RESEARCH ARTICLE

Haemophilus ducreyi Hfq Contributes to Virulence Gene Regulation as Cells Enter Stationary Phase

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ABSTRACT **To adapt to stresses encountered in stationary phase, Gram-negative bacteria utilize the alternative sigma factor RpoS. However, some species lack RpoS; thus, it is unclear how stationary-phase adaptation is regulated in these organisms. Here we defined the growth-phase-dependent transcriptomes of *Haemophilus ducreyi*, which lacks an RpoS homolog. Compared to mid-log-phase organisms, cells harvested from the stationary phase upregulated genes encoding several virulence determinants and a homolog of *hfq*. Insertional inactivation of *hfq* altered the expression of ~16% of the *H. ducreyi* genes. Importantly, there were a significant overlap and an inverse correlation in the transcript levels of genes differentially expressed in the *hfq* inactivation mutant relative to its parent and the genes differentially expressed in stationary phase relative to mid-log phase in the parent. Inactivation of *hfq* downregulated genes in the *flp-tad* and *lspB-lspA2* operons, which encode several virulence determinants. To comply with FDA guidelines for human inoculation experiments, an unmarked *hfq* deletion mutant was constructed and was fully attenuated for virulence in humans. Inactivation or deletion of *hfq* downregulated Flp1 and impaired the ability of *H. ducreyi* to form microcolonies, downregulated DsrA and rendered *H. ducreyi* serum susceptible, and downregulated LspB and LspA2, which allow *H. ducreyi* to resist phagocytosis. We propose that, in the absence of an RpoS homolog, Hfq serves as a major contributor of *H. ducreyi* stationary-phase and virulence gene regulation. The contribution of Hfq to stationary-phase gene regulation may have broad implications for other organisms that lack an RpoS homolog.**

IMPORTANCE **Pathogenic bacteria encounter a wide range of stresses in their hosts, including nutrient limitation; the ability to sense and respond to such stresses is crucial for bacterial pathogens to successfully establish an infection. Gram-negative bacteria frequently utilize the alternative sigma factor RpoS to adapt to stresses and stationary phase. However, homologs of RpoS are absent in some bacterial pathogens, including *Haemophilus ducreyi*, which causes chancroid and facilitates the acquisition and transmission of HIV-1. Here, we provide evidence that, in the absence of an RpoS homolog, Hfq serves as a major contributor of stationary-phase gene regulation and that Hfq is required for *H. ducreyi* to infect humans. To our knowledge, this is the first study describing Hfq as a major contributor of stationary-phase gene regulation in bacteria and the requirement of Hfq for the virulence of a bacterial pathogen in humans.**

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Upon entry into stationary phase or under conditions of nutrient deprivation, bacteria exhibit global changes in gene expression that result in altered virulence and increased tolerance to stresses (1). In *Escherichia coli* and many other Gram-negative bacteria, this global change in gene expression is regulated in part by the stationary-phase sigma factor RpoS (2–4). However, several Gram-negative pathogens lack an obvious homolog of RpoS; it is unclear how gene expression is coordinated upon entry into stationary phase in these organisms.

Hfq, first identified as a host factor required for Qβ phage replication in *E. coli*, is a highly conserved, homohexameric RNA-binding protein (5). By preferentially binding to A/U-rich regions in regulatory small RNAs (sRNAs) and mRNAs, Hfq enhances sRNA-mRNA interactions, resulting in altered mRNA stability or translation (5). Hfq affects mRNA stability and translation by multiple mechanisms: (i) prior to interaction with their mRNA targets, Hfq may directly regulate the stability of sRNAs either by protecting them from degradation or by facilitating their degra-

dation; (ii) Hfq may induce degradation of sRNA-mRNA complexes; (iii) Hfq in association with an sRNA may either repress translation by sequestering the ribosome-binding site or activate translation by exposing the translation initiation region; and (iv) Hfq may directly regulate the stability of mRNA transcripts by stimulating their degradation (5).

