The RNA-Binding Chaperone Hfq Is an Important Global Regulator of Gene Expression in *Pasteurella multocida* and Plays a Crucial Role in Production of a Number of Virulence Factors, Including Hyaluronic Acid Capsule

Marianne Mégroz,^a Oded Kleifeld,^b Amy Wright,^a David Powell,^c Paul Harrison,^c Ben Adler,^a Marina Harper,^a John D. Boyce^a

Department of Microbiology^a and Department of Biochemistry and Molecular Biology,^b Infection and Immunity Program, Monash Biomedicine Discovery Institute, Monash University, Clayton, Victoria, Australia; Monash Bioinformatics Platform, Monash University, Clayton, Victoria, Australia^c

The Gram-negative bacterium *Pasteurella multocida* is the causative agent of a number of economically important animal diseases, including avian fowl cholera. Numerous *P. multocida* virulence factors have been identified, including capsule, lipopolysaccharide (LPS), and filamentous hemagglutinin, but little is known about how the expression of these virulence factors is regulated. Hfq is an RNA-binding protein that facilitates riboregulation via interaction with small noncoding RNA (sRNA) molecules and their mRNA targets. Here, we show that a *P. multocida* *hfq* mutant produces significantly less hyaluronic acid capsule during all growth phases and displays reduced *in vivo* fitness. Transcriptional and proteomic analyses of the *hfq* mutant during mid-exponential-phase growth revealed altered transcript levels for 128 genes and altered protein levels for 78 proteins. Further proteomic analyses of the *hfq* mutant during the early exponential growth phase identified 106 proteins that were produced at altered levels. Both the transcript and protein levels for genes/proteins involved in capsule biosynthesis were reduced in the *hfq* mutant, as were the levels of the filamentous hemagglutinin protein PfhB2 and its secretion partner LspB2. In contrast, there were increased expression levels of three LPS biosynthesis genes, encoding proteins involved in phosphocholine and phosphoethanolamine addition to LPS, suggesting that these genes are negatively regulated by Hfq-dependent mechanisms. Taken together, these data provide the first evidence that Hfq plays a crucial role in regulating the global expression of *P. multocida* genes, including the regulation of key *P. multocida* virulence factors, capsule, LPS, and filamentous hemagglutinin.

Small noncoding RNA (sRNA) molecules play essential roles in regulating the production of a wide range of proteins, including those involved in virulence; quorum sensing; and the metabolism of carbon, amino acids, and iron (1–5). Typically, sRNA molecules are between 50 and 400 nucleotides long and act primarily by interacting with one or more target mRNAs via short imperfect base pairing (6, 7). Protein production can be controlled by sRNAs via multiple mechanisms, including inhibition of translation, activation of translation, and alteration of transcript degradation rates (8, 9). The regulation of bacterial protein expression via sRNAs often requires the activity of Hfq, an RNA-binding chaperone protein that belongs to the Sm-like RNA-binding protein family and displays a highly conserved core sequence (6, 10). Hfq monomers form a homohexameric ring-shaped structure that preferentially binds to A/U-rich sequences on target RNA molecules and mediates the various posttranscriptional regulatory mechanisms of sRNAs (11–13). Hfq can also directly protect sRNA molecules from RNase E-mediated degradation (14) and can have an sRNA-independent role in the regulation of mRNA decay, via direct interaction with poly(A) polymerase (15).

Hfq homologues have been identified in a range of Gram-positive and Gram-negative bacterial species, including many pathogenic species (16). Most *hfq* mutants display a broad range of pleiotropic phenotypes, suggesting a global role for Hfq in bacterial physiology (6, 17, 18). These pleiotropic effects have been observed more commonly in Gram-negative species, including the pathogens *Brucella abortus*, *Salmonella enterica*, *Yersinia pestis*, *Moraxella catarrhalis*, *Neisseria meningitidis*, and *Vibrio cholerae*, but also in some Gram-positive species such as *Listeria monocytogenes* (18–24).

Many *hfq* mutants show reduced growth rates, reduced virulence, and increased susceptibility to host defense mechanisms and environmental stresses (6, 16).

Pasteurella multocida is an encapsulated, Gram-negative, facultative anaerobe that is the causative agent of a number of important animal diseases, including fowl cholera (25). Fowl cholera can affect most avian species, including chickens, turkeys, ducks, and wild waterfowl, with infections resulting in high mortality rates and major economic losses to poultry industries worldwide (26, 27). *P. multocida* is a heterogeneous species, with strains being classified into five serogroups (serogroups A, B, D, E, and F) based on capsular composition (28) and into eight lipopolysaccharide (LPS) genotypes (L1 through to L8) based on the LPS outer core biosynthesis genes (29). A number of *P. multocida* virulence factors have been defined, including the polysaccharide capsule, LPS,

Received 10 February 2016 Accepted 11 February 2016

Accepted manuscript posted online 16 February 2016

Citation Mégroz M, Kleifeld O, Wright A, Powell D, Harrison P, Adler B, Harper M, Boyce JD. 2016. The RNA-binding chaperone Hfq is an important global regulator of gene expression in *Pasteurella multocida* and plays a crucial role in production of a number of virulence factors, including hyaluronic acid capsule. Infect Immun 84:1361–1370. doi:10.1128/IAI.00122-16.

Editor: G. H. Palmer

Address correspondence to John D. Boyce, john.boyce@monash.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.00122-16>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

filamentous hemagglutinin adhesins, iron-sequestering systems, and sialic acid uptake (30–33). The polysaccharide capsule is a critical virulence factor of *P. multocida* that allows the bacteria to evade host immune defense mechanisms (34, 35). Very little is known about how the expression of these virulence factors is regulated, as only one *P. multocida* regulatory protein, Fis, has been identified and characterized. Fis has been shown to be essential for the production of capsule, and a *fis* mutant displayed highly reduced expression levels of many genes, including those within the capsule biosynthesis locus and *pfhB2*, encoding filamentous hemagglutinin (36).

In this study, the role of Hfq in *P. multocida* gene expression and protein production was characterized by using an *hfq* mutant constructed in the *P. multocida* fowl cholera strain VP161. As Hfq is a crucial modulator of sRNA action, an understanding of the role of Hfq provides an initial indication of the significance of sRNAs in the regulation of *P. multocida* genes and will underpin future work on specific sRNAs in this bacterium. The VP161 *hfq* mutant displayed highly reduced hyaluronic acid (HA) capsule production as well as reduced *in vivo* fitness. RNA sequencing (RNA-Seq) and high-throughput quantitative liquid proteomics analyses indicated that Hfq plays an important role in controlling the expression of >100 genes/proteins. In particular, genes/proteins belonging to the capsule biosynthetic locus were expressed at reduced levels in the *hfq* mutant, consistent with the reduced levels of HA capsule production. These analyses also revealed that Hfq plays a role in the production of filamentous hemagglutinin and proteins involved in LPS biosynthesis. Taken together, these data indicate that Hfq plays an important role in the regulation of key *P. multocida* virulence factors and is essential for full *in vivo* fitness.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in lysogeny broth (LB), and *P. multocida* strains were grown in either heart infusion (HI) or brain heart infusion (BHI) broth (Oxoid). Liquid cultures were incubated at 37°C with shaking (200 rpm). Solid media were obtained by the addition of 1.5% agar. When required, the media were supplemented with antibiotics at the following concentrations: 10 µg/ml (*Escherichia coli*) or 2.5 µg/ml (*P. multocida*) tetracycline and 50 µg/ml kanamycin (*P. multocida*).

Measurement of bacterial growth rates in rich medium. Cultures of *P. multocida* strains grown overnight were subcultured at a 1:50 dilution in BHI broth and grown until cultures reached an optical density at 600 nm (OD₆₀₀) of 0.2 to 0.3. A 1-ml aliquot (standardized to an OD₆₀₀ of 0.2) of each culture was transferred to 50 ml of sterile BHI broth and incubated at 37°C with shaking. To determine the growth rate, optical density measurements of the liquid cultures (at 600 nm) were taken at regular intervals by using the WPA CO8000 Biowave cell density meter, until the late exponential growth phase was reached.

