Posttranscriptional Regulation of the *Yersinia pestis* Cyclic AMP Receptor Protein Crp and Impact on Virulence

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ABSTRACT **The cyclic AMP receptor protein (Crp) is a transcriptional regulator that controls the expression of numerous bacterial genes, usually in response to environmental conditions and particularly by sensing the availability of carbon. In the plague pathogen *Yersinia pestis*, Crp regulates the expression of multiple virulence factors, including components of the type III secretion system and the plasminogen activator protease Pla. The regulation of Crp itself, however, is distinctly different from that found in the well-studied *Escherichia coli* system. Here, we show that at physiological temperatures, the synthesis of Crp in *Y. pestis* is positively regulated at the posttranscriptional level. The loss of the small RNA chaperone Hfq results in decreased Crp protein levels but not in steady-state Crp transcript levels, and this regulatory effect occurs within the 5′ untranslated region (UTR) of the Crp mRNA. The posttranscriptional activation of Crp synthesis is required for the expression of *pla*, and decoupling *crp* from Hfq through the use of an exogenously controlled promoter and 5′ UTR increases Pla protein levels as well as partially rescues the growth defect associated with the loss of Hfq. Finally, we show that both Hfq and the posttranscriptional regulation of Crp contribute to the virulence of *Y. pestis* during pneumonic plague. The Hfq-dependent, posttranscriptional regulation of Crp may be specific to *Yersinia* species, and thus our data help explain the dramatic growth and virulence defects associated with the loss of Hfq in *Y. pestis*.**

IMPORTANCE **The Crp protein is a major transcriptional regulator in bacteria, and its synthesis is tightly controlled to avoid inappropriate induction of the Crp regulon. In this report, we provide the first evidence of Crp regulation in an Hfq-dependent manner at the posttranscriptional level. Our discovery that the synthesis of Crp in *Yersinia pestis* is Hfq dependent adds an additional layer of regulation to catabolite repression in this bacterium. Our work provides a mechanism by which the plague pathogen links not just the sensing of glucose or other carbon sources but also other signals that influence Crp abundance via the expression of small RNAs to the induction of the Crp regulon. In turn, this allows *Y. pestis* to fine-tune Crp levels to optimize virulence gene expression during plague infection and may allow the bacterium to adapt to its unique environmental niches.**

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Over the past two millennia, *Yersinia pestis*, the bacterium responsible for the disease plague, has been associated with significant morbidity and mortality (1, 2). When infected with *Y. pestis* via the respiratory route, individuals can develop primary pneumonic plague, a severe, rapidly progressing purulent exudative bronchopneumonia that, if untreated, is almost always fatal. In an intranasal mouse model of infection, the progression of primary pneumonic plague in the lungs is biphasic: the first half of the infection is characterized by the lack of a significant immune response (the anti-inflammatory phase) in the lungs that transitions rapidly to a highly inflammatory state (the proinflammatory phase) (3–5). This transition occurs approximately midway through the infection.

We have previously shown that the plasminogen activator Pla, an atypical aspartate protease located in the outer membrane of *Y. pestis* bacteria, is required to cause primary pneumonic plague (6). In the absence of Pla, the bacterial load in the lungs does not increase significantly over time, and yet *Y. pestis* is still able to escape the respiratory system to cause a systemic infection. In addition, Pla is needed for the pulmonary disease to transition to the proinflammatory stage but is not necessary to maintain the early stage of the infection (6). The major activity of Pla during infection is hypothesized to be the direct conversion of the mammalian zymogen protein plasminogen to the active plasmin form, thereby accelerating fibrinolysis (7, 8), although other recently identified targets of Pla proteolysis may also contribute to pathogenesis (9–11).

While *pla* is thought to be constitutively expressed, several reports have demonstrated multiple factors that affect Pla synthesis and activity. For instance, Pla protein levels are moderately influ-

enced by temperature, and higher temperatures result in increased Pla protein compared to the lower temperatures associated with the flea (12). In addition, the catalytic activity of Pla requires bound lipopolysaccharide (LPS), specifically of the "rough" form that has minimal or no O-antigen side chains (such as the LPS of *Y. pestis*) (13, 14). Furthermore, the transcription of *pla* is activated by the cyclic AMP (cAMP) receptor protein (Crp), a global transcription factor that is found in many pathogenic and nonpathogenic bacteria (15–17).

Crp regulates a large number of genes (over 100 genes in *Escherichia coli* and 292 genes in the 201 strain of *Y. pestis*) via a process known as catabolite repression (17, 18). Crp-regulated genes can often be identified by the presence of a conserved Crp box sequence within their promoter regions, and Crp, in conjunction with its coactivator molecule cAMP, binds to this sequence to activate or repress gene transcription (18, 19). Thus, Crp and cAMP together allow the bacterium to modulate the expression of numerous genes in order to appropriately meet the needs of the cell (20). This occurs through the coordinated regulation of the expression of factors involved in general housekeeping and metabolism, cell division, and pathogenesis, including virulence determinants such as the type I fimbriae of uropathogenic *E. coli* and the HapR regulon of *Vibrio cholerae* (18, 21–23). The regulation of virulence factors via catabolite repression is particularly important in *Yersinia* species, as Crp has been shown to influence not only *pla* gene expression but also the *sycO-ypkA-yopJ* operon of the Yop-Ysc type III secretion system (T3SS) in *Y. pestis*, the Ysa T3SS and flagellum of *Yersinia enterocolitica*, and the virulence-associated Csr small RNAs (sRNAs) in *Yersinia pseudotuberculosis* (15, 24–26). Indeed, mutants of all three pathogenic *Yersinia* species lacking *crp* are highly attenuated in animal models of infection, although this may be due in part to the severe growth defect associated with the loss of Crp in addition to the dysregulation of virulence factor expression (17, 24, 26, 27).

Both cAMP and Crp levels fluctuate due to changing environmental states or nutritional status, often via the sensing of carbon levels and through the effects of other regulatory proteins, such as Fis (28–30). For instance, in *E. coli*, Crp represses its own gene expression and that of the adenylate cyclase gene *cyaA* in response to glucose (31, 32). In *Y. pestis*, Crp is also a repressor of the *cyaA* gene, but the *crp* gene itself is not autoregulated; instead, *crp* is directly regulated via the PhoPQ two-component system (33, 34). This suggests that, while there are similarities between the regulation of catabolite repression in *Y. pestis* and *E. coli*, differences have emerged between the plague bacillus and other species in the mechanisms by which the Crp regulon is controlled, although as yet a posttranscriptional component has not been reported.

In a screen to discover additional factors that regulate Pla activity, we identified the gene encoding Hfq. Hfq is a chaperone of bacterial small, noncoding regulatory RNAs that enhances the interaction between sRNAs and their target mRNAs. This results in the up- or downregulation of target gene expression, usually through posttranscriptional mechanisms (35). Hfq often binds to the 5′ region of regulated mRNAs, and in conjunction with sRNAs, these interactions can have dramatic effects on translation, transcript stability, and protein activity (36). By acting as a homohexamer, Hfq contains at least 2 distinct RNA-binding surfaces which may explain why the protein is able to facilitate RNA-RNA interactions (35).

Hfq has been implicated in the virulence of multiple bacteria

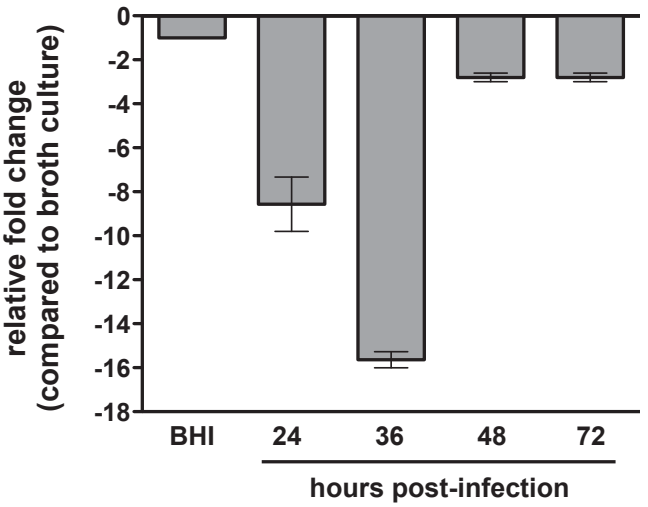

FIG 1 Changes in relative *pla* mRNA levels during the progression of primary pneumonic plague. C57BL/6 mice were infected via the intranasal (i.n.) route with *Y. pestis*, and the relative levels of the *pla* transcript in the lungs at the times indicated were compared to BHI broth after 12 h at 37°C (set at −1) by qRT-PCR. Data are representative of two independent experiments.

