQ does not directly interact with LcrF (34). Therefore, we suppose that LcrQ may downregulate LcrF expression by interacting with other proteins. To test this hypothesis, we screened proteins interacting with LcrQ using a bacterial two-hybrid system configured to contain a library of about 60 *ysc-yop* T3SS functional genes derived from the pYV plasmid but excluding genes involved in plasmid replication. Interestingly, LcrQ interacted with itself (Fig. 2A). Additionally, LcrQ interacted with SycH (pYV0020), SycE (pYV0024), LcrH (also known as SycD, pYV0056), and YscB (pYV0078) (Fig. 2A and B), which are customized T3S chaperones specific to the secreted Yops.

To understand the relevance of LcrQ-T3S chaperone interactions, we first overexpressed LcrQ in mutants lacking these T3S chaperones or their cognate Yop substrate. As shown in Fig. 2C, only deletion mutations of *yopD* (designated ΔyopD) or *lcrH* genes (ΔlcrH) abolished the downregulation function by LcrQ. Moreover, overexpression of LcrQ in the absence of YopD or LcrH could not inhibit the accumulation of *lcrF*-, *yscW*-, and *yopE*-specific mRNA (Fig. 3A and Fig. S1B). This suggests that the negative regulatory role of LcrQ depends upon the presence of functional YopD and LcrH.

During this analysis, it became evident that the intracellular level of LcrQ was much lower when overexpressed in the ΔyopD or ΔlcrH background than the wild-type (WT) background (Fig. 3B). Consistent with this, a large portion of LcrQ was secreted into
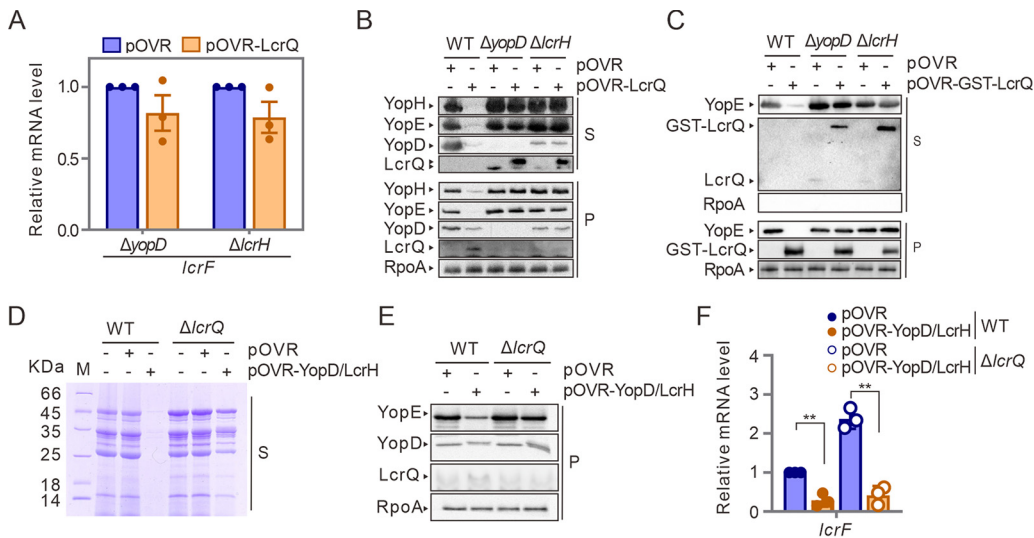
FIG 3 LcrQ coordinates with YopD/LcrH complex in repressing T3SS. (A) Effects of LcrQ overexpression on mRNA levels of *lcrF* in Δ*yopD* and Δ*lcrH* strains. The mRNA levels of *lcrF* in YpIII strains carrying pOVR plasmid were normalized to 1, respectively. (B) Effects of overexpressed LcrQ on Yops expression in cell pellets (P) and protein secretion (S) in YpIII WT, Δ*yopD*, and Δ*lcrH* strains. The T3SS-related proteins were detected using protein-specific antiserum. (C) Expression and secretion of Yops and LcrQ in GST-LcrQ-overexpressed strains. RpoA in supernatant was detected to exclude the possibility of contamination of cell lyses faction. (D and E) Repressive effects of overexpressed YopD/LcrH complex on Yops secretion (D) and Yops expression (E) in YpIII WT and Δ*lcrQ* strains. (F) Overexpression of YopD/LcrH complex on *lcrF* mRNA level in YpIII WT and Δ*lcrQ* strains. The *lcrF* mRNA level in WT strain carrying pOVR was normalized to 1. **, $P < 0.01$.