Ethics statement. All animal experiments were carried out in accordance with the provisions of the Prevention of Cruelty to Animal Act, 1986; the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (37); and Monash University Animal Welfare Committee guidelines and policies. The protocols were approved by the Monash Animal Research Platform 2 (MARF-2) Animal Ethics Committee (AEC) of Monash University (AEC number MARF/2011/066, Understanding How *Pasteurella multocida* Causes Disease in Animals).

DNA manipulations. Restriction digests, ligations, and PCR amplifications were performed by using enzymes obtained from NEB or Roche, according to the manufacturer's instructions. Plasmid DNA was prepared by using the NucleoSpin plasmid kit (Macherey-Nagel), while genomic DNA was prepared by using the HiYield genomic DNA minikit (Real

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant description	Source or reference
Strains		
<i>E. coli</i> DH5α	<i>deoR endA1 gyrA96 hsdR17</i> (r _K [−] m _K ⁺) <i>recA1 relA1 supE44 thi-1</i> (<i>lacZYA-argFV169</i>) φ80 <i>lacZ</i> ΔM15 F [−]	Bethesda Research Laboratories
<i>P. multocida</i> VP161	Serotype A:1; Vietnamese isolate from chickens	66
AL2234	VP161 <i>hyaD</i> TargeTron mutant; Kan ^r	This study
AL2521	VP161 <i>hfq</i> TargeTron mutant; Kan ^r	This study
AL2526	AL2521 containing pAL1108; Kan ^r Tet ^r	This study
AL2527	AL2521 containing pAL99T; Kan ^r Tet ^r	This study
Plasmids		
pAL99T	<i>P. multocida</i> - <i>E. coli</i> expression plasmid (Tet ^r); contains the constitutive <i>tpiA</i> promoter upstream of the cloning site; derivative of pAL99	38
pAL953	<i>P. multocida</i> -specific TargeTron plasmid (Kan ^r Spec ^r); intron targeted to the <i>gata</i> gene	38
pAL1069	<i>P. multocida</i> TargeTron plasmid for inactivation of <i>hyaD</i>	This study
pAL1104	<i>P. multocida</i> TargeTron plasmid for inactivation of <i>hfq</i>	This study
pAL1108	Wild-type <i>hfq</i> cloned into pAL99T (Tet ^r)	This study

Genomics). PCR amplification of DNA was performed by using *Taq* DNA polymerase (Roche) or Phusion high-fidelity DNA polymerase (NEB) on an Eppendorf Mastercycler. Amplified DNA was purified by using the NucleoSpin gel or PCR cleanup kit (Macherey-Nagel). The primers used in this study are listed in Table 2. Column-purified DNA samples were quantified by using the NanoDrop 1000 spectrophotometer (Thermo Scientific) and/or by gel electrophoresis. Sequencing reactions were performed by using Applied Biosystems Prism BigDye terminator mix, version 3.1. Electropherograms were generated on a capillary platform Applied Biosystems 3730 genetic analyzer and analyzed by using Vector NTI Advance, version 11.5 (Life Technologies).

Construction of *P. multocida* *hfq* and *hyaD* mutants. The TargeTron (Sigma-Aldrich) mutagenesis system was used to separately inactivate *hfq* and, as a control for HA assays, the capsular biosynthesis gene *hyaD* in *P. multocida* A:1 strain VP161, as described previously (38). The group II intron within *P. multocida* TargeTron plasmid pAL953 (Table 1) was retargeted to *hfq* or *hyaD* according to the TargeTron user manual and with specific primers designed by using the TargeTron design site (Sigma-Aldrich) (Table 2). The retargeted TargeTron plasmids pAL1104 (*hfq* targeted) and pAL1069 (*hyaD* targeted) were then used to separately transform *P. multocida* VP161 by electroporation, and transformants were selected on solid agar containing kanamycin. Following transformation, strains were cured of free-replicating plasmid as described previously (38), and putative *hfq* and *hyaD* mutants were identified by patching for Kan^r/Spec^r colonies. Colony PCRs using the TargeTron-specific primer EBS universal, together with an *hfq*- or *hyaD*-specific primer (BAP7400 and BAP2067, respectively) (Table 2), were used to initially identify TargeTron mutants (data not shown). To confirm that *P. multocida* *hfq* or *hyaD* mutants contained only a single TargeTron insertion, Southern hybridization was performed (data not shown), using a TargeTron intron-specific digoxigenin (DIG)-labeled probe. In addition, the exact position of the intron within the genome was determined by direct sequencing using genomic DNA isolated from each TargeTron mutant as the template with the intron-specific EBS universal primer (Table 2). Two mu-

TABLE 2 Primers used in this study

Primer	Sequence (5'–3')	Description
EBS Universal	GTAAACGACGCCAGT	Universal TargeTron primer, located ~250 bp from the 5' end of the intron
BAP2067	GGAAGGAACAGTTTCTCTGGATTG	Forward primer located within VP161 <i>hyaD</i>
BAP2679	TTGTGTGGAATTGTGAGCGGA	Primer located upstream of the <i>tpi</i> promoter and MCS ^a on pAL99T
BAP7153	AAAAAAGCTTATAATTATCCTTACTGAACCAGACTGTGCGCCCAGATAGGGTG	TargetTron IBS primer specific for inactivation of <i>hyaD</i>
BAP7154	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCAGACTGTAACTTACCTTCTTTGT	TargetTron EBS1d primer specific for inactivation of <i>hyaD</i>
BAP7155	TGAACGCAAGTTTCTAATTTTCGGTTTTCAGTCGATAGAGGAAAGTGTCT	TargetTron EBS2 primer specific for inactivation of <i>hyaD</i>
BAP7389	TGAACGCAAGTTTCTAATTTTCGATTCTTGTTCGATAGAGGAAAGTGTCT	TargetTron EBS2 primer specific for inactivation of <i>hfq</i>
BAP7390	AAAAAAGCTTATAATTATCCTTAACAAGCTCAGATGTGCGCCCAGATAGGGTG	TargetTron IBS primer specific for inactivation of <i>hfq</i>
BAP7391	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTCAGATTGTAACCTTACCTTCTTTGT	TargetTron EBS1d primer specific for inactivation of <i>hfq</i>
BAP7400	CAGAGGATCCTGTGTTTAGCTAGTTGTATC	Forward primer located upstream of VP161 <i>hfq</i> ; contains BamHI site
BAP7401	AGATGTCGACCACTTGAATAAATGTGTTC	Reverse primer located downstream of VP161 <i>hfq</i> ; contains SalI site

^a MCS, multiple-cloning site.

tants that generated unambiguous sequencing data identical to data for the correct target gene sequence (immediately adjacent to the intron insertion site) were selected for further study and designated AL2521 (VP161 *hfq* mutant) and AL2234 (VP161 *hyaD* mutant).

Complementation of the *hfq* mutant. For complementation of the *hfq* mutant, an intact copy of the *hfq* gene was amplified from VP161 genomic DNA by using primers BAP7400 and BAP7401 that contained BamHI and SalI restriction sites, respectively (Table 2). After digestion with BamHI and SalI, the amplified fragment was cloned into BamHI- and SalI-digested pAL99T (Table 1), such that the *P. multocida* constitutive promoter *tpiA* would drive the transcription of *hfq*. The ligated products were then used to transform *E. coli* strain DH5 α . *E. coli* transformants containing the correct plasmid were identified by colony PCR using pAL99T-specific and *hfq*-specific primers BAP2679 and BAP7401, respectively. DNA sequencing was used to confirm the fidelity of the *hfq* sequence, and one correct recombinant plasmid was selected and designated pAL1108. The vector pAL99T and the complementation plasmid pAL1108 were then used to separately transform the VP161 *hfq* mutant (AL2521) via electroporation, generating strains AL2527 and AL2526, respectively (Table 1).

Quantitative hyaluronic acid capsule assay. The amount of HA capsular material produced by each *P. multocida* strain was determined as described previously (34). Student's unpaired *t* test was used to assess the differences in HA production between strains.

Competitive *in vivo* growth assays. Competitive growth assays in mice were performed to quantify the relative *in vivo* fitness of the *hfq* mutant and complemented strains compared with wild-type strain VP161, as described previously (39). To identify each bacterial population, after overnight incubation of input and output samples on HI medium, individual colonies were patched onto HI medium and either HI medium containing kanamycin (to select for the *hfq* mutant) or HI medium containing kanamycin and tetracycline (to select for the *hfq* mutant containing the plasmid). Mutants with a competitive index (CI) of <0.5 were identified as having significantly reduced *in vivo* fitness, and Student's unpaired two-tailed *t* test was also used to assess the statistical differences in relative CIs between each of the tested strains.