(37), and as a pleiotropic mediator of sRNA-mRNA interactions, is known to affect the regulation of multiple virulence-associated genes (38–40). Both Hfq and sRNAs are significant contributors to the posttranscriptional regulation of gene expression in *Yersinia* species and are required for the virulence of *Y. pestis* and *Y. pseudotuberculosis* in multiple animal models of infection (41–44). This study describes the contribution of Hfq to the regulation of *pla* expression via the direct, posttranscriptional control of Crp synthesis and addresses the contribution of Hfq-mediated regulation of Crp to the virulence of *Y. pestis* during primary pneumonic plague.

RESULTS

Dynamic expression of *pla* during primary pneumonic plague. Previous studies have shown a modest increase in Pla protein levels when *Y. pestis* is cultured *in vitro* at 37°C compared to that at lower temperatures (12). As primary pneumonic plague is a biphasic syndrome and dependent on the activity of Pla during the proinflammatory phase of the disease (6), we hypothesized that *Y. pestis* also controls the expression of *pla* to coincide with the temporal requirement of the protease during the infection. Therefore, we examined the relative levels of the *pla* transcript in the lungs of mice at various times postinfection compared with transcript levels in bacteria when cultured *in vitro* in brain heart infusion (BHI) broth at 37°C. In the early phase of the infection, *pla* transcript levels are decreased by up to 16-fold compared to those in bacteria grown in broth culture, but by 48 h, *pla* transcript levels recover to approximately *in vitro* levels, and this expression pattern continues into the terminal phase of the infection (Fig. 1). Notably, transcript levels of the control gene *gyrB* remain relatively constant over the course of the infection compared with those of either *proS* or the 16S rRNA (see Table S1 in the supplemental material), thus demonstrating that *pla* expression is specifically and dynamically altered in a manner that is consistent with the Pla-dependent biphasic inflammatory response during primary pneumonic plague.

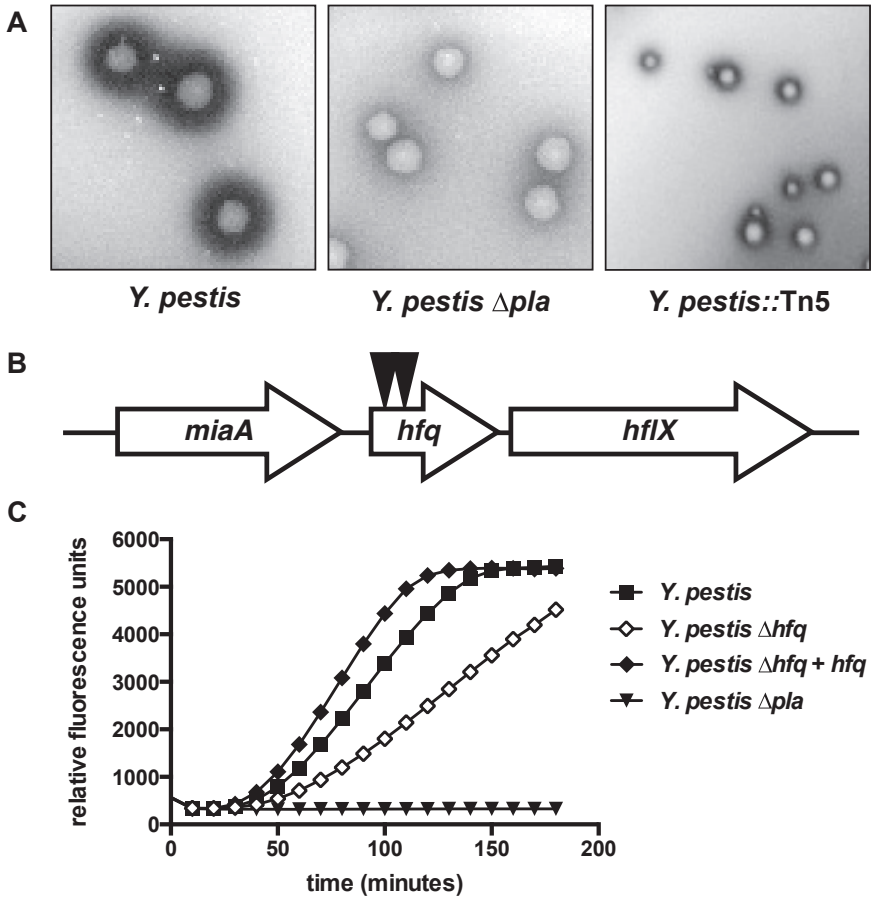

FIG 2 Hfq contributes to the regulation of Pla activity. (A) *Y. pestis* was grown on BHI agar and then overlaid with a top agar containing fat-free milk and human Glu-Plg. Activation of plg by Pla results in a zone of clearance surrounding the bacteria (left), while *Y. pestis* lacking Pla does not produce equivalent zones (middle). Reduced zones of clearance produced by one of the *Y. pestis*::Tn*5* mutants recovered from the screen (right). (B) *Y. pestis* was mutagenized with the transposon TnMod-RKm′, and mutants were assessed for altered zones of clearance. Two mutants with reduced zones of clearance had independent insertions (indicated by black triangles) in the gene encoding the small RNA chaperone Hfq. (C) The plg-activating ability of *Y. pestis*, an isogenic *Y. pestis* Δ*hfq* mutant, a Δ*hfq* strain complemented with a wild-type copy of *hfq* integrated onto the chromosome (Δ*hfq* + *hfq*), and a Δ*pla* mutant cultured at 37°C are shown. Data are representative of at least 3 independent experiments.

Decreased plasminogen activation by *Y. pestis* in the absence of Hfq. The changes in *pla* transcript levels *in vivo* led us to conduct a genetic screen for regulators of Pla activity. In this screen, *Y. pestis* was mutagenized with the Tn*5* transposon TnMod-RKm′ (45), plated onto BHI agar, and cultured at 37°C for 2 days. Then, colonies were overlaid with agar containing milk and human Glu-plasminogen (Plg) and incubated for an additional day. Since milk (casein) is a substrate for plasmin, changes in the ability of *Y. pestis* to activate plg can be assessed by examining altered zones of clearance surrounding the bacteria (Fig. 2A, left). This activity is dependent upon Pla (Fig. 2A, middle).

We screened approximately 20,000 mutants from 13 independent matings for altered zones of clearance. Of those, 169 mutants with reduced zones compared to those of the wild type (Fig. 2A, right) were rescreened for plg-activating ability in liquid culture. Among the mutants with confirmed reductions in Pla activity compared to that of wild-type bacteria were those with transposon insertions in *crp* and *cyaA*, confirming earlier reports (15). In addition, we identified two independent mutants with reduced ability to activate plg containing insertions in the gene encoding the small RNA chaperone Hfq (Fig. 2B). To examine this further, we used a strain of *Y. pestis* with a previously generated isogenic, unmarked deletion of *hfq* (46) and performed a plg activation assay with bacteria cultured at 37°C. The *hfq* mutant was unable to activate plg to the same extent as the wild-type strain, confirming the transposon insertion phenotype (Fig. 2C).