the supernatants of these mutants (Fig. 3B). Hence, it appears that a YopD-LcrH complex may inhibit LcrQ secretion. To explore this relationship, we appended the GST tag to the N terminus of LcrQ, which had been observed to abolish the secretion of YscM (a LcrQ homologue) (31). Surprisingly, a portion of GST-LcrQ was observed in the clear supernatant fractions of the Δ*yopD* or Δ*lcrH* strain, although not by the parental strain that contained functional YopD and LcrH (Fig. 3C). This is likely to be active secretion to the culture supernatant rather than by contamination of bacterial cellular material because cytoplasmic-located RpoA was not detected in our supernatant samples (Fig. 3C). Critically, GST-LcrQ trapped in the cytoplasm of the Δ*yopD* or Δ*lcrH* strain had no repressive effect on YopE synthesis (Fig. 3C), although it does repress both expression and secretion of YopE when overexpressed in the YpIII parental strain (34). Together, these data suggest that intracellular LcrQ functions through the YopD-LcrH complex, and this complex retains LcrQ in the bacterial cytoplasm.

Since intracellular LcrQ requires the presence of the YopD-LcrH complex for its negative regulatory role, we next tested if the repressive effect of intracellular YopD/LcrH requires the presence of LcrQ. Noticeably, overexpression of YopD and LcrH only slightly repressed Yops secretion and synthesis in a Δ*lcrQ* strain, whereas it caused a dramatic repression in the YpIII parental background (Fig. 3D and E). On the other hand, *lcrF*-specific mRNA was repressed in both the parental and the Δ*lcrQ* backgrounds upon YopD/LcrH overexpression (Fig. 3F). These data suggest that both LcrQ-dependent and independent pathways can promote the repressive effects of YopD-LcrH.

Mapping regulatory regions within LcrQ. In the absence of any predicted structural elements within LcrQ, we wanted to define regions that were important for its regulatory role. To facilitate this, we constructed an LcrQ-mCherry mutant library whereby 102 of 115 LcrQ residues were substituted for alanine. The remaining 12 pre-existing alanine residues and the methionine initiation codon were left unchanged. Fusion to mCherry enabled convenient monitoring of the recombinant LcrQ mutant expression level. A biosensor assay based upon the *lcrG* promoter transcriptionally
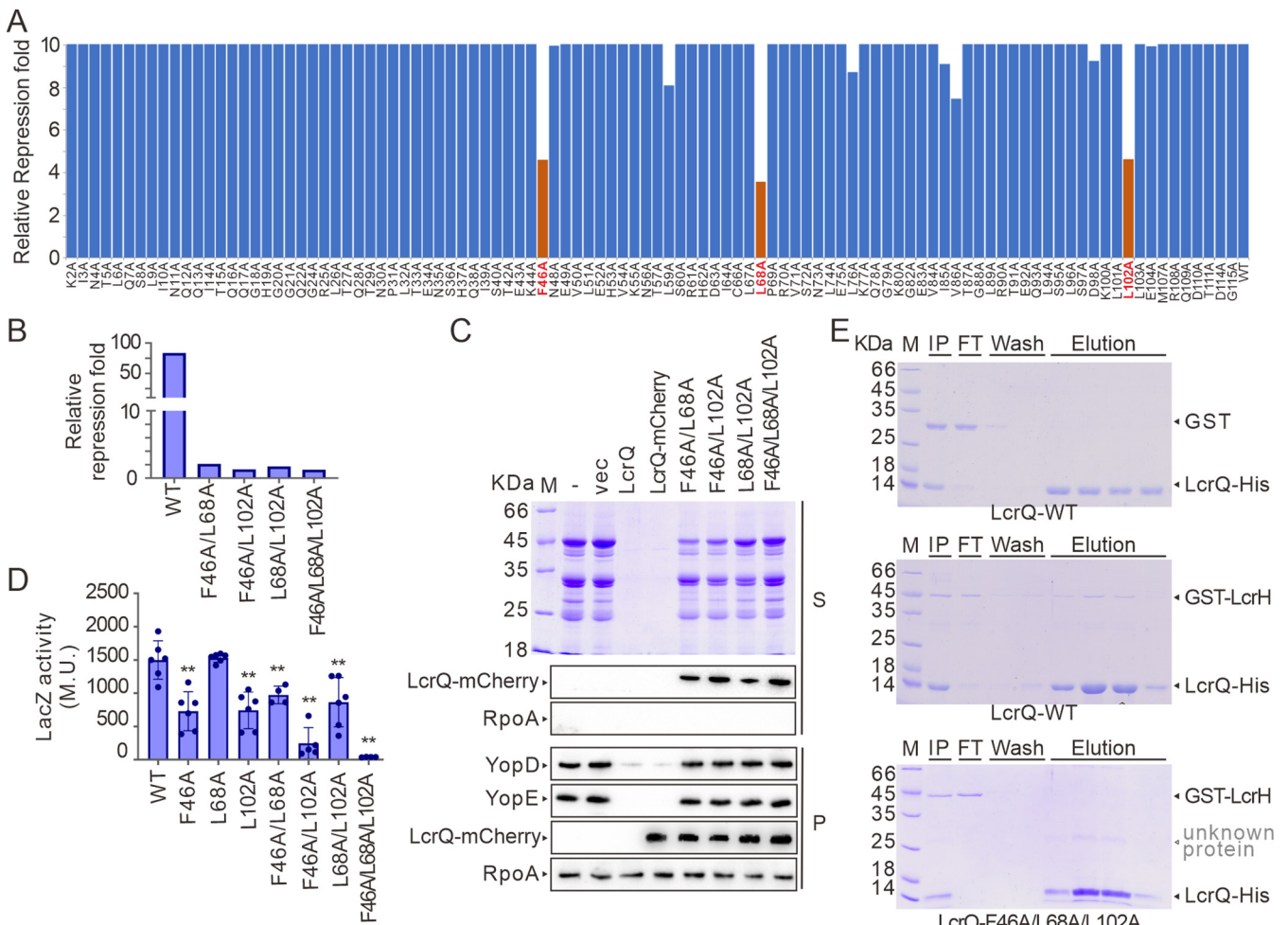
FIG 4 Residues F46, L68, and L102 are important for the negative regulatory role of LcrQ. (A) Scanning mutagenesis of *lcrQ* and correlation to diminished repression by the corresponding mutated product. The relative repression fold was calculated using the repression fold of LcrQ to *lcrG* promoter activity against the LcrQ protein level, which was monitored using mCherry fluorescence intensity. Data are average of three colonies. Mutations showed decreased repressive effects are indicated in red. (B and C) Measuring the effects of combinatory double or triple point mutations within *lcrQ* on the ability of LcrQ to repress *lcrG* promoter activity (B) and Yops expression and secretion (C). (D) Measuring the effects of point mutations within *lcrQ* on the ability of LcrQ to interact with LcrH in bacterial two-hybrid assays. **, $P < 0.01$. (E) Interaction of LcrQ WT or triple mutations (containing His tag) with GST-LcrH protein in pulldown assay using Ni-NTA. GST protein was used as a control.

