The Importance of the Small RNA Chaperone Hfq for Growth of Epidemic *Yersinia pestis*, but Not *Yersinia pseudotuberculosis*, with Implications for Plague Biology

Guangchun Bai,† Andrey Golubov,‡ Eric A. Smith,§ and Kathleen A. McDonough

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*Xersinia pestis*, the etiologic agent of plague, has only recently evolved from *Yersinia pseudotuberculosis*. *hfq* deletion caused severe growth restriction at 37°C in *Y. pestis* but not in *Y. pseudotuberculosis*. Strains from all epidemic plague biovars were similarly affected, implicating Hfq, and likely small RNAs (sRNAs), in the unique biology of the plague bacillus.

*Yersinia pestis*, the etiologic agent of plague, poses a continuing natural threat to human health, and recent concerns about its potential use as a biological threat agent have increased our need to understand the biology of this deadly bacterial pathogen. Molecular evidence indicates that *Y. pestis* evolved from *Yersinia pseudotuberculosis* within the last 20,000 years (1), and three-fourths of their genes remain at least 97% identical (8). The diseases caused by these two bacteria differ greatly, and only *Y. pestis* has the capacity for the complex process of flea-borne transmission (15, 24, 31). The extent to which biological differences between these distinct bacterial pathogens are caused by altered expression of conserved genes, rather than overt genetic differences, is not known.

Hfq functions as a posttranscriptional regulator by stabilizing small RNAs (sRNAs) and facilitating their interactions with mRNA targets (18, 20, 21, 35, 41, 43, 45, 48). Hfq’s pleiotropic role in gene regulation is well established (29, 42), and its importance as a regulator of virulence in pathogenic bacteria is being increasingly recognized (3, 9, 10, 12, 25, 27, 28, 33, 37, 38, 47). Hfq positively regulates the *Y. enterocolitica*-specific *yst* toxin gene (30) and is required for virulence of *Y. pseudotuberculosis* (34) and a Pestoides variant of *Y. pestis* (19). However, the role of Hfq in the epidemic *Y. pestis* biovars has not been explored.

Our interest in the regulation of differential gene expression led us to generate *hfq* deletion mutants of *Y. pestis* and *Y. pseudotuberculosis*. Preliminary studies suggested species-specific differences in the growth properties of these mutants. In this study, we report that epidemic *Y. pestis* strains are strongly dependent on Hfq for *in vitro* growth at 37°C but that *Y. pseudotuberculosis* strains are not. These results indicate the potential for significant differences in the roles of sRNAs in the physiology of these genetically related, but biologically distinct, bacterial pathogens.

** Growth and cell morphology of the *hfq* mutant strains. **The Hfq amino acid sequences are identical among eight sequenced strains of *Y. pestis* and four of *Y. pseudotuberculosis* (http://www.ncbi.nlm.nih.gov/Blast.cgi). This protein shares 98.6% identity with *Escherichia coli* Hfq over a 73-amino-acid (aa) N-terminal portion that is associated with RNA chaperone function (39). This shared region is also highly conserved in *Pseudomonas aeruginosa* Hfq, which contains only 82 aa (40).

Deletion of *hfq* has a moderate effect on *E. coli* cell morphology, tolerance of stress conditions, and growth rate in rich media, which is more pronounced at 25°C than at 37°C (42). We initially explored Hfq’s role in the growth of *Y. pestis* and *Y. pseudotuberculosis* by generating *hfq* knockout mutations in *Y. pestis* KIM6+ (Pgm+ pCD1+ pMT1+ pPs+) and KIM10+ (Pgm+ pCD1+ pMT1+ pPs+), as well as *Y. pseudotuberculosis* PTB52c (pYV′; serotype IB) and PTB54c (pYV′; serotype III). Mutations were generated by using homologous recombination to replace the entire *hfq* open reading frame (ORF) (306 bp) with a *cat* (chloramphenicol acetyltransferase) gene cassette amplified from pKD3 (11). Mutations were initially complemented with a single-copy *hfq* gene expressed from its native promoter and integrated into the chromosome 3’ to one of three tRNA^Asn^ genes using pSPC471, which contains the YP-HPI pathogenicity island’s attachment and integration sequences (6, 23) subcloned from pKR529 (generously provided by A. Rakin).