RNA extraction and purification, rRNA depletion, library preparation, and high-throughput RNA sequencing. *P. multocida* strains AL2521 (VP161 *hfq* mutant) and VP161 (wild type) were each grown in

BHI broth to the mid-exponential growth phase ($OD_{600} = 0.6$; $\sim 1.0 \times 10^9$ CFU/ml). A total of 2.5 ml of ice-cold killing buffer (containing 0.05 M Tris-HCl [pH 7.5], 15 mg/ml sodium azide, and 0.6 mg/ml chloramphenicol) was added to each 25-ml culture, and the mixed sample was cooled on ice before centrifugation at $9,000 \times g$ for 10 min (4°C). Pelleted cells were then resuspended in 1 ml RNAlater stabilization reagent (Qiagen) and incubated for 5 min at room temperature before centrifugation at $5,000 \times g$ for 10 min. Total RNA was purified by using the RNeasy minikit (Qiagen) with DNase treatment according to the manufacturer's instructions. RNA samples were quantified and analyzed for DNA contamination by using the Qubit kit (Invitrogen) and visualized by gel electrophoresis. rRNA was removed by using the Ribo-Zero magnetic kit (Illumina) according to the manufacturer's instructions. The TruSeq RNA sample preparation kit (Illumina) was used to construct cDNA libraries, with library validation, normalization, and pooling performed by Micromon Services (Monash University). All libraries were combined and sequenced on a single lane of a HiSeq 2000 instrument (Macrogen, South Korea). Mapping of the RNA-Seq reads to the draft VP161 genome was carried out by using CLC Genomics Workbench v 7.0 (CLC Bio), and statistical analyses were carried out by using voom and limma as described previously (40). Raw combined data sets of 50,071,021 and 47,130,114 total paired reads were generated for AL2521 and VP161, respectively. Of these, 17,718,215 and 14,903,935 paired reads (for AL2521 and VP161, respectively) aligned uniquely to the *P. multocida* VP161 genome ($\sim 30\%$ unambiguous reads for each combined data set), giving high coverage of the transcriptome. Genes were identified as being differentially expressed if they displayed a ≥ 2.0 -fold ($\geq 1.0\text{-log}_2$) change in expression across the triplicate replicates at a false discovery rate (FDR) of <0.05 . The differentially expressed VP161 genes identified were mapped to their closest orthologue in the fully annotated reference genome of *P. multocida* strain Pm70 (41) so that overrepresented gene ontology groups and pathway associations could be determined via BioCyc (42), as we reported previously (40).

Quantitative proteomics and mass spectrometry. Cultures of each *P. multocida* strain were grown to either the early exponential ($OD_{600} = 0.2$) or mid-exponential ($OD_{600} = 0.6$) growth phase and then centrifuged at $9,400 \times g$ for 10 min. The pelleted cells were washed twice in 100 mM Tris-HCl (pH 7.5) and lysed via the addition of 0.35 ml of lysis buffer (4% SDS, 100 mM dithiothreitol [DTT], 100 mM Tris-HCl [pH 7.5]) and

FIG 1 Hyaluronic acid (HA) polysaccharide capsule produced by *Pasteurella multocida* strains. (A) Amounts of HA produced at the mid-exponential growth phase by wild-type strain VP161 (WT), the *hfq* mutant, the complemented *hfq* mutant (*hfq[hfq]*), and the *hfq* mutant with the empty vector (*hfq[vector]*). (B) Amounts of HA detected (per 10^9 cells) in wild-type (●) and *hfq* mutant (▲) cells at the early exponential ($OD_{600} = 0.20$ to 0.25), mid-exponential ($OD_{600} = 0.65$ to 0.75), and late exponential ($OD_{600} = 1.10$ to 1.40) growth phases. The average amount of HA under each condition is indicated by the horizontal bars, $\pm$ standard deviations of the means. *, *P* value of <0.05 as determined by an unpaired *t* test.

incubation for 10 min at 99°C . The protein concentration was determined by using the Bradford assay (43). Proteins (150 μg /sample) were purified and trypsin digested by using a filter-aided sample preparation (FASP) protein digestion kit (Expedeon) as described previously (44), except that triethyl ammonium bicarbonate was used instead of ammonium bicarbonate. The digested proteins were isotopically labeled by dimethylation using heavy and light formaldehyde as described previously (45). Heavy and light samples were then mixed and desalted by using an Empore 4-mm/1-ml extraction disk cartridge (C_{18} -SD) (3M). Desalted samples were concentrated under a vacuum and resuspended in 0.1% formic acid and 2% acetonitrile. Peptides were resolved and analyzed by nano-liquid chromatography (LC)–tandem mass spectrometry using a Dionex ultra-high-performance liquid chromatography system coupled to a Thermo Scientific Q-Exactive Orbitrap mass spectrometer (located at the Monash Biomedical Proteomics Facility). Proteins were identified and quantified with MaxQuant (46) and Perseus software. For differential expression testing, the protein expression ratios were log transformed. These transformed ratios were roughly normally distributed, so limma (47) was used to fit and test for the significance of differential expression. Those proteins showing a ≥ 2.0 -fold (≥ 1.0 -log₂) change in expression across the triplicate replicates at an FDR of <0.05 were considered to be differentially expressed.

Accession numbers. The RNA-Seq data are available at the NCBI Gene Expression Omnibus under accession number [GSE77721](#). The proteomics data have been deposited in ProteomeXchange via the PRIDE database under accession number PXD003586.

RESULTS

The *hfq* mutant produces reduced hyaluronic acid capsule. To investigate the role of Hfq in *P. multocida*, an *hfq* mutant was generated in the highly virulent strain VP161. The mutant grew indistinguishably from the wild-type strain in BHI liquid broth at 37°C *in vitro* (see Fig. S1 in the supplemental material), but on solid medium, it produced smaller and less mucoid colonies than those of wild-type strain VP161, consistent with reduced HA capsular expression. The amount of HA was quantified in mid-exponential-phase cultures of wild-type strain VP161, the *hfq* mutant (AL2521), the complemented *hfq* mutant (AL2526), and the *hfq* mutant containing the vector only (AL2527). The *hfq* mutant produced significantly less HA than did the wild-type strain,

and HA production was restored to near-wild-type levels when the mutant was complemented with an intact copy of *hfq* (Fig. 1A). The control *hyaD* mutant produced no detectable HA (data not shown).

The amount of HA capsule produced by the *hfq* mutant was also assessed at different growth phases *in vitro*. The wild-type strain produced small amounts of HA at the early exponential growth phase, maximal amounts of HA at the mid-exponential growth phase, and intermediate levels of HA at the late exponential growth phase (Fig. 1B). No detectable HA was produced by the *hfq* mutant at the early exponential growth phase, and significantly reduced levels of HA were produced at the mid- and late exponential growth phases (Fig. 1B). Again, the *hyaD* acapsular control strain produced no detectable HA at any of the tested growth phases (data not shown). These data indicate that capsule production in *P. multocida* is growth phase dependent, and inactivation of *hfq* results in significantly reduced capsule expression during all tested growth phases *in vitro*.

The *hfq* mutant displays reduced *in vivo* fitness in mice. As the HA capsule is a known virulence factor of *P. multocida* serogroup A strains (34), competitive growth assays were performed in mice to compare the *in vivo* fitness of the wild-type strain with that of the *hfq* mutant, the *hfq* mutant complemented with an intact copy of *hfq*, and the *hfq* mutant containing the vector only. The *hfq* mutant displayed a 4-fold reduction in *in vivo* growth compared to that of the wild-type parent strain VP161 ($CI = 0.25 \pm 0.05$) (Fig. 2). Importantly, this loss of *in vivo* fitness was restored to near-wild-type levels when the *hfq* mutant was provided with an intact copy of *hfq* *in trans* ($CI = 0.68 \pm 0.06$) (Fig. 2). These data indicate that the *P. multocida* VP161 Hfq is important for *in vivo* fitness in mice.