fused to promoterless *lacZ* was established as a screen for the repressive effect of LcrQ on T3SS expression. The repressive effect was determined by calculating the ratio of the fold repression relative to the respective LcrQ mutant expression level. As seen in Fig. 4A and Table S1, the relative repression fold of the three mutants, $LcrQ_{F46A}$, $LcrQ_{L68A}$, and $LcrQ_{L102A}$, was considerably lower than observed for all other variants, including wild-type LcrQ. Hence, these three residues are important for the full repressive function of LcrQ. Interestingly, no single mutant totally abolished the repressive role of LcrQ (Fig. 4A). As a consequence, we constructed the F46A, L68A, and L102A mutations in double and triple combinations. This generated stable LcrQ variants with far greater regulatory defects, with the triple mutation combination, $LcrQ_{F46A, L68A, L102A}$, being particularly defective (Fig. 4B). As expected, ectopic overexpression of this stable $LcrQ_{F46A, L68A, L102A}$ variant failed to repress the accumulation of *lcrF*- and *yopE*-specific mRNA levels (Fig. S2) and the synthesis and secretion of Yops (Fig. 4C) under T3SS-permissive conditions. Hence, this scanning mutagenesis approach has identified crucial LcrQ residues that support its negative regulatory role.

Having identified LcrH as a novel regulatory target of LcrQ, we next examined if the single, double, and triple mutant combinations of LcrQ influenced the interaction with LcrH. Initially using the bacterial two-hybrid system, we found that $LcrQ_{L68A}$ maintained an ability
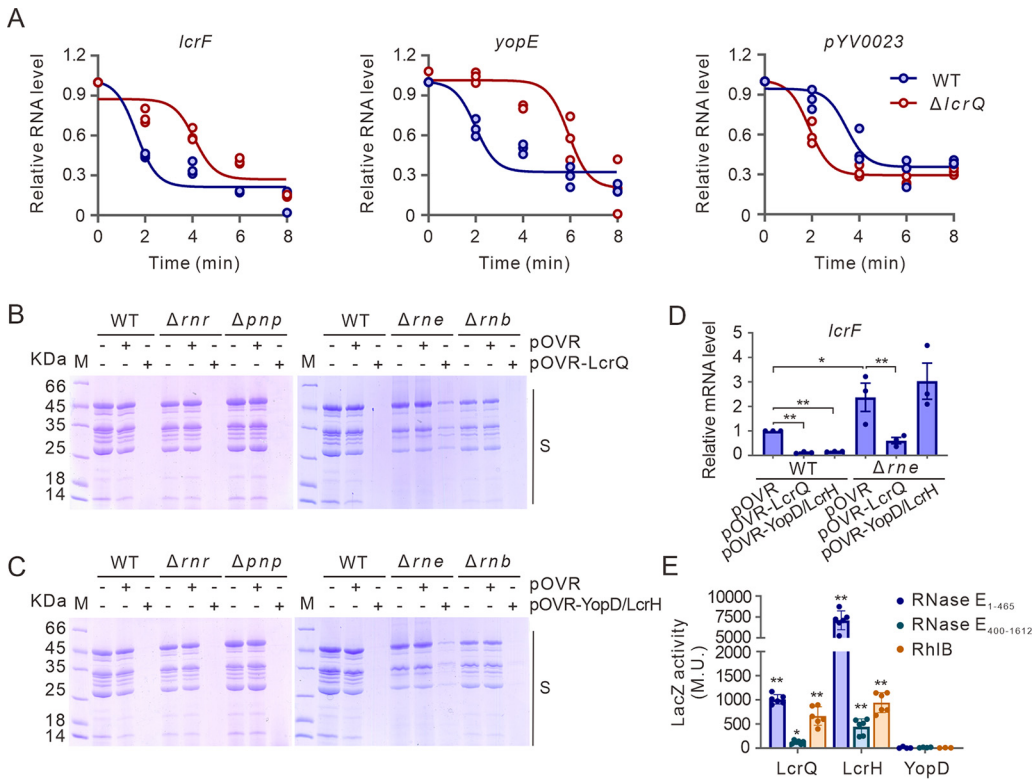
FIG 5 RNase E participates in the negative regulation of LcrF by LcrQ/YopD/LcrH complex. (A) mRNA stability of *lcrF*, *yopE*, and *pYV0023* in YpIII WT and Δ*lcrQ* strains. (B and C) Effects of overexpressed LcrQ (B) or YopD/LcrH complex (C) on Yops secretion in different RNase mutants (Δ*rnr*, Δ*pnp*, Δ*rne*, or Δ*rnb*). (D) *lcrF* mRNA levels in YpIII or Δ*rne* with overexpression of LcrQ or the YopD/LcrH complex. (E) Interaction of LcrQ, LcrH, or YopD with RNase E and RhlB proteins in a bacterial two-hybrid assay. RNase E was separated into two fragments, RNase E_{1-465} and RNase $E_{400-1612}$, in this assay. *, $P < 0.05$; **, $P < 0.01$.