Hfq-dependent, transcriptional regulation of *pla*. To determine if the absence of Hfq affects Pla protein levels, we cultured wild-type *Y. pestis*, Δ*hfq Y. pestis*, a Δ*hfq* strain of *Y. pestis* complemented with a wild-type copy of *hfq* integrated onto the chromosome (Δ*hfq* + *hfq Y. pestis*), and Δ*pla Y. pestis* for 6 h at 37°C and performed immunoblot analyses on cell lysates with an anti-Pla antibody. We observed that the loss of Hfq reduces Pla protein abundance compared to that in wild-type bacteria (Fig. 3A). To establish if Hfq has an effect on the steady-state levels of *pla* mRNA, we then compared the relative levels of the *pla* transcript between the wild-type, Δ*hfq*, and Δ*hfq* + *hfq* strains cultured under the same conditions. The level of *pla* mRNA in the Δ*hfq* strain was reduced by 7.5-fold compared to that of wild-type *Y. pestis*, demonstrating that Hfq affects the expression and/or stability of

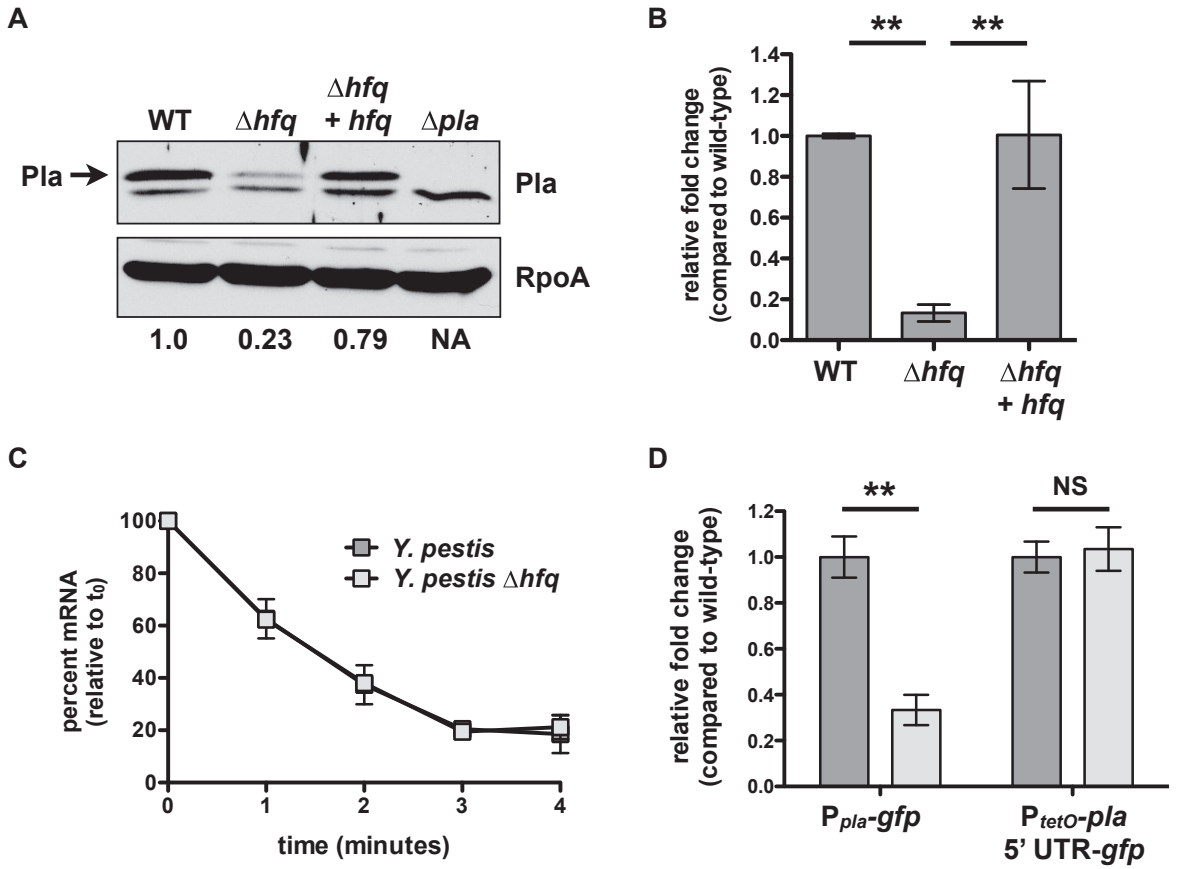

FIG 3 Indirect, transcriptional control of *pla* via Hfq. (A) Wild-type, Δ*hfq*, Δ*hfq* + *hfq*, and Δ*pla Y. pestis* were grown at 37°C, and equal concentrations of cell lysates were separated by SDS-PAGE followed by immunoblot analysis with an anti-Pla or anti-RpoA antibody. Pla is indicated with an arrow; the lower band is nonspecific. Immunodetection of RpoA is shown as a loading control. The relative density of the Pla band compared with the wild type is shown below the RpoA panel. NA, not applicable. Data are representative of 3 independent experiments. (B) Steady-state levels of the *pla* transcript after 6 h at 37°C. Strains of *Y. pestis* were grown in triplicate, and the relative fold change of the *pla* transcript in the Δ*hfq* and Δ*hfq* + *hfq* strains compared to that of the wild-type bacteria (set at 1) was determined by qRT-PCR using the $\Delta\Delta C_T$ method. Data are representative of 3 independent experiments. (C) Half-life of *pla* mRNA. Wild-type and Δ*hfq Y. pestis* were cultured as described above, and at time 0 rifampin was added to prevent *de novo* RNA synthesis. Percent remaining mRNA at each time point was measured by qRT-PCR and compared with time 0. *pla* half-life: 1.66 ± 0.27 min for the wild type, 1.70 ± 0.24 min for the Δ*hfq* mutant, difference not significant. Data are representative of 2 independent experiments. (D) Wild-type or Δ*hfq Y. pestis* strains with the chromosomal-integrated P_{pla}-*gfp* or P_{tetO}-*pla* 5′ UTR-*gfp* reporter constructs were cultured at 37°C for 6 h, and fold change in fluorescence compared with that of the wild type (set at 1), normalized to the optical density of the culture, was determined. For the P_{tetO}-*pla* 5′ UTR-*gfp* reporters, ATc was added at time 0. **, $P < 0.005$; NS, not significant. Data represent the combination of 3 independent experiments. For all panels, error bars indicate standard deviation of the mean.

pla at the transcript level (Fig. 3B) and providing a potential explanation for the reduced abundance of the Pla protein under our conditions.

To test if the reduced steady-state transcript levels of *pla* in the absence of Hfq are due to changes in *pla* mRNA stability or half-life ($t_{1/2}$), we cultured wild-type and Δ*hfq Y. pestis* for 6 h at 37°C, after which rifampin was added to the cultures to inhibit the further initiation of *de novo* transcription. We found no significant difference in *pla* transcript levels between the wild-type and Δ*hfq* strains (Fig. 3C), indicating that the loss of Hfq has no impact on the stability of the *pla* mRNA.

As direct effects on protein production mediated by Hfq and sRNAs typically occur within the 5′ untranslated region (UTR) of regulated transcripts (38), we generated two green fluorescence protein (GFP)-based reporter constructs to measure Hfq-dependent, posttranscriptional or transcriptional and posttranscriptional activity from the *pla* promoter and/or 5′ UTR. To measure transcriptional and posttranscriptional effects, we cloned 500 nucleotides (nt) upstream of the *pla* translational start site, including 27 nt of the *pla* coding sequence (CDS) immediately upstream of the *gfp* CDS (P_{pla}-*gfp*). To measure solely posttranscriptional effects at the *pla* 5′ UTR, this region (15) and 27 nt of the *pla* CDS were cloned downstream of a modified P_{tetO} promoter that lacks the P_{tetO} 5′ UTR, followed by the CDS for *gfp* (P_{tetO}-*pla* 5′ UTR-*gfp*). The transcription of this construct is controlled by the addition of anhydrotetracycline (ATc), and thus any differences in GFP fluorescence can be attributed to effects at the *pla* 5′ UTR. Both reporters were integrated onto the chromosomes of wild-type and Δ*hfq Y. pestis*, bacteria were cultured for 6 h at 37°C (and in the case of the P_{tetO}-*pla* 5′ UTR-*gfp* reporter strains, ATc was added at time 0), and fluorescence was measured and normalized to the optical densities of the cultures. Although GFP fluorescence from the P_{pla}-*gfp* reporter was significantly reduced in the Δ*hfq* strain compared to that in the wild type, we found that there was no difference in fluorescence between the strains carrying the P_{tetO}-*pla* 5′ UTR-*gfp* reporter (Fig. 3D). This