Global RNA expression changes in the *P. multocida* *hfq* mutant. Hfq is an RNA chaperone that has been shown in many other bacterial species to be essential for the action of most *trans*-encoded regulatory sRNA molecules (16). Hfq and associated sRNAs can act at the level of mRNA stability or at the level of translational

FIG 2 The *hfq* mutant is attenuated for growth in mice. Shown are competitive growth indices for the *P. multocida* strains in a mouse infection model. Each data point shows the individual CI for one pair of strains in one mouse. The average CI for each pair of competing strains is indicated by the horizontal bars, $\pm$ standard deviations of the means. * indicates a *P* value of <0.05 as determined by a two-tailed unpaired *t* test.

efficiency (11). To identify changes in transcript abundance, the transcriptomes of the wild-type strain and the *hfq* mutant (grown to the mid-exponential growth phase) were compared by using high-throughput RNA-Seq. The RNA-Seq reads were mapped to the *P. multocida* VP161 genome, and 128 genes were identified as being differentially expressed. More than 85% of the differentially expressed genes (109 of 128 genes) showed increased expression levels compared to those in the wild type (see Table S1 in the supplemental material), suggesting that Hfq and its associated sRNAs generally act to decrease transcript levels, in keeping with the most common mode of sRNA/Hfq action at the transcriptional level, which is to increase RNase E turnover of transcripts.

Functional groups overrepresented in the upregulated gene set included those involved in cytochrome complex assembly (GO: 0017004; 6 genes; $P = 2 \times 10^{-6}$), organic substance transport (GO:0071702; 13 genes; $P = 6 \times 10^{-4}$), nitrogen compound transport (GO:0071705; 7 genes; $P = 8 \times 10^{-4}$), and protein-binding transcription factors (GO:0000988; 3 genes; $P = 0.001$). Among the upregulated genes were *pcgB*, *pcgD*, PM1042 (PMVP_1156, PMVP_1157, and PMVP_1057, respectively), encoding proteins involved in LPS biosynthesis (48; M. Harper and J. D. Boyce, unpublished data), and *hsf* and PM0855 (PMVP_0693 and PMVP_0855, respectively), predicted to encode the Hsf adhesin and a Flp pilus-like adhesin, respectively (39). The periplasmic nitrate reductase (*nap*) genes (*napFDAGHBC*) were all strongly upregulated (7.5- to 11.6-fold) in the *hfq* mutant, as were a number of genes encoding other putative electron transport proteins, including *ccmABCDEF* (2.0- to 4.1-fold) and *nrfBCD* (3-fold). In addition, numerous genes encoding proteins involved in heat shock and stress responses were also expressed at higher levels in the *hfq* mutant, including *htpX*, *uspG*, *htrA*, *dnaK*, *rpoH*, *clpB*, *rseC*, *rseB*, *mclA*, and *rpoE* (PMVP_0444, PMVP_0450, PMVP_0713, PMVP_0715, PMVP_1638, PMVP_1759, PMVP_1836, PMVP_1837, PMVP_1838, and PMVP_1839, respectively). An association of Hfq with the regulation of stress response genes/proteins has been seen in other bacteria, including *Y. pestis*, *Clostridium difficile*, and *S. enterica* (20, 49, 50).

Nineteen genes displayed decreased transcript levels in the *hfq* mutant compared to the wild-type strain (see Table S2 in the supplemental material). Functional groups overrepresented in this set

included polysaccharide transport (GO:0015774; two genes; $P = 4 \times 10^{-4}$) and pyridoxal phosphate metabolism (GO:0042822; two genes; $P = 0.001$). Importantly, 5 of the 10 capsule biosynthesis genes showed significantly reduced transcript expression in the *hfq* mutant ($P < 0.0001$) (see Table 5), correlating with the reduced HA capsule production observed for the *hfq* mutant.

Global changes in protein production in the *P. multocida* *hfq* mutant. Hfq often regulates protein production by altering mRNA translational efficiency via sRNA binding (7). Therefore, the proteomes of the VP161 *hfq* mutant and wild-type strain VP161 were compared during both early exponential and mid-exponential growth phases by using quantitative high-throughput liquid proteomics. Totals of 1,147 and 1,041 proteins were identified during early exponential and mid-exponential growth, respectively. During the early exponential growth phase of the *hfq* mutant, 85 proteins were detected at increased levels and 21 proteins were detected at reduced levels compared to those in the wild-type strain (see Tables S3 and S4, respectively, in the supplemental material). During the mid-exponential growth phase, 78 proteins were identified as being differentially expressed in the *hfq* mutant: 49 at increased levels (see Table S5 in the supplemental material) and 29 at decreased levels (see Table S6 in the supplemental material). Comparing the data from both growth phases, 37 proteins were expressed at increased levels at both the early exponential and mid-exponential growth phases (Table 3), and 8 proteins were expressed at decreased levels at both growth phases (Table 4). Five of the capsule biosynthesis proteins (HyaE, HyaD, HyaC, HexD, and HexC) showed between 10- and 20-fold-reduced production at the early exponential growth phase and between 1.8- and 2.7-fold-reduced production at the mid-exponential growth phase. These data correlated well with the decreased transcript levels observed for the capsule biosynthesis genes as measured by RNA-Seq (Table 5) and with the reduced HA capsule levels expressed by this strain. In addition, the known *P. multocida* virulence factor PfhB2 and its secretion partner LspB2 were also present at reduced levels in the *hfq* mutant at both growth phases (Table 4). In contrast, the levels of production of the hemoglobin-binding proteins PMVP_0265 (PM0300) and PMVP_0304 (PM0337) were increased in the *hfq* mutant at both the early exponential and mid-exponential growth phases (Table 3).

Comparison of the proteomic and transcriptomic data. The proteomic analysis of the *hfq* mutant at the mid-exponential growth phase (see above) identified 78 proteins as being differentially expressed, with 49 being identified at increased levels and 29 at decreased levels. Of the 49 proteins produced at higher levels in the *hfq* mutant, 13 also showed increased transcript levels as measured by RNA-Seq; all 13 of these proteins also showed increased levels (as measured by proteomics) at the early exponential growth phase (see Table S7 in the supplemental material). The 13 genes/proteins that showed increased expression levels by both techniques included 2 outer membrane lipoproteins (Plp4 and PlpB), 3 components of the periplasmic nitrate reductase system, the universal stress protein UspG, and the oligopeptide transporter DppA. Similarly, of the 29 proteins measured at reduced levels in the *hfq* mutant by high-throughput proteomics, 6 were also downregulated as determined by RNA-Seq (see Table S8 in the supplemental material), and 5 of these also showed decreased expression at the early exponential growth phase (see Table S8 in the supplemental material). The six genes/proteins determined to be downregulated in the *hfq* mutant by both proteomics and tran-

TABLE 3 Proteins showing increased production in the *P. multocida* *hfq* mutant strain in comparison to wild-type strain VP161 during both early exponential and mid-exponential growth phases as measured by high-throughput liquid proteomics