to engage with LcrH to a level observed for wild-type LcrQ (Fig. 4D). On the other hand, the single (LcrQ$_{F46A}$ and LcrQ$_{L102A}$) and double (LcrQ$_{F46A, L68A}$, and LcrQ$_{L68A, L102A}$) mutant variants decreased the LcrQ-LcrH interaction as judged by a 2- to 3-fold reduction in reporter output (Fig. 4D). Furthermore, the double (LcrQ$_{F46A, L102A}$) and triple (LcrQ$_{F46A, L68A, 102A}$) mutation variants abrogated much of the interaction with LcrH (Fig. 4D). Critically, this was not due to protein instability because the fluorescence intensity of LcrQ$_{F46A, L102A}$ and LcrQ$_{F46A, L68A, 102A}$ in fusion with mCherry was comparable to wild-type LcrQ (Table S2). To further confirm these findings, we established a pulldown assay using strains producing His-tagged LcrQ variants together with either GST alone or a GST-LcrH fusion. GST-LcrH could be successfully coeluted with wild-type His-LcrQ but not with the His-LcrQ$_{F46A, L68A, L102A}$ variant (Fig. 4E). Crucially, GST alone did not coelute with either His-LcrQ variant (Fig. 4E). Moreover, neither GST-LcrH nor GST alone could bind to Ni-nitrilotriacetic acid (Ni-NTA) in the absence of His-LcrQ (Fig. S3). Taken all together, these data suggest that the residues at positions 46 and 102 are critical for interacting with LcrH, and this interaction permits LcrQ to exert a negative regulatory role. Intriguingly, we also identified position 68 to influence this LcrQ regulatory capacity, but this may occur independently of the LcrQ-LcrH pathway.

RNase E contributes to negative regulation of LcrF through LcrQ and YopD-LcrH interactions. Since LcrQ cooperates with YopD-LcrH complex and the YopD-LcrH complex regulates T3SS posttranscriptionally (21, 23), we next tested if LcrQ also participates in posttranscriptional regulation. Consistent with our hypothesis, deletion of *lcrQ* increased the stability of *lcrF*- and *yopE*-specific mRNA, but not mRNA of the control fragment pYV0023 encoding a likely transposase remnant (Fig. 5A). To examine whether RNA decay factors are also involved in this negative regulatory circuit, we

overexpressed LcrQ and YopD with LcrH in four different RNase mutant strains, Δ*rne*, Δ*pnp*, Δ*rnr*, and Δ*rnb* (36). As seen in Fig. 5B and C, the repressive impact on Yops secretion normally caused by accumulation of either LcrQ or the YopD-LcrH was diminished specifically in the Δ*rne* strain lacking RNase E production. This correlated with the observation that *lcrF*-specific mRNA was higher in this mutant than the WT strain (Fig. 5D). Crucially, overexpression of LcrQ in the Δ*rne* strain was less effective at repressing *lcrF* mRNA levels (4-fold reduction) than in the WT strain (9-fold) (Fig. 5D). Moreover, YopD/LcrH overexpression in the Δ*rne* strain had no repressive impact on *lcrF*-specific mRNA levels compared to the WT strain (Fig. 5D). Hence, the RNase E mRNA decay factor influences the negative role of LcrQ and YopD-LcrH complex.