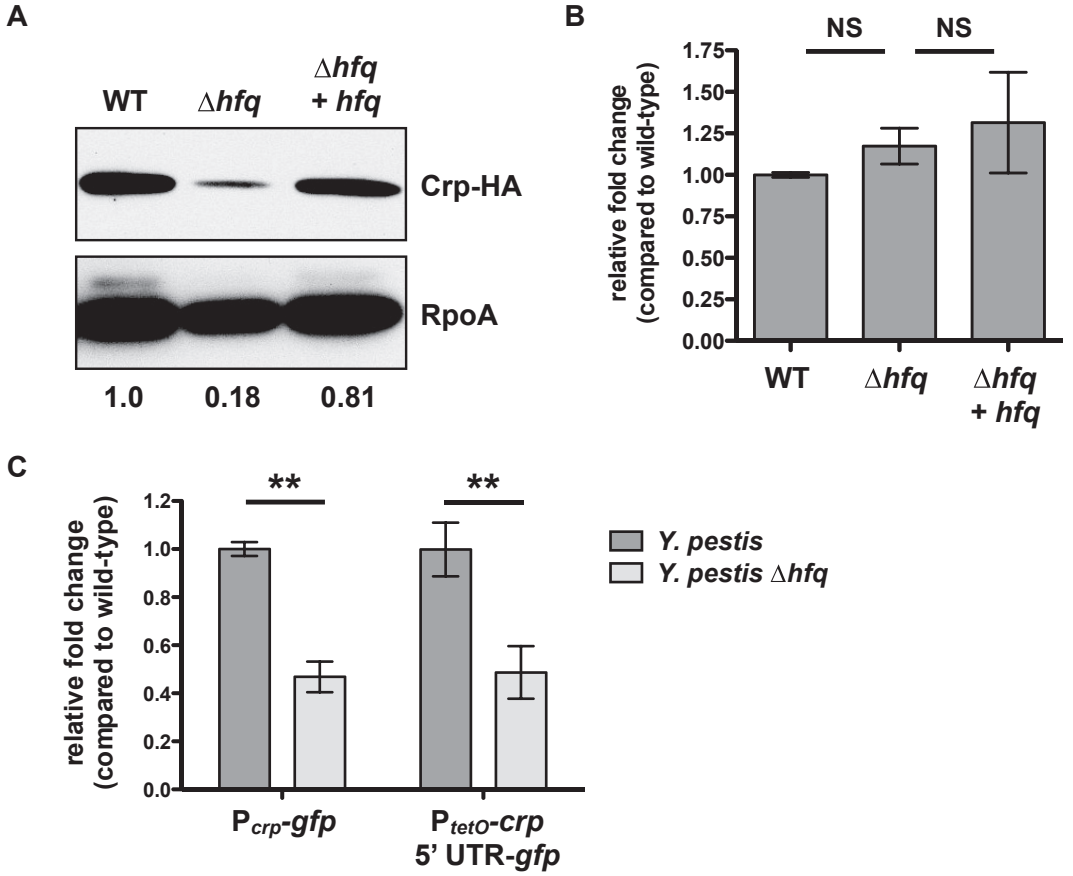

FIG 4 Hfq-dependent, posttranscriptional control of Crp. (A) Wild-type, Δ*hfq*, and Δ*hfq* + *hfq Y. pestis* strains carrying an HA-tagged version of the *crp* gene were cultured for 6 h in BHI broth at 37°C, and whole-cell lysates were analyzed by immunoblotting with an anti-HA antibody. RpoA (bottom) is shown as a loading control. The relative density of the Crp-HA band compared with that of the wild type is shown below the RpoA panel. Data are representative of at least 3 independent experiments. (B) Steady-state levels of the *crp* transcript after 6 h at 37°C. Strains of *Y. pestis* were grown in triplicate, and the relative fold change of the *crp* transcript in the Δ*hfq* and Δ*hfq* + *hfq* strains compared to that of the wild-type bacteria (set at 1) was determined by qRT-PCR using the $\Delta\Delta C_T$ method. Data represent the combination of 3 independent experiments. (C) Wild-type or Δ*hfq Y. pestis* strains with the chromosomal-integrated P_{crp}-*gfp* or P_{tetO}-*crp* 5′ UTR-*gfp* reporter constructs were cultured at 37°C for 6 h, and fold change in fluorescence compared with that of the wild type (set at 1), normalized to the optical density of the culture, was determined. For the P_{tetO}-*crp* 5′ UTR-*gfp* reporters, ATc was added at time 0. **, $P < 0.005$. Data represent the combination of 3 independent experiments. For all panels, error bars indicate standard deviation of the mean.

indicates that Hfq does not contribute to the posttranscriptional regulation of *pla* at the 5′ UTR. In total, the data presented in Fig. 3 indicate that the Hfq-dependent effects on *pla* are likely at the transcriptional rather than the posttranscriptional level and therefore indirect.

Hfq-dependent, posttranscriptional regulation of *crp*. Based on these findings, we hypothesized that Hfq may contribute to the regulation of a factor upstream of Pla. Previous studies have demonstrated that the transcription of *pla* is directly activated by Crp (15, 17). Therefore, to test if Hfq contributes to the regulation of Crp, we replaced the native *crp* gene with a C-terminal hemagglutinin (HA)-tagged version by allelic exchange in the wild-type, Δ*hfq*, and *hfq*-complemented strains of *Y. pestis*. These bacteria were cultured for 6 h at 37°C, and whole-cell lysates were examined by immunoblot analysis with an anti-HA antibody. We found that the loss of Hfq results in decreased Crp protein levels compared to that of the wild type (Fig. 4A). To determine the impact of Hfq on the steady-state levels of the *crp* transcript, we performed quantitative reverse transcription-PCR (qRT-PCR) on RNA extracted from the same cultures. Unlike that observed for *pla*, here the loss of Hfq has no effect on the relative level of the *crp* mRNA compared to that of the wild type (Fig. 4B), indicating that Hfq does not affect the turnover of this transcript in *Y. pestis*. Thus, the data from Fig. 4A and B together suggest that Hfq contributes to the posttranscriptional regulation of Crp.

To assess this more directly, we generated transcriptional and posttranscriptional (P_{crp}-*gfp*) and posttranscriptional (P_{tetO}-*crp* 5′ UTR-*gfp*) GFP reporter constructs for *crp* in the same manner as described for *pla*. To create the posttranscriptional reporter, we first mapped the transcriptional start site of the *Y. pestis* CO92 *crp* mRNA to 79 nt upstream of the translation start site (ATG) by the technique of 5′/3′ rapid amplification of cDNA ends (RACE) (43). The reporter constructs were integrated in single copy onto the chromosomes of the wild-type and Δ*hfq* strains of *Y. pestis*, and fluorescence was measured after 6 h at 37°C (ATc was added to the P_{tetO}-*crp* 5′ UTR-*gfp* reporter strains at time 0). For both the P_{crp}-*gfp* and P_{tetO}-*crp* 5′ UTR-*gfp* reporters, fluorescence intensity was significantly lower in the absence of Hfq than in the wild type (Fig. 4C). This indicates that Hfq promotes Crp synthesis through posttranscriptional regulatory effects at the 5′ UTR and/or prox-