VP161 locus tag	Predicted function and/or product	Pm70 protein name	Early exponential growth		Mid-exponential growth	
			Expression ratio (log ₂)	FDR	Expression ratio (log ₂)	FDR
PMVP_0194	Periplasmic dipeptide transporter	DppA	2.59	2.82E−04	2.44	1.06E−04
PMVP_0198	Dipeptide ABC transporter, ATP-binding protein	DppF	2.21	4.28E−03	1.71	1.39E−03
PMVP_0229	Murein L,D-transpeptidase, provisional	PM0270	1.57	4.66E−04	1.73	4.44E−04
PMVP_0253	Predicted membrane-bound lysozyme inhibitor of the c type	None	3.30	6.48E−04	2.06	2.53E−04
PMVP_0265	Hemoglobin/haptoglobin-binding protein	PM0300	1.79	5.84E−04	1.28	2.32E−03
PMVP_0267	Mrp putative ATPase	Mrp	1.18	5.19E−04	1.32	1.77E−03
PMVP_0292	Transglycosylase SLT domain protein	PM0326	1.56	1.69E−03	2.85	1.85E−03
PMVP_0304	Hemoglobin-binding protein with TonB receptor	PM0337	1.49	6.40E−03	1.49	9.02E−04
PMVP_0305	Hemoglobin-binding protein with TonB receptor	PM0337	1.83	1.75E−03	1.32	3.53E−03
PMVP_0327	MscS family potassium efflux protein, KefA	PM0358	1.07	8.84E−04	1.00	7.98E−03
PMVP_0376	Formate dehydrogenase accessory protein	FdhE	1.29	1.16E−03	1.00	3.62E−02
PMVP_0449	TRAP ^a transporter/C ₄ -dicarboxylate ABC transporter	PM0473	1.58	5.48E−04	1.78	8.79E−04
PMVP_0450	Universal stress protein, UspG	PM0474	1.21	5.03E−04	1.35	1.74E−03
PMVP_0488	Conserved protein with no known function	PM0519	2.10	4.44E−04	1.81	1.77E−03
PMVP_0516	Glucose-1-phosphate adenyltransferase	GlgC	1.46	5.48E−04	1.56	3.39E−02
PMVP_0541	Branched-chain amino acid aminotransferase	IlvE	1.39	5.84E−04	1.10	5.18E−03
PMVP_0561	Lipoprotein	Plp4	1.77	4.44E−04	1.31	1.85E−03
PMVP_0629	Tellurite resistance protein	TehB	3.50	8.84E−04	2.18	4.17E−03
PMVP_0756	ABC-ATPase-MoxR-like	None	1.18	1.58E−02	1.47	9.02E−04
PMVP_0837	Histidinol-phosphate aminotransferase	HisH1	1.26	1.01E−03	1.41	1.47E−03
PMVP_0852	Protein within FliP/tight adhesion locus, homology to RpeC	PM0853	2.00	7.94E−04	1.74	5.98E−04
PMVP_0939	Membrane-bound lytic murein transglycosylase	PM0928	1.66	7.94E−04	1.99	3.96E−04
PMVP_0948	Aspartate kinase, monofunctional class	PM0937	1.33	5.19E−04	1.21	2.57E−03
PMVP_0992	Predicted membrane/periplasmic protein of unknown function	PM0979	2.08	2.82E−04	2.05	4.04E−04
PMVP_1069	Dihydrodipicolinate synthase	DapA	1.11	7.94E−04	1.01	8.68E−03
PMVP_1208	Type I DNase HsdR (HtpX peptidase M48 superfamily)	PM1190	1.34	7.27E−03	1.51	8.79E−04
PMVP_1233	Transglutaminase-like superfamily domain protein	PM1211	1.41	8.19E−04	1.98	2.08E−03
PMVP_1614	GTA ^b type/β-D-1,6-glucosyl transferase	PM1562	2.46	4.44E−04	2.45	1.06E−04
PMVP_1616	Large conductance mechanosensitive channel protein	MscL	2.12	7.32E−03	2.55	2.42E−04
PMVP_1649	Periplasmic nitrate reductase precursor	NapA	1.59	8.84E−04	1.29	1.81E−03
PMVP_1652	Periplasmic nitrate reductase	NapB	1.12	2.91E−02	1.75	5.47E−03
PMVP_1760	Thioredoxin	Trx	1.87	4.88E−03	1.73	1.33E−02
PMVP_1787	Outer membrane lipoprotein 2 precursor	PlpB	1.32	5.03E−04	1.09	9.91E−03
PMVP_1863	Nitrate/nitrite response regulator protein	NarP	2.51	2.82E−04	2.05	2.53E−04
PMVP_1872	SrfB homologue, uncharacterized protein conserved in bacteria	PM1819	1.21	2.04E−03	1.21	7.88E−03
PMVP_1879	Protein of unknown function	PM1826	1.86	4.66E−04	1.58	8.79E−04
PMVP_1962	Periplasmic oligopeptide-binding protein precursor	OppA	1.26	5.19E−04	1.17	3.72E−03
PMVP_2095	Glutamate dehydrogenase	GdhA	2.01	1.75E−03	2.71	1.06E−04

^a TRAP, tripartite ATP-independent periplasmic.^b GTA, glycosyltransferase A.

scriptomics analyses included three capsule biosynthesis proteins, the filamentous hemagglutinin secretion partner LspB, the global regulator of carbon metabolic flux FruR (51), and the imidazole glycerol phosphate synthase, which is a key enzyme that links amino acid and nucleotide biosynthesis pathways (52). Interestingly, 59 of the 78 proteins identified as being differentially expressed in the *hfq* mutant by proteomics analysis were not determined to be differentially expressed by transcriptomics analysis. It is likely that many of these proteins are regulated by Hfq-sRNA interactions that act posttranscriptionally, a common mechanism of sRNA action (7).

DISCUSSION

Inactivation of *hfq* in different bacterial species has resulted in a wide range of phenotypic effects. For some species, such as *Bru-*

cella melitensis, *Legionella pneumophila*, and *Salmonella enterica*, *hfq* mutants show a reduced growth rate with a longer lag phase in certain *in vitro* media (53–55). In other bacterial species, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Haemophilus influenzae* (24, 56, 57), and *P. multocida* (as shown in this study for strain VP161), the growth rate *in vitro* in rich medium is unaffected by *hfq* inactivation. Despite this normal *in vitro* growth rate, competition assays in mice revealed that the *P. multocida hfq* mutant showed significantly reduced fitness *in vivo*. Reduced *in vivo* fitness has also been demonstrated for *hfq* mutant strains of *Y. pestis*, *Klebsiella pneumoniae*, *Francisella tularensis*, and *Actinobacillus pleuropneumoniae* (20, 58–60).

One of the critical *P. multocida* virulence factors is the polysaccharide capsule (34). The *P. multocida hfq* mutant showed significantly reduced HA capsular polysaccharide (CPS) production

TABLE 4 Proteins showing decreased production in the *P. multocida* *hfq* mutant strain in comparison to wild-type strain VP161 during both early exponential and mid-exponential growth phases as measured by high-throughput liquid proteomics

VP161 locus tag	Predicted function and/or product	Pm70 protein name	Early exponential growth		Mid-exponential growth	
			Expression ratio (log ₂)	FDR	Expression ratio (log ₂)	FDR
PMVP_0001	LspB	LspB2	−2.74	8.84E−04	−1.83	1.85E−03
PMVP_0002 ^a	Filamentous hemagglutinin	PfhB2	−2.81	4.44E−04	−1.85	1.92E−03
PMVP_0003 ^a	Filamentous hemagglutinin	PfhB2	−2.82	4.66E−04	−1.82	2.21E−03
PMVP_0004 ^a	Filamentous hemagglutinin	PfhB2	−2.70	4.44E−04	−1.77	3.58E−03
PMVP_0005 ^a	Filamentous hemagglutinin	PfhB2	−2.19	8.19E−04	−1.81	1.14E−02
PMVP_0006 ^a	Filamentous hemagglutinin	PfhB2	−1.69	5.03E−04	−1.86	1.85E−03
PMVP_0218	Spermidine/putrescine ABC transporter, periplasmic-binding protein	PotD1	−1.88	6.96E−04	−1.58	8.79E−04
PMVP_0767	Capsule biosynthesis, HyaE	HyaE	−4.17	2.82E−04	−1.11	2.63E−02
PMVP_0768	Capsule biosynthesis, HyaD	PM0775	−4.35	2.82E−04	−1.06	1.95E−02
PMVP_0769	Capsule biosynthesis, HyaC	PM0776	−3.86	1.22E−03	−1.41	5.60E−03
PMVP_0771	Capsule transport, HexD	HexD	−3.29	4.50E−04	−1.01	1.33E−02
PMVP_0869	DNA-binding transcriptional regulator, fructose repressor	FruR	−1.99	1.00E−02	−1.31	4.81E−03

^a These features likely represent broken sections of one gene (*pfbB2*) that have not been fully assembled in the draft VP161 genome.

(up to 14-fold) at all tested growth phases. Transcriptomic and proteomic analyses of the *P. multocida* *hfq* mutant confirmed the phenotypic data, showing highly reduced transcript and protein levels for many of the capsule biosynthesis genes/proteins (Table 5). Thus, Hfq plays a crucial role in positively regulating capsule production. The reduced level of capsule biosynthesis gene transcripts suggests that Hfq-mediated regulation acts primarily at the level of transcription; however, we cannot rule out an additional role for posttranscriptional mechanisms. In contrast to the effect of Hfq on *P. multocida* capsule production, a *K. pneumoniae* *hfq* mutant is hypermucoid, with increased levels of K2-specific CPSs (58). The reduction in capsule production in the *P. multocida* *hfq* mutant is likely to play a major role in the reduced *in vivo* fitness observed for this strain.