We wondered if this association was through a direct interaction between these proteins. Using a bacterial two-hybrid system assay, we found that YopD did not show any direct interaction with RNase E, but LcrQ and LcrH can both interact with RNase E and its associated protein RhlB (Fig. 5E). Importantly, the regulatory-deficient LcrQ mutants F46A, L68A and L102A, in either single, double, or triple combination, could all still interact with RNase E or RhlB (Fig. S4). Hence, RNase E or RhlB do not compete with LcrH for the same binding sites on LcrQ. Taken altogether, these data indicate that RNase E is an important contributor to Ysc-Yop T3SS downregulation by LcrQ and YopD-LcrH control in pathogenic *Yersinia*. Further, it is likely that RNase E works through interactions with LcrQ and LcrH.

DISCUSSION

A number of studies have highlighted the important regulatory role played by LcrQ in the control of Ysc-Yop T3SS by *Yersinia* (21, 31, 34). However, detailed knowledge of the molecular mechanism is lacking. In this study, we demonstrated that LcrQ inhibits expression of *yop* genes by downregulating the expression of *lcrF* encoding the master transcriptional regulator LcrF. This regulatory process depends on the presence of a posttranscriptional regulatory complex composed of YopD and LcrH. Furthermore, we demonstrated that coupling between LcrQ and this complex is achieved through a direct interaction of LcrQ with LcrH. Finally, these two proteins can both interact with RNase E, suggesting LcrQ, YopD/LcrH, and RNase E may combine to regulate T3SS in *Yersinia*.

Previous studies had indicated that the negative regulatory role of LcrQ may require the presence of the YopD-LcrH complex (22, 35), but no direct mechanism underlying this possible relationship had been demonstrated experimentally. Moreover, additional studies using an *in vitro* translation system demonstrated that YopQ translation repression by the YopD-LcrH complex required the LcrQ homologue, YscM1 (13, 27). Herein, we bridge all these studies by identifying that LcrQ interacts with LcrH to facilitate the negative regulatory role of the YopD-LcrH complex. Critically, stable LcrQ variants unable to physically interact with LcrH could no longer exert a repressive role on the T3SS. These findings are supported by the observation that YscM interacts with LcrH in *Y. enterocolitica* (37, 38). We speculate that the purpose of this interaction might be to influence mRNA stability. The basis for this idea stems from observing that both LcrH and LcrQ interact with RNase E and its associated protein RhlB. We propose a model that suggests this interaction facilitates *lcrF* mRNA degradation (Fig. 6). Our future experiments will strive to confirm this coupling. Interestingly, previous studies with YopD have indicated a role in mRNA stability (24–26). In fact, the recent work of Kusmierek and colleagues indicates that this process involves an intricate array of RNA binding proteins and degradation factors (26). Our work corroborates and extends these findings by suggesting that the mRNA stability function attributed to YopD may actually depend upon LcrQ-LcrH, which acts as a molecular scaffold to recruit RNase E in the vicinity of YopD (Fig. 6).

RNase E, which recognizes a specific AU-rich RNA motif (39, 40), is an established regulator of T3SSs in different bacteria. However, the effects can be either repression, such as in *Yersinia* (26, 41) and enterohemorrhagic *Escherichia coli* (EHEC) (42, 43), or
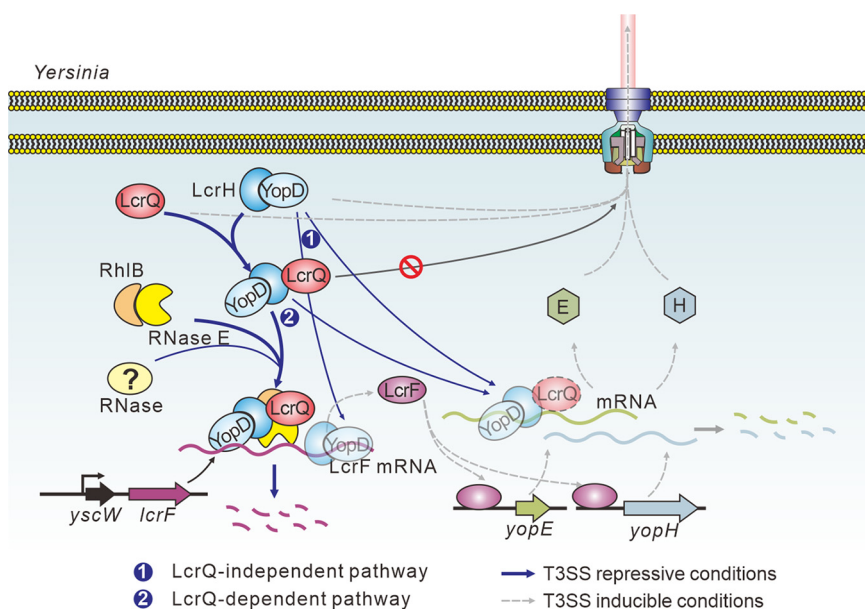
FIG 6 Proposed model for the role of LcrQ in regulating *Yersinia* T3SS. Under T3SS-inducible conditions, the master regulator LcrF activates the transcription of *yop* genes. The synthesized Yop proteins are then secreted outside *Yersinia* cells through the T3SS (indicated by dotted lines). Under T3SS-repressive conditions, the intracellular YopD-LcrH complex represses the expression of T3SS genes via a pathway that is either independent of LcrQ (1) or dependent on LcrQ (2). The LcrQ-dependent pathway also involves RNase E and its associated protein RhlB and possibly some other uncharacterized RNases. This involvement occurs via direct protein-protein interactions involving LcrQ with LcrH as well as LcrQ/LcrH with RNase E and RhlB. Importantly, the interaction between YopD-LcrH with LcrQ inhibits the secretion of LcrQ (⊘). LcrQ trapped in the cytoplasm subsequently promotes the repressive effect of the YopD-LcrH-LcrQ complex in a feedback pathway.