Bacterial growth in brain heart infusion (BHI) media was measured visually on agar plates or by optical density (OD) in liquid cultures at 28 and 37°C. Cell morphology was also examined by microscopy at selected time points. Growth rates and morphology of the *Y. pseudotuberculosis* *hfq* mutants were similar to those of wild-type (WT) cells at either 28°C or 37°C (Fig. 1 and 2). In contrast, the *Y. pestis* mutants exhibited significant temperature-dependent phenotypes. Growth of the *Y. pestis* KIM6+ and KIM10+ *hfq* mutants was only moderately reduced at 28°C relative to that of the WT, but they showed little to no growth on either solid or liquid BHI medium at 37°C (Fig. 1). The *Y. pestis* *hfq* mutants also formed...
lengthened rods, which were significantly different from the sphere and short-rod morphology of WT, particularly at 37°C (Fig. 2).

Growth and morphology of the Y. pestis hfq mutants were largely restored by addition of a single-copy WT hfq expressed from its immediate upstream promoter (Fig. 1 and 2). However, we noted that complementation in Y. pestis was incomplete, and the slight growth defect in Y. pseudotuberculosis PTB52c was not affected by hfq addition. Similar difficulties in achieving complete hfq complementation have been reported previously for E. coli (42), but the basis of this defect is not known. We investigated whether complementation was limited by either inadequate levels of Hfq or lack of hfq coexpression with adjacent genes.

Transcription start sites (TSS) for hfq in Y. pestis and Y. pseudotuberculosis were determined by primer extension analyses. The TSS mapped to a position 90 nucleotides (nt) upstream of the translation start site, confirming that hfq is expressed from its immediate upstream promoter (Fig. 3A and B). However, hfq and hflX mRNA levels in the complemented strain were lower than those in the WT (Fig. 3C). Further reverse transcription-PCR (RT-PCR) analyses showed that hfq was also transcribed as part of a larger overlapping operon with miaA and hflX (Fig. 3B and C), as it is in E. coli (42). We reasoned that expression from this additional promoter upstream of miaA could increase hfq levels in WT cells. A new hfq-complementing strain was generated using a multiple-copy pCRII-based plasmid (Invitrogen), and increased hfq levels were confirmed by Northern blot analysis (data not shown). This multicopy construct fully restored the growth defect of Y.

FIG. 1. Comparison of growth of hfq mutants with solid (A) and liquid (B) media. (A) WT, hfq null mutant (hfq), and complemented hfq mutant (com) strains of Y. pestis KIM6+ and Y. pseudotuberculosis PTB52c were streaked on BHI plates and incubated for 2 days at 28°C or 37°C, as indicated. (B) Growth curves of WT, hfq mutant (hfq), and complemented mutant (hfq-C) strains of Y. pestis KIM6+ and Y. pseudotuberculosis PTB52c in BHI broth at 28°C or 37°C, measured by optical density. With both solid and liquid media, Y. pestis KIM10+ results were similar to those for Y. pestis KIM6+ strains, while results for Y. pseudotuberculosis PTB54c were similar to those for PTB52c (not shown).
pestis hfq mutants at both 28°C and 37°C (Fig. 3D and E), indicating that Hfq alone is sufficient for complementation, provided that it is expressed at adequate levels.

Generality of the plague bacillus’ dependence on Hfq for growth. We were struck by the biological differences between Y. pestis and Y. pseudotuberculosis with respect to their dependence on Hfq for growth at 37°C. Other investigators recently reported less dependence on Hfq for growth using an enzootic Pestoides (sometimes called Microtus [49]) Y. pestis strain (19). The previous study did not measure growth at late time points or on solid media, where we observed the most dramatic growth restriction. However, strain differences may also be relevant. The enzootic Pestoides strains, which are virulent only for small rodents, are thought to represent an evolutionary intermediate between Y. pseudotuberculosis and epidemic Y. pestis strains (14, 17). The phenotypic, biochemical, and genetic profiles of Pestoides strains combine features that are otherwise considered specific for either Y. pseudotuberculosis or Y. pestis lineages (2, 4). KIM, the strain that we used, belongs to the Mediavalis biovar, one of three Y. pestis biovars associated with epidemic plague and virulence for large mammals (including humans) as well as small rodents. We therefore extended our study to determine whether KIM’s strong physiological dependence on Hfq also occurred in other epidemic plague biovars.

hfq mutants were generated from representative strains from the remaining two epidemic biovars, CO92 R73 (Pgm+/Lcr−/pFra+/pPst−) (biovar Orientalis) and Kuma D11 (Pgm+/Lcr−/Pst+/Fra+/Gly+/pMT2+/pPCP2+) (biovar Antigua).