We have previously shown that the global transcriptional regulator Fis is essential for the expression of *P. multocida* capsule biosynthesis genes (36). Our proteomic and transcriptomic analyses indicated that Fis was produced at unchanged (proteomics data) or slightly increased (transcriptomics data) levels (1.8-fold; FDR = 0.03) in the *hfq* mutant compared to the wild-type strain,

implying that the loss of capsule expression in the *hfq* mutant was not the result of a loss of Fis production. However, Fis may play a role in the regulation of *hfq* expression, as RNA-Seq analysis of a *P. multocida* VP161 *fis* mutant during the early exponential growth phase revealed a 2.2-fold decrease in *hfq* expression (FDR = 0.001) compared to that of the wild-type strain (Harper and Boyce, unpublished). These data suggest that Fis positively regulates Hfq, which in turn positively regulates capsule expression. However, mutation of *fis* alone has a much larger effect on capsule expression than mutation of *hfq* alone, suggesting that Fis primarily acts separately from Hfq.

P. multocida expresses two filamentous hemagglutinins, PfhB1 and PfhB2, that are important for *in vivo* fitness (31, 61). Our proteomic analyses showed that the loss of Hfq leads to a reduction in the amounts of PfhB2 and the secretion partner LspB2 during both early exponential and mid-exponential growth. It is possible that the reduced expression of PfhB2 may have contributed, along with reduced capsule expression, to the reduced *in vivo* fitness of the *hfq* mutant. Our transcriptomic analyses showed that genes encoding these proteins were also likely downregulated

TABLE 5 Differential expression of capsule biosynthesis genes/proteins in the *P. multocida* *hfq* mutant strain in comparison to wild-type strain VP161

VP161 locus tag	Pm70 name	Gene name	RNA-Seq data for mid-exponential growth		Proteomics data for early exponential growth		Proteomics data for mid-exponential growth	
			Expression ratio (log ₂)	FDR	Expression ratio (log ₂)	FDR	Expression ratio (log ₂)	FDR
PMVP_0765	<i>phyB</i>	<i>phyB</i>	−1.35	1.02E−03	ND ^a	ND	0.12	9.24E−01
PMVP_0766	<i>phyA</i>	<i>phyA</i>	−1.86	7.35E−04	−0.91	5.18E−02	−0.65	2.03E−01
PMVP_0767	<i>hyaE</i>	<i>hyaE</i>	−1.78	2.16E−03	−4.17	2.82E−04	−1.11	2.63E−02
PMVP_0768	PM0775	<i>hyaD</i>	−1.84	2.08E−03	−4.35	2.82E−04	−1.06	1.95E−02
PMVP_0769	PM0776	<i>hyaC</i>	−1.41	7.48E−03	−3.86	1.22E−03	−1.41	5.60E−03
PMVP_0770	PM0777	<i>hyaB</i>	−0.67	2.90E−02	ND	ND	ND	ND
PMVP_0771	<i>hexD</i>	<i>hexD</i>	−0.60	5.78E−02	−3.29	4.50E−04	−1.01	1.33E−02
PMVP_0772	<i>hexC</i>	<i>hexC</i>	−0.68	4.22E−02	−3.44	4.44E−04	−0.88	1.90E−02
PMVP_0773	<i>hexB</i>	<i>hexB</i>	−0.62	3.90E−02	ND	ND	ND	ND
PMVP_0774	<i>hexA</i>	<i>hexA</i>	−0.46	4.09E−02	0.30	6.42E−01	−0.71	6.89E−02

^a ND indicates that the level of the protein was not measured under the specified conditions.

(PfhB2 was 1.4-fold downregulated, with an FDR of 0.056, and LspB2 was 2.3-fold downregulated, with an FDR of 0.01), indicating that the Hfq-associated regulation of these genes is also likely to be at the transcriptional level.

LPS is another crucial *P. multocida* virulence factor; strains expressing truncated LPS are highly attenuated for virulence (62, 63). Three genes involved in LPS biosynthesis (*pcgB*, *pcgD*, and *PM1042*) were expressed at increased levels in the *hfq* mutant. *PcgB* and *PcgD* are required for the biosynthesis and transfer of phosphocholine residues to the terminal galactose residues of the LPS produced by strain VP161 (48), and a functional *PM1042* protein is required for the addition of phosphoethanolamine to lipid A (Harper and Boyce, unpublished). Both phosphoethanolamine and phosphocholine are important charged LPS substituents that can affect the interaction of host antimicrobial peptides with the bacterial surface (48). Numerous genes/proteins involved in electron transport were also upregulated in the *hfq* mutant, including *napFDAGHBC*, *ccmABCDEF*, and *nrfBCD*. Interestingly, the Nap system is predicted to reduce nitrate to nitrite during anaerobic growth (64), and a previous microarray study of *P. multocida* (strain X73) showed that the expression of the *nap* genes was significantly upregulated during *in vivo* growth in the chicken host, a relatively anaerobic niche (65). Thus, appropriate regulation of these genes appears critical for full *in vivo* fitness.

In conclusion, this study defined a clear role for Hfq in the regulation of *P. multocida* gene and protein expression and allowed us to identify genes controlled (either directly or indirectly) by Hfq-sRNA interactions at both the transcriptional and translational levels. Together, transcriptional and proteomic analyses of the *hfq* mutant supported our hypothesis that Hfq is involved in the regulation of virulence- and fitness-associated genes of *P. multocida*. Hfq is clearly involved in regulating the production of the known *P. multocida* virulence factors HA capsule, filamentous hemagglutinin, and LPS as well as accessory virulence genes such as those encoding iron uptake proteins, stress response proteins, and bacterial adhesins. We are currently focusing on identifying specific sRNA-mRNA interactions and in particular the specific mechanism(s) by which Hfq/sRNA action regulates the expression of HA capsule, LPS, and filamentous hemagglutinin.

ACKNOWLEDGMENTS

We thank Deanna Deveson Lucas for assistance with RNA-Seq sample preparation and Marietta John for construction of the VP161 *hyaD* mutant.

This work was supported in part by the Australian Research Council.