activation such as with *Pseudomonas aeruginosa* (44). There remains a lack of detail surrounding the action of RNase E in these different modes of regulation; to fill these knowledge gaps is worthy of further studies. Our data indicate that RNase E is an important contributor to Ysc-Yop T3SS downregulation by LcrQ and YopD-LcrH control in pathogenic *Yersinia*. However, we also observed that the repressive effects of LcrQ and YopD/LcrH were not completely abolished in our Δ*rne* strain (Fig. 5). This is not so surprising given the multifactorial nature of RNase E function. For example, the basis of our Δ*rne* strain is an incomplete deletion caused by a 3′ truncation of the *rne* gene (36). It is evident that the nature of the *rne* mutation, coupled to the expression of other RNases in the organism, can affect the phenotypes displayed by *rne* mutants with respect to RNA degradosome assembly, mRNA turnover, maturation of rRNA and tRNA precursors, processing and degradation of regulatory RNAs, as well as rRNA quality control (45). Any of these situations may be at play in our *Yersinia* Δ*rne* background. Moreover, unidentified factors, such as additional RNA binding proteins, may also be involved in the regulatory roles of LcrQ and YopD/LcrH. Hence, further studies of our Δ*rne* mutant will likely identify additional players in the posttranscriptional regulation of *lcrF* expression and its impact on T3SS control by pathogenic *Yersinia*.

Interestingly, others implicate one other RNA stability factor, PNPase, in the control of T3SS in *Yersinia* (46, 47). In particular, secretion of YopE and YopD were inhibited in the absence of PNPase, but only upon a short exposure of bacteria to T3SS-inducing conditions (47). Intriguingly, prolonged exposure did not result in any defect, and this is consistent with our data (Fig. 5). Subsequently, however, PNPase was found to posttranscriptionally regulate *lcrF* expression through YopD (26). Yet, in our hands, an over-expressed YopD-LcrH complex still strongly repressed *ysc-yop* T3SS in our Δ*pnp* mutant. These discrepancies probably reflect subtle genetic differences between the specific strains used in the various studies, which are impacted by the relative

expression levels of the various RNases comprising the RNA degradosome. It also suggests that the role of PNPase in this regulatory process may not be a dominant feature in all *Yersinia* strains.

Another aspect of this study was the observation that YopD-LcrH complex can retain cytoplasmic pools of LcrQ. This is probably a consequence of the direct interaction between LcrQ and LcrH. This corroborates specific secretion of LcrQ occurring from regulatory-deficient mutants of *yopD* and *lcrH* when grown in the nonpermissive secretion conditions of plus Ca^{2+} (25, 48, 49). Interestingly, reciprocal experiments showed that YopD was specifically secreted in a Δ*lcrQ* strain grown in the same nonpermissive conditions (21, 29). This suggests that LcrQ may also retain critical cytoplasmic levels of YopD. The accumulation of cytoplasmic levels of both LcrQ and the YopD-LcrH complex would facilitate the repression of T3SS under noninducible conditions (Fig. 6). As LcrQ secretion is an obvious checkpoint in orchestrated control of Yop synthesis and secretion, an analysis of the LcrQ secretor domain is warranted. Precedent for the value of this type of study comes from an analysis of the equivalent YopD secretor domain that revealed features setting it aside from a classical T3SS substrate signal, including possible *yopD* translation control mechanisms (50).