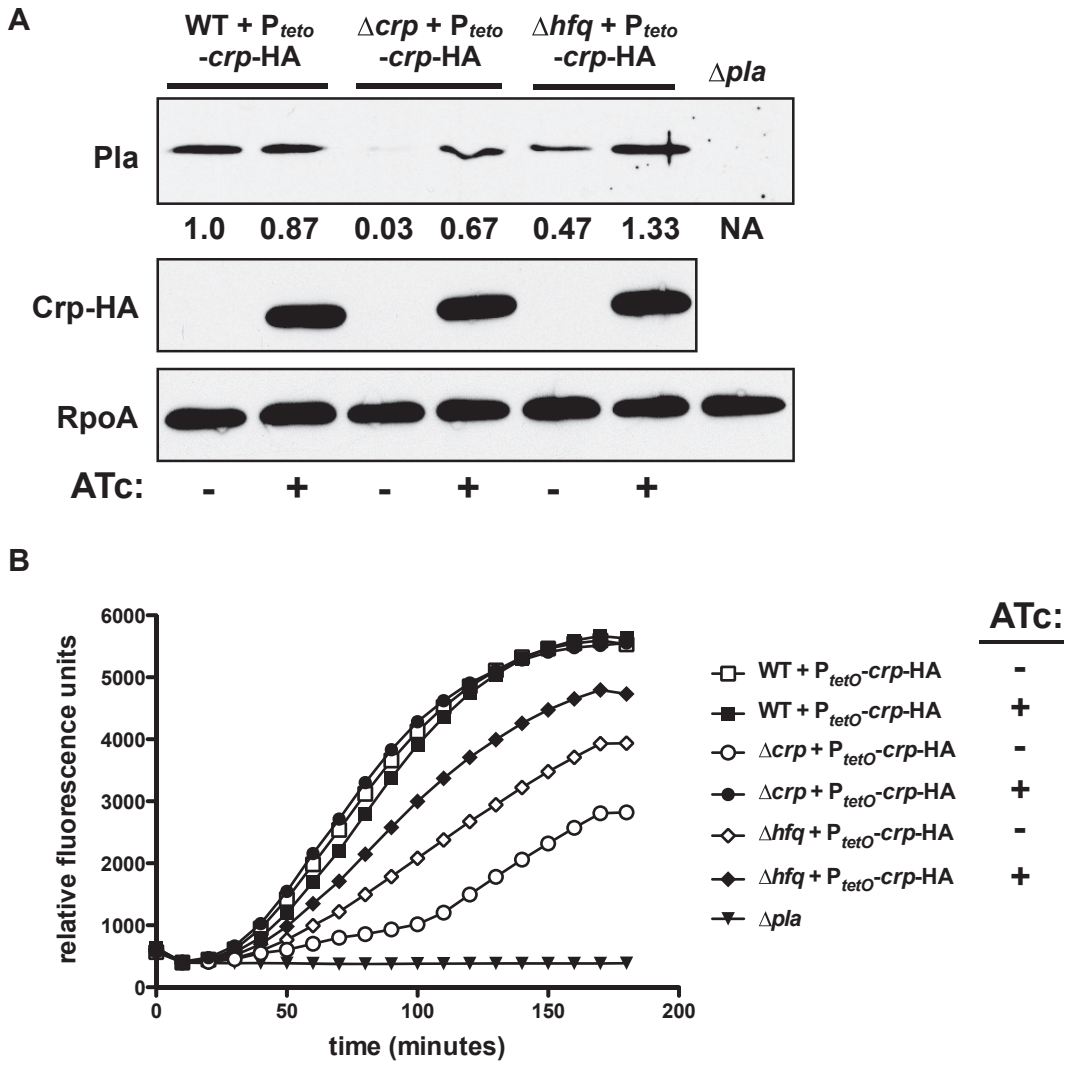

FIG 5 Hfq-decoupled expression of *crp* increases Pla synthesis and activity. (A) Wild-type, Δ*crp*, and Δ*hfq Y. pestis* strains, all containing a chromosomal-integrated P_{tetO}-*crp*-HA construct, were cultured in the presence or absence of ATc for 6 h at 37°C, and cell lysates were analyzed by immunoblotting for Pla (top), the HA tag (middle), or RpoA (as a loading control; bottom). The Δ*pla* strain is shown as a negative control for the presence of Pla. The relative density of the Pla band compared with that of the wild type is shown below the Pla panel. Data are representative of 3 independent experiments. (B) A plg activation assay was performed as described in Fig. 2C on the same strains as described above in the presence or absence of ATc. Data are representative of 3 independent experiments.

imal region of the *crp* CDS. A kinetic analysis of this effect over the growth curve of *Y. pestis* shows that fluorescence produced from the P_{tetO}-*crp* 5′ UTR-*gfp* reporter in the wild-type strain rapidly increases until approximately 6 h postinduction, while fluorescence in the Δ*hfq* bacteria increases between 2 and 4 h to approximately 40 to 50% of the normalized signal intensity of the wild type (see Fig. S1 in the supplemental material).

Decoupling *crp* expression from Hfq increases Pla levels and activity. If the Hfq-dependent posttranscriptional regulation of Crp subsequently influences the expression of *pla*, we hypothesized that unlinking Crp from the regulatory effects mediated by Hfq would result in increased Pla protein levels and activity. To test this, we generated a construct in which *crp* expression is decoupled from Hfq by linking the *crp* CDS to the P_{tetO} promoter and *tetO* 5′ UTR, a promoter and UTR that are unaffected by the absence of Hfq (46). This construct was integrated in a single copy onto the chromosomes of wild-type, Δ*hfq*, and Δ*crp* strains of *Y. pestis*. In the absence of ATc, Pla protein levels are reduced in both mutant strains compared to those of the wild type as measured by immunoblotting, confirming that both Hfq and Crp positively influence the production of Pla (Fig. 5A). In the presence of ATc, however, Pla protein levels in the Δ*hfq* and Δ*crp* strains are increased over −ATc conditions, demonstrating that the defect in Pla levels in the absence of Hfq can be compensated for by the increased production of Crp, although we cannot rule out that the residual levels of endogenous Crp in the Hfq mutant may contribute to Pla synthesis indirectly through the regulation of another factor via Hfq or Crp. Consistent with this, we confirmed by immunoblotting that Crp-HA is produced by these same bacteria only in the presence of ATc. As expected, the addition of ATc to the same strains lacking the inducible P_{tetO}-*crp*-HA construct has no impact on plg activation (see Fig. S2).

We then tested if the expression of *crp*-HA in the Hfq mutant could increase the Pla-dependent plg-activating ability of *Y. pestis*.

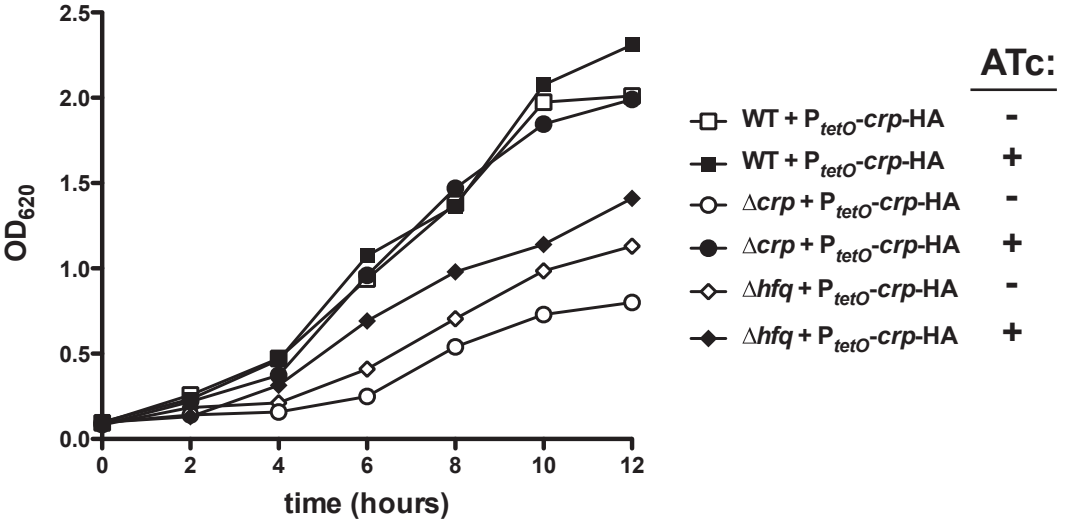

FIG 6 Induction of Crp synthesis partially restores the growth of Δ*hfq Y. pestis* in rich broth. The same bacterial strains containing the P_{tetO}-*crp*-HA construct as described in Fig. 5 were cultured in BHI broth with shaking at 37°C for 12 h in the presence or absence of ATc, and at the indicated times the OD_{620} was measured. The growth of the strains containing the P_{tetO}-*crp*-HA construct in the absence of ATc was equivalent to the parent strains without the construct, and the addition of ATc had no impact on the growth of wild-type bacteria (not shown). Data are representative of 3 independent experiments.

Here, we cultured the same *Y. pestis* strains as described above in the presence or absence of ATc for 6 h at 37°C and performed a plg activation assay. We found that the addition of ATc to the wild-type strain has no influence on plg activation compared to that of the equivalent strain in the absence of ATc (Fig. 5B), confirming our previous observations and demonstrating that excess Crp does not adversely affect Pla activity. The addition of equivalent amounts of ATc to the Δ*crp* P_{tetO}-*crp*-HA strain fully restores plg activation to wild-type levels, indicating that Pla activity can be controlled by manipulating Crp levels in this strain. The addition of ATc to the Δ*hfq* P_{tetO}-*crp*-HA strain is able to partially restore Pla activity, although not to the same extent as wild-type bacteria or the Δ*crp* P_{tetO}-*crp*-HA strain in the presence of ATc (Fig. 5B). In total, the data presented in Fig. 5 indicate that, although the Hfq-dependent regulation of Crp contributes to Pla protein production, there may be additional Hfq-regulated factors beyond Crp that are involved in the ability of Pla to maximally activate plg.