REFERENCES

- Romby P, Vandenesch F, Wagner EGH. 2006. The role of RNAs in the regulation of virulence-gene expression. *Curr Opin Microbiol* 9:229–236. <http://dx.doi.org/10.1016/j.mib.2006.02.005>.
- Lenz DH, Mok KC, Lilley BN, Kulkarni RV, Wingreen NS, Bassler BL. 2004. The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* 118:69–82. <http://dx.doi.org/10.1016/j.cell.2004.06.009>.
- Sonnleitner E, Abdou L, Haas D. 2009. Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 106:21866–21871. <http://dx.doi.org/10.1073/pnas.0910308106>.
- Sharma CM, Papenfort K, Pernitzsch SR, Mollenkopf HJ, Hinton JCD, Vogel J. 2011. Pervasive post-transcriptional control of genes involved in amino acid metabolism by the Hfq-dependent GcvB small RNA. *Mol Microbiol* 81:1144–1165. <http://dx.doi.org/10.1111/j.1365-2958.2011.07751.x>.
- Massé E, Gottesman S. 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc Natl Acad Sci U S A* 99:4620–4625. <http://dx.doi.org/10.1073/pnas.032066599>.
- Sobrero P, Valverde C. 2012. The bacterial protein Hfq: much more than a mere RNA-binding factor. *Crit Rev Microbiol* 38:276–299. <http://dx.doi.org/10.3109/1040841X.2012.664540>.
- Vogel J, Luisi BF. 2011. Hfq and its constellation of RNA. *Nat Rev Microbiol* 9:578–589. <http://dx.doi.org/10.1038/nrmicro2615>.
- Gottesman S, Storz G. 2011. Bacterial small RNA regulators: versatile roles and rapidly evolving variations. *Cold Spring Harb Perspect Biol* 3:a003798. <http://dx.doi.org/10.1101/cshperspect.a003798>.
- Bobrovskyy M, Vanderpool CK. 2013. Regulation of bacterial metabolism by small RNAs using diverse mechanisms. *Annu Rev Genet* 47:209–232. <http://dx.doi.org/10.1146/annurev-genet-111212-133445>.
- Moller T, Franch T, Hojrup P, Keene DR, Bächinger HP, Brennan RG, Valentin-Hansen P. 2002. Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. *Mol Cell* 9:23–30. [http://dx.doi.org/10.1016/S1097-2765\(01\)00436-1](http://dx.doi.org/10.1016/S1097-2765(01)00436-1).
- Schumacher MA, Pearson RF, Moller T, Valentin-Hansen P, Brennan RG. 2002. Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J* 21:3546–3556. <http://dx.doi.org/10.1093/emboj/cdf322>.
- De Lay N, Schu DJ, Gottesman S. 2013. Bacterial small RNA-based negative regulation: Hfq and its accomplices. *J Biol Chem* 288:7996–8003. <http://dx.doi.org/10.1074/jbc.R112.441386>.
- Brennan RG, Link TM. 2007. Hfq structure, function and ligand binding. *Curr Opin Microbiol* 10:125–133. <http://dx.doi.org/10.1016/j.mib.2007.03.015>.
- Folichon M, Arluisson V, Pellegrini O, Huntzinger E, Régnier P, Hajnsdorf E. 2003. The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res* 31:7302–7310. <http://dx.doi.org/10.1093/nar/gkg915>.
- Mohanty BK, Maples VF, Kushner SR. 2004. The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol* 54:905–920. <http://dx.doi.org/10.1111/j.1365-2958.2004.04337.x>.
- Chao Y, Vogel J. 2010. The role of Hfq in bacterial pathogens. *Curr Opin Microbiol* 13:24–33. <http://dx.doi.org/10.1016/j.mib.2010.01.001>.
- Tsui HCT, Leung HCE, Winkler ME. 1994. Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12. *Mol Microbiol* 13:35–49. <http://dx.doi.org/10.1111/j.1365-2958.1994.tb00400.x>.
- Robertson GT, Roop RMI. 1999. The *Brucella abortus* host factor I (HF-I) protein contributes to stress resistance during stationary phase and is a major determinant of virulence in mice. *Mol Microbiol* 34:690–700. <http://dx.doi.org/10.1046/j.1365-2958.1999.01629.x>.
- Figueroa-Bossi N, Lemire S, Maloriot D, Balbontin R, Casadesús J, Bossi L. 2006. Loss of Hfq activates the σ E-dependent envelope stress response in *Salmonella enterica*. *Mol Microbiol* 62:838–852. <http://dx.doi.org/10.1111/j.1365-2958.2006.05413.x>.
- Geng J, Song Y, Yang L, Feng Y, Qiu Y, Li G, Guo J, Bi Y, Qu Y, Wang W, Wang X, Guo Z, Yang R, Han Y. 2009. Involvement of the post-transcriptional regulator Hfq in *Yersinia pestis* virulence. *PLoS One* 4:e6213. <http://dx.doi.org/10.1371/journal.pone.0006213>.
- Attia AS, Sedillo JL, Wang W, Liu W, Brautigam CA, Winkler W, Hansen EJ. 2008. *Moraxella catarrhalis* expresses an unusual Hfq protein. *Infect Immun* 76:2520–2530. <http://dx.doi.org/10.1128/IAI.01652-07>.
- Fantappiè L, Metruccio MME, Seib KL, Oriente F, Cartocci E, Ferlicca F, Giuliani MM, Scarlato V, Delany I. 2009. The RNA chaperone Hfq is involved in stress response and virulence in *Neisseria meningitidis* and is a pleiotropic regulator of protein expression. *Infect Immun* 77:1842–1853. <http://dx.doi.org/10.1128/IAI.01216-08>.
- Ding Y, Davis BM, Waldor MK. 2004. Hfq is essential for *Vibrio cholerae* virulence and downregulates σ E expression. *Mol Microbiol* 53:345–354. <http://dx.doi.org/10.1111/j.1365-2958.2004.04142.x>.
- Christiansen JK, Larsen MH, Ingmer H, Søgaard-Andersen L, Kallipolitis BH. 2004. The RNA-binding protein Hfq of *Listeria monocytogenes*: role in stress tolerance and virulence. *J Bacteriol* 186:3355–3362. <http://dx.doi.org/10.1128/JB.186.11.3355-3362.2004>.
- Boyce JD, Harper M, Wilkie IW, Adler B. 2010. *Pasteurella*, p 325–346. In Gyles CL, Prescott JF, Songer JG, Thoen CO (ed), *Pathogenesis of bacterial infections of animals*, 4th ed. Blackwell Publishing, Ames, IA.
- Blackall PJ, Mifflin JK. 2000. Identification and typing of *Pasteurella*