Interestingly, we show that the negative regulatory function of YopD/LcrH was not completely abolished in the absence of LcrQ (Fig. 3D and E). However, the negative regulatory function of LcrQ was completely abolished in the absence of YopD-LcrH (Fig. 3B). This suggests that the regulatory role of LcrQ is strictly dependent on the presence of the YopD-LcrH complex, but the YopD-LcrH complex can function through both LcrQ-dependent and independent mechanisms. Our model of posttranscriptional regulation of *lcrF* expression reflects the involvement of these two pathways (Fig. 6). At this point, the reason for these two pathways and the relative contribution of each to regulatory control is not known. The LcrQ-independent nature of YopD function is thought to manifest itself in the form of translation inhibition of Yop synthesis by direct binding to *yop* mRNA (24), association with the 30S ribosomal subunit (27), and hijacking of global RNA regulators (26). However, these findings could be reinvestigated in light of LcrQ dependency.

Finally, we identified the LcrQ$_{L68A}$ variant that had decreased ability to repress Yops synthesis and secretion despite maintaining an interaction with LcrH, RNase E, and RhlB. Although our interaction assay does not measure productive binding, we suggest that the phenotype associated with the LcrQ$_{L68A}$ variant implies that LcrQ-dependent regulation must incorporate additional regulatory targets. In this context, we and others showed that LcrQ and/or YscM1/YscM2 can also directly interact with several other T3S chaperones, including SycH, SycE, SycO, and SycB (37, 51; this study). Furthermore, we demonstrated herein that LcrQ has potential to bind to itself. Despite the established importance of the LcrQ-SycH interaction to efficient LcrQ secretion (31, 33), roles for the other interactions in T3SS biogenesis, function, and regulation are not well established. However, all these interactions have potential to function in this regulatory process. Having access to the regulatory-deficient LcrQ$_{L68A}$-producing mutant may provide an important genetic tool to revisit the biological consequences of these binding phenomena.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. The *Y. pseudotuberculosis* YpIII and its derivate strains used in this study were cultured in YLB medium (1% tryptone, 0.5% NaCl, and 0.5% yeast extract) at 26°C. *E. coli* strains were grown in LB medium and incubated at 37°C for amplifying plasmids or at 20°C for protein expression. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (30 μg/ml) were supplemented to the medium when needed. All bacterial strains and plasmids used in this study are listed in Table S3 in the supplemental material.

Plasmid construction. All oligonucleotides used in this study are listed in Table S4. To construct the LcrQ overexpression plasmid, the *lcrQ* gene was cloned into the pOVR plasmid (34) between the PstI and KpnI sites to obtain the plasmid designated pOVR-LcrQ. A *gst*-encoding region was amplified and inserted upstream of the *lcrQ* gene in pOVR-LcrQ. To overexpress the YopD-LcrH complex, the *yopD* and *lcrH* genes were both amplified and overlapped into one fragment using a ribosomal binding region as an internal linker. This overlapped fragment was then cloned into the pOVR plasmid. Clones composed

of various promoter-*lacZ* transcriptional fusions were constructed based on the pZT plasmid as described earlier (23). The promoter and 5′ UTR of *yopH* or *yopE* genes (35) were cloned upstream of promoterless *lacZ* using a ClonExpress II one step cloning kit (Vazyme). For the bacterial two-hybrid assay (52), genes were cloned into pKT25 or pUT18 using the ClonExpress II one step cloning kit (Vazyme).

Yops extraction and Western blotting assay. The Yops produced by various YpIII strains were extracted as previously described (34, 53). Briefly, overnight cultures of YpIII strains in YLB were diluted (1:20) into Ca^{2+}-depleted medium (20 mM $MgCl_2$ and 5 mM EGTA) and cultured at 26°C for another 2 h. After that, cultures were transferred to 37°C and incubated for 4 h. Bacterial cell pellets were harvested by centrifugation. For each strain, an 8.1-ml supernatant fraction was carefully removed and then filtrated by a 0.22-μm filter to avoid bacterial contamination. Trichloroacetic acid (TCA) and acetone were used for protein precipitation from supernatant samples. The weights of bacterial cell pellets were determined for normalizing protein levels in bacterial pellets and supernatants. Proteins were dissolved in SDS-loading buffer and resolved by SDS-PAGE. For Western blotting, proteins resolved in SDS-PAGE were transferred into a polyvinylidene difluoride (PVDF) membrane (Millipore) by a semidry method. The membrane was then blocked with 5% nonfat milk. Protein-specific antiserum previously recovered from immunized rabbits (53) was diluted 1,000-fold and used to detect the protein levels of Yops. Mouse anti-Flag monoclonal antibody (1:2,000; Sigma) was used to detect the LcrF levels when it was fused with Flag tag. As appropriate, horseradish peroxidase (HRP)-labeled goat anti-rabbit or anti-mice IgG (1:10,000; Beyotime) was used as the secondary antibody. Enhanced chemiluminescence reagent (Bio-Rad) was used for signal generation. Image detection and collection used a ChemiDoc imaging system, and analysis was performed by the Image Lab software.