Hfq-dependent regulation of Crp promotes the growth of *Y. pestis* in rich medium. The loss of Hfq from *Y. pestis* results in a significant growth defect compared to growth of wild-type bacteria when cultured *in vitro* in rich medium, such as BHI broth (41, 46, 47). Upon generating a Δ*crp* mutant of *Y. pestis*, we also noted a large growth defect under the same conditions (Fig. 6), although the growth defect is greater than that of the Hfq mutant. Considering the impact of Hfq on the regulation of Crp, we hypothesized that these phenomena may be linked, in that the growth defect of the Δ*hfq* mutant may be due in part to the dysregulation of Crp synthesis. To test this, we used the same P_{tetO}-*crp*-HA-inducible strains described for experiments in Fig. 5 to assess whether the induction of Crp in the Hfq mutant could restore the growth of this strain. Bacteria were cultured at 37°C with shaking in BHI broth, and the optical density at 620 nm (OD_{620}) was measured every 2 h. We found that, as expected, the addition of ATc to the Δ*crp* P_{tetO}-*crp*-HA strain restores growth to that of the wild-type strain (Fig. 6). The addition of ATc to the Δ*hfq* P_{tetO}-*crp*-HA strain increases the growth rate of this strain over vehicle alone but does not fully restore growth to that of the wild type. Thus, these data indicate that the growth defect associated with the loss of Hfq from *Y. pestis* is in part due to the reduced synthesis of Crp but that other Hfq-regulated factors also participate in the full growth of the plague bacillus *in vitro*.

Contribution of Hfq and the posttranscriptional regulation of Crp to primary pneumonic plague. Hfq has been implicated in the virulence of numerous pathogenic bacteria, and as a pleiotropic participant in gene regulation, Hfq may have effects on multiple factors involved in virulence. Therefore, in order to test the contribution of Hfq to the virulence of *Y. pestis* during primary pneumonic plague, we intranasally infected C57BL/6 mice with $pCD1^+$ wild-type or Δ*hfq Y. pestis*. While all mice infected with the wild-type strain succumbed to the infection by day 4, none of the *Y. pestis* Δ*hfq*-infected mice died, even after 7 days (Fig. 7A). In addition, we examined the kinetics of infection by enumerating CFUs in the lungs at various times postinfection and found that the *Y. pestis* Δ*hfq* mutant is unable to persist in the pulmonary compartment (see Fig. S3).

As previous work has demonstrated the contribution of Crp to the virulence of *Y. pestis* (17, 27), we hypothesized that the attenuation of the Hfq mutant may be in part due to the dysregulation of Crp. To test this, we generated strains of $pCD1^+$ *Y. pestis* carrying the P_{tetO}-*crp*-HA construct as described above, but in this case, the gene encoding the *tet* repressor TetR was not included. In the absence of TetR, the addition of ATc to induce gene expression is not required, and thus transcription from the P_{tetO} promoter is constitutive. Production of Crp-HA from both the Δ*hfq* and Δ*crp* strains of *Y. pestis* carrying the constitutive P_{tetO}-*crp*-HA construct was confirmed by immunoblotting (see Fig. S4). Mice were intranasally infected with wild-type *Y. pestis*, the Δ*hfq* and Δ*crp* strains of *Y. pestis* carrying the constitutive P_{tetO}-*crp*-HA, or the equivalent mutants carrying the Tn*7* vector only. After 48 h, mice were sacrificed and CFUs in the lungs were enumerated. Three of 4 mice infected with the Δ*hfq* strain containing the vector only had no bacteria in the lungs (below the limit of detection), while the

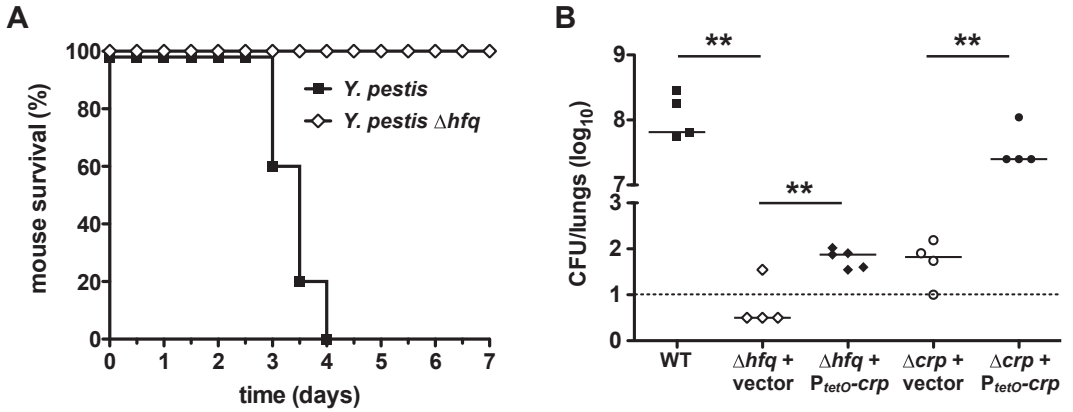

FIG 7 Contribution of Hfq and the posttranscriptional control of Crp to pneumonic plague. (A) Survival of mice infected via the i.n. route with wild-type or Δ*hfq Y. pestis*. (B) Impact of decoupling Crp synthesis from Hfq on *Y. pestis* CFUs in the lungs. Mice were infected via the i.n. route with wild-type *Y. pestis* or the Δ*hfq* or Δ*crp* strains of *Y. pestis* carrying either the constitutively expressed P_{tetO}-*crp*-HA construct or the Tn*7* vector only. After 48 h, CFUs in the lungs were determined. Each point represents the numbers of bacteria recovered from a single mouse. The limit of detection is indicated by a dashed line. Symbols below the limit of detection represent mice that did not have detectable numbers of bacteria. A solid line indicates the median of CFU recovered. **, $P < 0.005$. Data are representative of 2 independent experiments.

Δ*crp* strain had a median of 55 CFUs (Fig. 7B). On the other hand, mice infected with wild-type *Y. pestis* or the Δ*crp* P_{tetO}-*crp*-HA strain had a median of 5.5×10^7 or 2.5×10^7 CFUs in the lungs, respectively. Although full virulence was not restored to the *Y. pestis* Δ*hfq* strain carrying P_{tetO}-*crp*-HA, we found that the constitutive production of Crp in the Δ*hfq* mutant significantly increased the number of CFUs in the lungs compared to that of the same Δ*hfq* strain carrying the vector insertion only. Thus, our results indicate that a portion of the attenuation associated with the loss of Hfq is directly attributable to the dysregulation of Crp synthesis.

DISCUSSION

The influence of catabolite repression on gene expression via Crp and cAMP is well known, and many decades of research, particularly in *E. coli*, have established the paradigm by which this occurs. The expression, synthesis, and activity of both *crp* and *cyaA* must be appropriately controlled for the optimal physiology of the cell, and multiple studies have identified a variety of mechanisms by which these events occur. For instance, in *E. coli*, both *crp* expression and Crp activity (via cAMP binding) are regulated by the levels of extracellular glucose; indeed, Crp can positively or negatively affect its own expression in an autoregulatory feedback circuit (28, 48). Extracellular osmolarity also influences *crp* expression and Crp activity, linking not just nutrient levels but also salt concentration with Crp-dependent gene expression (49). In addition, Crp itself is acetylated in *E. coli*, suggesting that posttranslational modifications may also influence Crp activity (50, 51).

While Crp is a repressor of the *cyaA* gene in *Y. pestis*, *crp* expression is not autoregulated, suggesting that Crp levels themselves may be less sensitive to glucose levels in the plague pathogen than in *E. coli* (34, 52). In addition, Zhang et al. recently showed that the two-component response regulator PhoP directly activates the transcription of both *crp* and *cyaA* (33). Thus, considering the distinct environments that *Y. pestis* inhabits compared to *E. coli* or even other bacterial pathogens, these data suggest the possibility that divergent mechanisms of *crp* regulation have evolved that are specific or unique to *Yersinia* species. Indeed, the dependence of *crp* expression on the PhoPQ regulatory system indicates that *Y. pestis* has adapted multiple mechanisms to link catabolite repression with other environmental cues, such as low Mg^{2+} or the presence of antimicrobial peptides (53). This report adds to our understanding of Crp biology in the plague pathogen by demonstrating that at physiological temperatures, *Y. pestis* positively regulates the synthesis of Crp at the posttranscriptional level via the activity of the sRNA chaperone Hfq. This additional level of regulation to control Crp synthesis may be confined to a limited number of bacterial species, as Hfq-dependent, posttranscriptional mechanisms of Crp regulation have not been previously described, despite the many years of study of both Hfq and catabolite repression. Furthermore, these data explain why inducible promoters such as P_{BAD} and P_{lacO} are not fully functional in the *hfq* mutant of *Y. pestis* (not shown), since these promoters rely on Crp as a coactivator for induction (54).