- multocida*: a review. Avian Pathol 29:271–287. <http://dx.doi.org/10.1080/03079450050118395>.
27. Wilson BA, Ho M. 2013. *Pasteurella multocida*: from zoonosis to cellular microbiology. Clin Microbiol Rev 26:631–655. <http://dx.doi.org/10.1128/CMR.00024-13>.
 28. Carter GR. 1952. The type specific capsular antigen of *Pasteurella multocida*. Can J Med Sci 30:48–53.
 29. Harper M, John M, Turni C, Edmunds M, St Michael F, Adler B, Blackall PJ, Cox AD, Boyce JD. 2015. Development of a rapid multiplex PCR assay to genotype *Pasteurella multocida* strains by use of the lipopolysaccharide outer core biosynthesis locus. J Clin Microbiol 53:477–485. <http://dx.doi.org/10.1128/JCM.02824-14>.
 30. Harper M, Boyce JD, Adler B. 2012. The key surface components of *Pasteurella multocida*: capsule and lipopolysaccharide. Curr Top Microbiol Immunol 361:39–51. http://dx.doi.org/10.1007/82_2012_202.
 31. Tatum FM, Yersin AG, Briggs RE. 2005. Construction and virulence of a *Pasteurella multocida* fhaB2 mutant in turkeys. Microb Pathog 39:9–17. <http://dx.doi.org/10.1016/j.micpath.2005.05.003>.
 32. Bosch M, Garrido E, Llagostera M, Pérez De Rozas AM, Badiola I, Barbé J. 2002. *Pasteurella multocida* exbB, exbD and tonB genes are physically linked but independently transcribed. FEMS Microbiol Lett 210:201–208. <http://dx.doi.org/10.1111/j.1574-6968.2002.tb11181.x>.
 33. Vimr ER. 2013. Unified theory of bacterial sialometabolism: how and why bacteria metabolize host sialic acids. ISRN Microbiol 2013:816713. <http://dx.doi.org/10.1155/2013/816713>.
 34. Chung JY, Wilkie I, Boyce JD, Townsend KM, Frost AJ, Ghoddsi M, Adler B. 2001. Role of capsule in the pathogenesis of fowl cholera caused by *Pasteurella multocida* serogroup A. Infect Immun 69:2487–2492. <http://dx.doi.org/10.1128/IAI.69.4.2487-2492.2001>.
 35. Boyce JD, Adler B. 2000. The capsule is a virulence determinant in the pathogenesis of *Pasteurella multocida* M1404 (B:2). Infect Immun 68:3463–3468. <http://dx.doi.org/10.1128/IAI.68.6.3463-3468.2000>.
 36. Steen JA, Steen JA, Harrison P, Seemann T, Wilkie I, Harper M, Adler B, Boyce JD. 2010. Fis is essential for capsule production in *Pasteurella multocida* and regulates expression of other important virulence factors. PLoS Pathog 6:e1000750. <http://dx.doi.org/10.1371/journal.ppat.1000750>.
 37. National Medical Health and Research Council. 2004. Australian code of practice for the care and use of animals for scientific purposes, 7th ed. National Medical Health and Research Council, Canberra, Australia.
 38. Harper M, St Michael F, John M, Vinogradov E, Steen JA, van Dorsten L, Steen JA, Turni C, Blackall PJ, Adler B, Cox AD, Boyce JD. 2013. *Pasteurella multocida* Heddlston serovar 3 and 4 strains share a common lipopolysaccharide biosynthesis locus but display both inter- and intras-train lipopolysaccharide heterogeneity. J Bacteriol 195:4854–4864. <http://dx.doi.org/10.1128/JB.00779-13>.
 39. Harper M, Boyce JD, Wilkie IW, Adler B. 2003. Signature-tagged mutagenesis of *Pasteurella multocida* identifies mutants displaying differential virulence characteristics in mice and chickens. Infect Immun 71:5440–5446. <http://dx.doi.org/10.1128/IAI.71.9.5440-5446.2003>.
 40. Henry R, Crane B, Powell D, Deveson Lucas D, Li Z, Aranda J, Harrison P, Nation RL, Adler B, Harper M, Boyce JD, Li J. 2015. The transcriptomic response of *Acinetobacter baumannii* to colistin and doripenem alone and in combination in an *in vitro* pharmacokinetics/pharmacodynamics model. J Antimicrob Chemother 70:1303–1313. <http://dx.doi.org/10.1093/jac/dku536>.
 41. May BJ, Zhang Q, Li LL, Paustian ML, Whittam TS, Kapur V. 2001. Complete genomic sequence of *Pasteurella multocida*, Pm70. Proc Natl Acad Sci U S A 98:3460–3465. <http://dx.doi.org/10.1073/pnas.051634598>.
 42. Caspi R, Altman T, Billington R, Dreher K, Foerster H, Fulcher CA, Holland TA, Keseler IM, Kothari A, Kubo A, Krummenacker M, Latendresse M, Mueller LA, Ong Q, Paley S, Subhraveti P, Weaver DS, Weerasinghe D, Zhang P, Karp PD. 2014. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic Acids Res 42:D459–D471. <http://dx.doi.org/10.1093/nar/gkt1103>.
 43. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. [http://dx.doi.org/10.1016/0003-2697\(76\)90527-3](http://dx.doi.org/10.1016/0003-2697(76)90527-3).
 44. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. 2009. Universal sample preparation method for proteome analysis. Nat Methods 6:359–362. <http://dx.doi.org/10.1038/nmeth.1322>.
 45. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJR. 2009. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat Protoc 4:484–494. <http://dx.doi.org/10.1038/nprot.2009.21>.
 46. Cox J, Mann M. 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26:1367–1372. <http://dx.doi.org/10.1038/nbt.1511>.
 47. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. 2015. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:e47. <http://dx.doi.org/10.1093/nar/gkv007>.
 48. Harper M, Cox A, St Michael F, Parnas H, Wilkie I, Blackall PJ, Adler B, Boyce JD. 2007. Decoration of *Pasteurella multocida* lipopolysaccharide with phosphocholine is important for virulence. J Bacteriol 189:7384–7391. <http://dx.doi.org/10.1128/JB.00948-07>.
 49. Boudry P, Gracia C, Monot M, Caillet J, Saujet L, Hajnsdorf E, Dupuy B, Martin-Verstraete I, Soutourina O. 2014. Pleiotropic role of the RNA chaperone protein Hfq in the human pathogen *Clostridium difficile*. J Bacteriol 196:3234–3248. <http://dx.doi.org/10.1128/JB.01923-14>.
 50. Ansong C, Yoon H, Porwollik S, Mottaz-Brewer H, Petritis BO, Jaitly N, Adkins JN, McClelland M, Heffron F, Smith RD. 2009. Global systems-level analysis of Hfq and SmpB deletion mutants in *Salmonella*: implications for virulence and global protein translation. PLoS One 4:e4809. <http://dx.doi.org/10.1371/journal.pone.0004809>.
 51. Ramseier TM, Bledig S, Michotey V, Feghali R, Saier MH, Jr. 1995. The global regulatory protein FruR modulates the direction of carbon flow in *Escherichia coli*. Mol Microbiol 16:1157–1169. <http://dx.doi.org/10.1111/j.1365-2958.1995.tb02339.x>.
 52. Douangamath A, Walker M, Beismann-Driemeyer S, Vega-Fernandez MC, Sterner R, Wilmanns M. 2002. Structural evidence for ammonia tunneling across the (βα)8 barrel of the imidazole glycerol phosphate synthase bienzyme complex. Structure 10:185–193. [http://dx.doi.org/10.1016/S0969-2126\(02\)00702-5](http://dx.doi.org/10.1016/S0969-2126(02)00702-5).
 53. Cui M, Wang T, Xu J, Ke Y, Du X, Yuan X, Wang Z, Gong C, Zhuang Y, Lei S, Su X, Wang X, Huang L, Zhong Z, Peng G, Yuan J, Chen Z, Wang Y. 2013. Impact of Hfq on global gene expression and intracellular survival in *Brucella melitensis*. PLoS One 8:e71933. <http://dx.doi.org/10.1371/journal.pone.0071933>.
 54. McNealy TL, Forsbach-Birk V, Shi C, Marre R. 2005. The Hfq homolog in *Legionella pneumophila* demonstrates regulation by LetA and RpoS and interacts with the global regulator CsrA. J Bacteriol 187:1527–1532. <http://dx.doi.org/10.1128/JB.187.4.1527-1532.2005>.
 55. Sittka A, Pfeiffer V, Tedin K, Vogel J. 2007. The RNA chaperone Hfq is essential for the virulence of *Salmonella* Typhimurium. Mol Microbiol 63:193–217. <http://dx.doi.org/10.1111/j.1365-2958.2006.05489.x>.
 56. Bohn C, Rigoulay C, Boulou P. 2007. No detectable effect of RNA-binding protein Hfq absence in *Staphylococcus aureus*. BMC Microbiol 7:10. <http://dx.doi.org/10.1186/1471-2180-7-10>.
 57. Hempel RJ, Morton DJ, Seale TW, Whitby PW, Stull TL. 2013. The role of the RNA chaperone Hfq in *Haemophilus influenzae* pathogenesis. BMC Microbiol 13:134. <http://dx.doi.org/10.1186/1471-2180-13-134>.
 58. Chiang MK, Lu MC, Liu LC, Lin CT, Lai YC. 2011. Impact of Hfq on global gene expression and virulence in *Klebsiella pneumoniae*. PLoS One 6:e22248. <http://dx.doi.org/10.1371/journal.pone.0022248>.
 59. Meibom KL, Forslund AL, Kuoppa K, Alkhuder K, Dubail I, Dupuis M, Forsberg Å, Charbit A. 2009. Hfq, a novel pleiotropic regulator of virulence-associated genes in *Francisella tularensis*. Infect Immun 77:1866–1880. <http://dx.doi.org/10.1128/IAI.01496-08>.
 60. Subashchandrabose S, Leveque RM, Kirkwood RN, Kiupel M, Mulks MH. 2013. The RNA chaperone hfq promotes fitness of *Actinobacillus pleuropneumoniae* during porcine pleuropneumonia. Infect Immun 81:2952–2961. <http://dx.doi.org/10.1128/IAI.00392-13>.
 61. Fuller TE, Kennedy MJ, Lowery DE. 2000. Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature-tagged mutagenesis. Microb Pathog 29:25–38. <http://dx.doi.org/10.1006/mpat.2000.0365>.
 62. Boyce JD, Harper M, St Michael F, John M, Aubry A, Parnas H, Logan SM, Wilkie IW, Ford M, Cox AD, Adler B. 2009. Identification of novel glycosyltransferases required for assembly of the *Pasteurella multocida* A:1 lipopolysaccharide and their involvement in virulence. Infect Immun 77:1532–1542. <http://dx.doi.org/10.1128/IAI.01144-08>.
 63. Harper M, Cox AD, Adler B, Boyce JD. 2011. *Pasteurella multocida*

- lipopolysaccharide: the long and the short of it. *Vet Microbiol* 153:109–115. <http://dx.doi.org/10.1016/j.vetmic.2011.05.022>.
64. Cole J. 1996. Nitrate reduction to ammonia by enteric bacteria: redundancy, or a strategy for survival during oxygen starvation? *FEMS Microbiol Lett* 136:1–11. <http://dx.doi.org/10.1111/j.1574-6968.1996.tb08017.x>.
65. Boyce JD, Wilkie I, Harper M, Paustian ML, Kapur V, Adler B. 2002. Genomic scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host. *Infect Immun* 70:6871–6879. <http://dx.doi.org/10.1128/IAI.70.12.6871-6879.2002>.
66. Wilkie IW, Grimes SE, O’Boyle D, Frost AJ. 2000. The virulence and protective efficacy for chickens of *Pasteurella multocida* administered by different routes. *Vet Microbiol* 72:57–68. [http://dx.doi.org/10.1016/S0378-1135\(99\)00187-X](http://dx.doi.org/10.1016/S0378-1135(99)00187-X).