Growth of all Y. pestis mutants was severely restricted on BHI plates at 37°C (Fig. 4). As with the KIM mutants, the Kuma and CO92 mutants showed little to no growth on BHI plates at 37°C (Fig. 4C). The Y. pestis Kuma hfq mutant, from the oldest epidemic biovar, grew slowly even at 28°C (Fig. 4A), taking up to 24 h to reach WT stationary-phase ODs at 600 nm (OD600) (not shown). The Kuma hfq mutant’s limited growth in liquid BHI medium at 37°C was similar to that of the KIM mutants (Fig. 4B), and the OD600 of the KIM and Kuma mutants remained at 0.2 to 0.3 for up to 24 h (data not shown). However, the Y. pestis CO92 hfq mutant showed a significant
increase in OD 600 after 5 h at 37°C and formed sporadic colonies on plates. Regrowth experiments with recovered bacteria (not shown) suggested that this increased OD 600 was due to outgrowth of spontaneous suppressor mutants, consistent with the suppressor colonies that we observed on BHI plates (Fig. 4C).

We further explored this suppressor phenomenon by quantitating the relative frequencies of spontaneous suppressor mutations for each of the mutants after overnight growth at 28°C. Y. pestis CO92 hfq mutant suppressor clones were present at levels ~100-fold (Kuma) to ~1,000-fold (KIM) higher than with the other mutants (Fig. 4D). These results show that the essential nature of hfq for Y. pestis’ multiplication at 37°C is a general phenomenon that occurs across all three epidemic plague biovars. However, cross-biovar differences, particularly with respect to suppressor frequencies, suggest that there is continued genetic plasticity associated with this phenotype.

**Biological implications of the plague bacillus’ dependence on Hfq for growth.** Y. pestis’ dependence on Hfq for in vitro growth at 37°C was surprising to us, particularly compared with...
Hfq’s relative lack of importance for *Y. pseudotuberculosis* growth. In this respect, *Y. pseudotuberculosis* more closely resembles other bacterial pathogens, in which significant phenotypic defects due to *hfq* deletion manifest only under host-associated stress conditions rather than standard in vitro laboratory growth (for a review, see reference 9). For example, a *Salmonella hfq* deletion strain is highly attenuated during mouse infection and defective for intracellular growth, but it had only a slight in vitro growth restriction at 37°C (37). Likewise, *Vibrio cholerae* and *Brucella abortus* *hfq* mutants are significantly compromised for growth in vivo but not in vitro (12, 33).

The *Y. pestis hfq* mutant’s limited growth at 37°C suggests that Hfq is essential for plague infection of a mammalian host, which is consistent with recent studies in which *hfq* null Pestoides *Y. pestis* and *Y. pseudotuberculosis* showed decreased survival in macrophages and mice (19, 34). However, the different *Hfq*-dependent phenotypes observed in this study strongly suggest that sRNAs also play a unique role in *Y. pestis* biology and possibly in plague evolution. RNA regulators are associated with “evolvability” (46), and it is tempting to speculate that they have played a role in the divergence of *Y. pestis* from *Y. pseudotuberculosis*. Hfq interacts with sRNAs and mRNAs associated with both core genome and laterally transferred gene sequences, facilitating cross-regulation and incorporation of newly acquired genes into existing regulatory interactions (9, 37).

FIG. 4. Growth of *hfq* mutants generated from different *Y. pestis* biovars. (A and B) Growth curves of *Y. pestis hfq* mutant strains compared with their respective WT strains at 28°C (A) and 37°C (B). (C) Growth comparison of different strains on BHI plates for 48 h at 28°C or 37°C. Note that the growth of the CO92 *hfq* mutant at 37°C is actually due to sporadic suppressor colonies. (D) Relative suppressor frequencies for different *Y. pestis* mutants following overnight growth in BHI broth. Diluted cultures were spread on BHI plates in duplicate, followed by incubation at either 28°C or 37°C. The frequency of suppressor mutants was calculated as the ratio of CFU at 37°C to that at 28°C. All data shown are representative of two biological repeats.

Nonetheless, *Y. pestis* undergoes Lcr-associated growth restriction much more abruptly than *Y. pseudotuberculosis*, despite the presence of nearly identical Lcr plasmids (5, 7). This indicates that there are other important growth-associated physiological differences between these bacteria. This is consistent with functional inactivation of more than 10% of the *Y. pestis* genome relative to *Y. pseudotuberculosis*, including at least one mutation in *aspA* that further distinguishes epidemic from Pestoides *Y. pestis* strains (4, 8). We propose that Hfq is involved in some of the compensatory gene regulation associated with these gene inactivations.
Elucidation of Hfq’s unusual role in Y. pestis growth will provide new insights into the unique biology of the plague bacillus and the physiological factors that distinguish this deadly pathogen from its enteric relatives and progenitors.

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