Protein purification and GST pulldown assay. *E. coli* strain BL21(DE3) was used for protein purification. The pET21a-LcrQ, pET21a-LcrQ3m, pGEX-KG, and pGEX-KG-LcrH plasmids were transformed into BL21(DE3) and the strains grown at 37°C in LB and incubated to an optical density of 0.4 at a wavelength of 600 nm. IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.3 mM was used for protein production. Ni-NTA was used for His-LcrQ and His-LcrQ$_{F46A, L68A, L102A}$ (His-LcrQ3m) purification, and glutathione Sepharose was used for GST and GST-LcrH purification. For the pulldown assay, His-LcrQ, GST-LcrH, His-LcrQ3m, and GST-LcrH were incubated at 37°C for 1 h. Ni-NTA was used to trap the complex via the His tag. The combinations of His-LcrQ and GST alone, as well as His-LcrQ3m and GST alone, were used as negative controls.

YpIII mutant construction. YpIII mutants or strains with integration of Flag tag at the 5′ end or 3′ end of the *lcrF* gene were constructed using the suicide plasmid pDM4 (54) as previously described (55). Briefly, an ~500-bp fragment upstream and downstream of the region to be deleted was amplified, joined together by the two-step overlap PCR procedure, and then cloned into pDM4 plasmid. The pDM4 derivative was then transformed into *E. coli* S17-1λ*pir* by chemical transformation and then conjugated into YpIII by conjugal mating. Allelic exchange by homologous recombination was screened as previously described (55).

RNA isolation and qRT-PCR. The culture conditions of strains were the same as used for Yops extraction. The TRIzol reagent (Ambion) was used for RNA isolation. The reverse transcription-quantitative PCR (qRT-PCR) assay was performed as described (56). Briefly, 2 μg DNase I (Promega)-treated RNA was used in reverse transcription assay with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). SYBR green supermix and CFX Connect fluorescence quantitative PCR detection system (Bio-Rad) were used in quantification assay. The copy number of 16S rRNA was used for normalization. For each gene expression analysis, at least three biological repetitions were performed, and each repetition contains two technical replicates.

RNA stability assay. The overnight cultures of YpIII strains in YLB were diluted (1:20) into fresh YLB with 20 mM $MgCl_2$ and cultured at 26°C for 2 h, after which they were transferred to 37°C and incubated for a further 2 h. Rifampin was then added to a final concentration of 500 μg/ml. After determined time points (0 min, 2 min, 4 min, 6 min, and 8 min), samples were collected in the presence of 0.2 volumes of stop buffer (5% water-saturated phenol, 95% ethanol) and snap frozen in liquid nitrogen. RNA was isolated as described above, and the mRNA stability was detected by gene-specific qRT-PCR, also as described above.

Bacteria two-hybrid assay. The adenylate cyclase-based bacterial two-hybrid system was used to detect protein-protein interactions (52). *E. coli* BTH101 was cotransformed with various pKT25 and pUT18 derivatives. Three colonies from each transformation were used for testing the β-galactosidase activity using ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (Songon) as the substrate. The empty plasmid pair of pKT25 and pUT18 was used as the negative control, and the pKT25-Zip and pUT18-Zip plasmid pair was used as the positive control. The β-galactosidase activity was examined according to previous descriptions (57).

LcrQ mutant library screening. For LcrQ point mutation library construction, the *lcrQ* gene was first translationally fused at the C terminus with mCherry and cloned into the pBAD22 plasmid (58). The site-directed point mutations of LcrQ were performed by following the protocol provided by QuikChange site-directed mutagenesis kit (Stratagene). All the altered amino acids were mutated to alanine (Ala). This mutant library was cotransformed with the pZT-*lcrG*p plasmid (34) into the $\Delta lcrQ$ mutant to test the repressive effect of the LcrQ protein. The β-galactosidase activity was monitored to indicate the *lcrG* promoter activity. The fluorescence intensity of mCherry (excitation and emission wavelengths are 587 nm and 610 nm, respectively) was measured by a microplate reader (Biotek) to indicate the expression level of the LcrQ variants. Three colonies were tested for each strain harboring a unique LcrQ

variant. The relative fold repression of *lcrG*p by the LcrQ variants was calculated on the basis of *lcrG* promoter activity against the LcrQ expression level.

Statistical analysis. All data for the β-galactosidase activity assays were shown as mean $\pm$ standard deviation (SD) of the results of multiple independent experiments. Statistical analyses were performed using the unpaired Student's *t* test (two-tailed) between each of two groups.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 2.9 MB.

FIG S2, TIF file, 1.2 MB.

FIG S3, TIF file, 0.8 MB.

FIG S4, TIF file, 2.6 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, DOCX file, 0.02 MB.

TABLE S3, DOCX file, 0.04 MB.

TABLE S4, DOCX file, 0.04 MB.

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