An alignment of the *E. coli* strain MG1655 and *Y. pestis* CO92 *crp* 5′ UTR sequences reveals that, except for 15 of the 17 most proximal nucleotides to the translational start codon, no significant homology exists between the two, even though the *crp* CDS between the species are 84% identical. In addition, the *E. coli crp* 5′ UTR is 167 nt in length, while the equivalent *Y. pestis* CO92 sequence is only 79 nt long. An mFOLD analysis (55) of the *crp* 5′ UTR and proximal coding region transcript used to generate the P_{tetO}-*crp* 5′ UTR-*gfp* reporter predicts a secondary structure with extensive base pairing, including the region of the putative ribosome-binding site (see Fig. S5). Considering that the regulatory effect of Hfq occurs within the *Y. pestis crp* 5′ UTR, these differences may explain the adaptation of Crp synthesis in *Y. pestis* to include a posttranscriptional layer. As Hfq usually acts in conjunction with sRNAs to regulate protein synthesis, the *Yersinia*-specific *crp* 5′ UTR may enable sRNAs to bind to this region of the transcript and alleviate possible inhibitory secondary structures, such as that observed for the *E. coli rpoS* transcript (56, 57), whereas other bacteria may lack this mechanism of regulation due to the differences in the UTR.

In many species, Hfq contributes to the regulation of a large number of genes; however, the regulons controlled through Hfq

are distinct between various genera and species, as are the subsets of sRNAs encoded by different bacteria. For example, we found that approximately 80% of the putative sRNAs expressed by *Y. pseudotuberculosis*, the most recent ancestor of modern *Y. pestis* strains (58), are unique to *Yersinia* species and not found in other bacteria, such as *E. coli* or *Salmonella* (43). This suggests that, although there is overlap between some sRNAs and regulated genes between closely related species, many bacteria have adapted individual posttranscriptional mechanisms of gene regulation to meet the specific needs of the cell, be it due to differences in environmental niche, nutrient availability, or stress conditions.

In particular, *Y. pestis* has adapted to a lifestyle in which it is thought to exist predominantly as a parasite—the plague bacillus cycles between infection of an arthropod vector and a mammal and is not believed to spend significant time outside a host (59). During infection of the flea, *Y. pestis* expresses genes required for survival, colonization, and transmission by the flea, while other genes that are required for mammalian infection are downregulated (60). Conversely, upon entry into a mammalian host, flea-specific genes are downregulated and a different subset of genes is expressed. These include many mammalian virulence factors, including the Yop-Ysc T3SS, the F1 antigen pilus, the antiphagocytic pH 6 antigen, and Pla (3, 61). Not only are the levels of Pla protein reduced at lower temperatures compared to those under physiological conditions (12), here we show that *pla* is also differentially regulated during pneumonic plague in a manner that is consistent with its requirement during the latter stage of the respiratory infection, when Pla is needed to induce the proinflammatory phase of disease (6). As Crp acts as a direct positive transcriptional regulator of *pla*, and both Hfq and Crp are required for the full virulence of *Y. pestis*, the data presented here suggest the possibility that *Y. pestis* may optimize metabolic and virulence gene expression via the posttranscriptional regulation of Crp synthesis to link catabolite repression with Hfq/sRNA-dependent environmental sensing. These regulatory connections could work in both directions, as Heroven et al. recently showed that in *Y. pseudotuberculosis*, Crp influences the expression of the Hfq-independent sRNAs CsrB and CsrC (26).

Multiple regulatory pathways may synergize to induce the Crp regulon at the appropriate time and/or location. This might occur as follows: first, transcription of *crp* (and *cyaA*) is induced in a PhoPQ-dependent manner in response to external signals such as ion concentrations or the presence of antimicrobial peptides, to result in "priming" of the *crp* mRNA for translation. Full Crp synthesis, however, would not proceed until a second signal is detected that results in the expression of Hfq-dependent sRNA(s) that interacts with the *crp* 5′ UTR. Finally, once Crp is synthesized, sensing of the appropriate nutrient conditions would allow for the production of cAMP by CyaA, thus linking the levels of carbon/glucose with optimal Crp-dependent gene expression, but only after both the PhoPQ system and the required sRNAs have been activated and expressed. While the overproduction of Crp does not seem to adversely affect wild-type *Y. pestis* under our conditions, it is possible that excessive Crp induction during mammalian or flea infection could be detrimental to the bacterium and that these or other regulatory pathways may prevent this from occurring *in vivo*. The kinetics of the posttranscriptional regulation of Crp presented in Fig. S1 suggest that the sRNA(s) that activates Crp synthesis may become limiting as the bacteria transition into stationary phase, for instance.

During infection, these regulatory mechanisms could allow *Y. pestis* not only to modulate highly energetic processes such as type III secretion but also to induce virulence gene expression only when specific conditions are met. While it is not yet known what specific signals stimulate these changes in gene expression during pneumonic plague, it is possible that a low-level induction of the inflammatory response (6) may be sufficient to initiate the process. For example, *Salmonella* specifically stimulates gastrointestinal inflammation during infection in part to acquire nutrients (62), which consequently could alter the availability of carbon and thus the expression of the Crp regulon. Alternatively, variations in oxygenation, temperature, or even bacterial load during infection could serve as signals for expression of the sRNA(s) that influence the induction of Crp in *Y. pestis*. Our data suggest that, whatever these signal(s) may be, they are likely to posttranscriptionally stimulate Crp synthesis, thereby allowing for increased *pla* expression and presumably other components of the Crp regulon during infection.

Indeed, the posttranscriptional regulation of Crp in *Y. pestis* contributes significantly to virulence during pneumonic plague, as decoupling Crp synthesis from Hfq partially restores bacterial levels in a Δ*hfq* mutant. Not surprisingly, other Hfq-dependent, Crp-independent genes must also be required for disease, as the loss of Hfq results in the clearance of bacteria from the lungs over time. Indeed, our data are among the first to define the specific factors directly regulated through Hfq that are needed during plague infection. However, additional studies are needed to further delineate the genes within the Hfq regulon that are required for virulence during pneumonic plague, the sRNA(s) that contributes to the posttranscriptional regulation of Crp, and the host signals that *Y. pestis* senses to influence the proper transcriptional and posttranscriptional control of both Crp and other factors while in the mammalian host.

MATERIALS AND METHODS

Reagents, bacterial strains, and growth conditions. All chemicals were obtained from the Sigma Chemical Company (St. Louis, MO) unless otherwise indicated. Bacterial strains are listed in Table S2 in the supplemental material, plasmids are described in Table S3, and oligonucleotide sequences are given in Table S4. The fully virulent, wild-type *Y. pestis* strain CO92 and the avirulent, pCD1-negative derivative were obtained previously from the U.S. Army, Fort Detrick, MD. The presence or absence of pCD1, pMT1, pPCP1, and the *pgm* locus was confirmed by PCR. Unless otherwise indicated, all experiments were conducted with the pCD1$^-$ derivative of *Y. pestis* CO92. Experiments with select agent strains of *Y. pestis* were performed in CDC-approved biosafety level 3 (BSL3)/animal BSL3 (ABSL3) laboratories at Northwestern University. *Y. pestis* was routinely cultivated on BHI agar (Difco) at 26°C for 2 to 3 days. For liquid cultures, *Y. pestis* was grown in BHI broth at 26°C in a roller drum overnight before being diluted to an OD_{620} of 0.1 to 0.2 in 10 to 15 ml BHI broth in a 125-ml Erlenmeyer flask or in a roller drum and cultured with shaking at 250 rpm at 37°C, unless otherwise indicated. Ampicillin (100 μg/ml) or kanamycin (50 μg/ml) was added to the medium as needed. For animal infections, *Y. pestis* was cultured as described above at 37°C with the addition of 2.5 mM $CaCl_2$.

Animal experiments. All animal experiments were approved by the Northwestern University ACUC. Pathogen-free, 6- to 8-week-old female C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were housed in HEPA-filtered barrier units kept inside a ventilated animal cage rack for the duration of the experiments. Mice were given food and water *ad libitum* and were housed at ambient temperature with alternating 12-hour periods of light and dark. pCD1$^+$ *Y. pestis* and

derivatives were prepared as described above, washed once in sterile phosphate-buffered saline (PBS), and maintained at 37°C. Mice were anesthetized with ketamine and xylazine and inoculated by the intranasal route with approximately 1×10^4 CFU of the indicated bacterial strains in a volume of 20 μl as described previously (3, 43). Actual numbers of CFU inoculated were determined by plating serial dilutions on BHI agar. At various times, animals were sacrificed with an overdose of sodium pentobarbital. For CFU determination experiments, lungs were surgically removed and homogenized in PBS, and serial dilutions were plated on BHI agar. For survival experiments, mice were monitored twice daily for 7 days, and any surviving mice at the end of the experiment were euthanized as described above. For the analysis of gene expression *in vivo*, lungs were removed at the indicated times and immediately submerged in an excess of RNAlater RNA stabilization solution (Ambion) before proceeding.

RNA extraction and quantitative RT-PCR. For experiments described for Fig. 1, pCD1$^+$ *Y. pestis* was cultured under the same conditions used to prepare the bacteria for infection, and after 12 h, bacteria were mixed with 2 volumes of RNAprotect-bacteria RNA stabilization solution (Qiagen). For all other experiments, pCD1$^-$ strains were cultured at 37°C for 6 h, and RNA was stabilized as described above. Total RNA was then purified from lung tissue or cultured bacteria with the RiboPure RNA extraction kit (Ambion), treated with DNase (Ambion), and cDNA synthesized using the Superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen). qRT-PCR for the target genes was performed in triplicate with the SYBR green dye (Bio-Rad) by using the primer sets listed in Table S4 in the supplemental material. The calculated threshold cycle was normalized to the C_T of the *gyrB* gene from the same cDNA sample before calculating the fold change using the $\Delta\Delta C_T$ method (63); in Table S1, the C_T of *gyrB* was normalized to either *proS* or the 16S rRNA. Statistical analyses were performed using Student's *t* test.

Transposon mutagenesis and screen for altered Pla activity. Mutagenesis was performed by electroporating the pTnMod-RKm plasmid carrying the Tn*5* minitransposon into pCD1$^-$ *Y. pestis* (45); transposon mutants were plated onto BHI agar containing kanamycin and incubated at 37°C for 2 days. Plates were then overlaid with a top agar containing 5% skim milk and 200-μg human Glu-Plg (Haematologic Technologies) and incubated at 37°C for an additional day. Colonies that showed altered zones of clearance were purified and examined in a liquid *plg* activation assay as described below.

Mutagenesis, HA tagging, and Tn*7*-based chromosomal integration. Unmarked, isogenic mutants of *hfq* and *crp* were generated by lambda red recombination in the fully virulent or pCD1$^-$ strains of *Y. pestis* CO92 as described previously (43, 46). Regions of homology upstream and downstream of the genes were PCR-amplified using the primer sets listed in Table S4. The kanamycin resistance cassette used for the selection of recombinants was excised as described earlier (46).

The *crp* gene was replaced by allelic exchange in pCD1$^-$ strains of *Y. pestis* with a variant carrying the sequence for the HA tag on the 3′ end immediately preceding the stop codon, as described previously (46), with the primers listed in Table S4 in the supplemental material. Incorporation of the HA tag at the appropriate locus in kanamycin-sensitive colonies was confirmed by PCR.

Construction of ATc-inducible *crp*-HA strains was performed similarly as described (46). Here, the CDS for *crp* was amplified by PCR from the *Y. pestis* genome using a 3′ primer containing the HA sequence immediately prior to the stop codon, and the P_{tetO} promoter and 5′ UTR was PCR amplified from the plasmid pWL213 (6) before subsequently joining the fragments using the technique of splicing by overlap extension (SOE)-PCR. The resulting product was then subcloned into the Tn*7*-based integration plasmid pWL212 (6).

All Tn*7*-based constructs destined for integration onto the chromosome of *Y. pestis* were introduced to the *att*Tn*7* site contained within the *glmS-gstS* intergenic region via the Tn*7* site-specific transposon as described (46, 64). The kanamycin resistance cassette was resolved as described above, and integration was confirmed by PCR.

Plasminogen activation assays. The plg-activating ability of *Y. pestis* was assessed as described previously (6). Strains were grown for 6 h at 37°C before being diluted to 1×10^6 CFU in PBS and combined with purified human Glu-Plg (Haematologic Technologies; 4 μg) and the chromogenic substrate D-AFK-ANSNH-iC$_4$H$_9$-2HBr (SN5; Haematologic; 50 μM) in a total volume of 200 μl PBS. Reaction mixtures were incubated in triplicate for 3 h at 37°C, and the fluorescence at 460 nm was measured every 10 to 11 min in a Molecular Devices SpectraMax M5 microplate reader. For experiments measuring the effect of *crp* expression on plg activation, ATc (0.25 μg/ml) was added to the cultures at time 0, as appropriate. Data shown are representative of at least 3 independent experiments.

mRNA half-life determination. The decay rate and half-life of the *pla* transcript were determined essentially as described (46). *Y. pestis* strains were cultured in triplicate for 6 h at 37°C, following which rifampin (50 μg/ml) was added to the cultures to prevent *de novo* transcription. Aliquots of bacteria were removed immediately (time 0) and every minute thereafter for 4 min and immediately mixed with 2 volumes of RNAprotect-bacteria reagent. Relative transcript levels at each time point were measured by qRT-PCR as described above, and the half-life of the mRNA was determined by plotting the relative fold change compared with time 0 for each strain on a semilog plot. The first 4 data points were then fit with a linear curve, and the equation $t_{1/2} = 0.693/k$ was used, where k = the slope of the line. Data are represented as a percentage of mRNA remaining over time. Statistical analysis was performed using Student's *t* test.

Transcriptional start site determination. The transcriptional start site of the *crp* mRNA was performed using RACE as previously described (43). The oligonucleotides used for RACE are listed in Table S4. In each case, the generated PCR products of correct size were directly cloned into the plasmid pCR2.1 (Invitrogen). Plasmids were sequenced using M13 forward and reverse primers.

GFP assays. To construct P_{pla}-*gfp* and P_{crp}-*gfp* reporters, the promoter regions of *pla* and *crp* (500 bp upstream of the ATG and including the proximal 27 bp of the *pla* or *crp* CDS) were amplified by PCR. The gene for GFP was also PCR-amplified, and the resulting products were subsequently joined by SOE-PCR before being subcloned into pUC18R6K-min-Tn*7*T-kan. To construct the P_{tetO}-*pla* 5′ UTR-*gfp* and P_{tetO}-*crp* 5′ UTR-*gfp* reporters, the P_{tetO} sequence lacking the 5′ UTR (54) was PCR amplified from pWL213, and the 5′ UTR of *pla* (15) or *crp* was amplified from the genome of *Y. pestis* (77 bp upstream of the ATG of *pla* and including the proximal 27 bp of the *pla* CDS, or 79 bp upstream of the ATG of *crp* and including the proximal 27 bp of the *crp* CDS). These products were subsequently joined by SOE-PCR and cloned into pWL212. The reporters were then integrated onto the chromosome of *Y. pestis* as described above.

Strains carrying the GFP reporters were assessed for relative fluorescence at 37°C in triplicate as described previously (46). ATc (0.25 μg/ml) was added to the cultures where appropriate. Background fluorescence subtraction and normalization were performed as described (46). Statistical differences were determined by Student's *t* test.

Immunoblot analyses. For analysis of proteins by immunoblotting, bacteria were cultured as described above (at time 0, ATc was added to a concentration of 0.25 μg/ml unless otherwise indicated, when appropriate). Aliquots of the cultures were removed and centrifuged at 5,000 × *g* for 10 min at 4°C, and bacterial pellets were washed once with PBS and incubated for 30 min on ice with lysozyme (50 μg/ml). Cells were then sonicated using 3 30-s pulses, and lysates were centrifuged at 10,000 × *g* for 10 min at 4°C. Protein concentrations were measured by the Bradford assay (Bio-Rad), and equal concentrations of lysates were mixed with reducing sample buffer, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with antibodies to the HA tag (Roche), Pla (65), or RpoA.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01038-13/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.3 MB.
Figure S5, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.
Table S4, PDF file, 0.1 MB.

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