Hfq controls a wide variety of pathogenesis-related phenotypes in many bacteria, including motility, quorum sensing, biofilm formation, host cell adherence, invasion and intracellular survival, resistance to antimicrobial peptides, multidrug resistance, persister cell formation, and virulence (6–11). Hfq also affects a number of stress- and stationary-phase-related phenotypes due to its ability to regulate RpoS; mutant strains that lack *hfq* are defective in RpoS-mediated stress responses and stationary-phase adaptation (10, 12). Even in bacteria that lack RpoS, there is compelling evidence that Hfq contributes to stress- and stationary-phase-related phenotypes. For example, a *Brucella abortus hfq* mutant is more sensitive to a variety of stresses in stationary phase, and a *Francisella novicida hfq* mutant exhibits increased cell density at the transition to stationary phase (11, 13). In organisms that lack an RpoS homolog, whether Hfq serves as a major contributor of stationary-phase gene regulation is currently unknown.

Haemophilus ducreyi is a Gram-negative, obligate human pathogen that causes chancroid. Chancroid is a sexually transmitted genital ulcer disease (GUD) that manifests as painful genital ulcers and regional lymphadenopathy. Chancroid is now rare in the United States; cases are generally associated with contact with commercial sex workers in areas of endemicity. Chancroid is a prevalent GUD in the resource-poor countries of Africa, Asia, and Latin America (14). Due to syndromic management of sexually transmitted infections and lack of surveillance programs, the global prevalence of chancroid is now unknown (15). Apart from causing morbidity as a GUD, chancroid also facilitates the acquisition and transmission of human immunodeficiency virus type 1 (HIV-1) (16). In addition to chancroid, *H. ducreyi* also causes a nonsexually transmitted chronic lower limb ulceration syndrome that is reported from the South Pacific (17–19).

H. ducreyi lacks any recognized environmental or animal reservoir; humans are the only known hosts for this organism. In the human host, *H. ducreyi* associates with macrophages and neutrophils in an abscess and primarily remains extracellular (20, 21). While the doubling time of *H. ducreyi* in nutrient-rich broth is approximately 2 h, the estimated minimal doubling time in the human skin is 16.5 h (22). Thus, *H. ducreyi* encounters a variety of stresses, likely including nutrient limitation *in vivo*. To combat these stresses and successfully establish infection in the human host, *H. ducreyi* likely modifies its gene expression, physiological state, and virulence traits. However, *H. ducreyi* lacks an obvious homolog of RpoS, which controls the general stress response and stationary-phase adaptation in other organisms; how *H. ducreyi* regulates its gene expression in response to stationary phase is unclear.

We previously reported a comparison of the transcriptome sequencing (RNA-Seq)-based transcriptomes of the parent strain 35000HP, an isogenic *cpxA* deletion mutant, and an isogenic *cpxR* deletion mutant grown to the mid-log, transition, and stationary phases of growth (23). In this study, we characterized the gene expression differences at different growth phases in the parent strain 35000HP using the previously reported data and explored the contribution of Hfq to *H. ducreyi* stationary-phase gene regulation and virulence in humans. We found that cells harvested from stationary phase had increased expression of genes encoding several virulence determinants as well as a homolog of *hfq*. There was an overlap and an inverse relationship in the expression patterns of genes affected by inactivation of *hfq* and genes differentially expressed in stationary phase relative to mid-log phase. Finally, an *hfq* deletion mutant was fully attenuated for virulence in humans; inactivation or deletion of *hfq* downregulated several *H. ducreyi* virulence determinants.

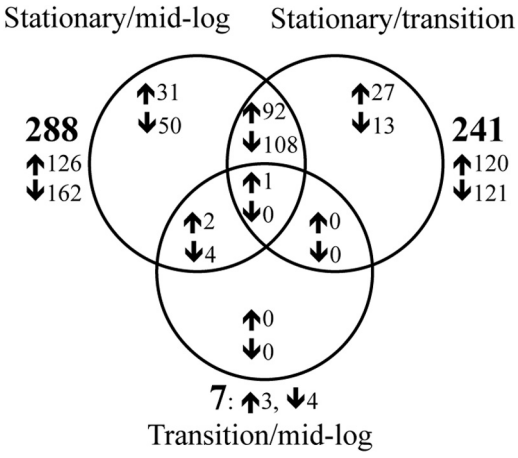

FIG 1 Venn diagram showing the overlap in differential gene expression across different growth phases. The up- and downregulated genes are indicated by up (↑) and down (↓) arrows, respectively. The total number of differentially regulated genes in different comparisons is indicated in bold outside the Venn diagram. Stationary/mid-log, genes differentially expressed in stationary phase relative to mid-log phase; Stationary/transition, genes differentially expressed in stationary phase relative to transition phase; Transition/mid-log, genes differentially expressed in transition phase relative to mid-log phase.

RESULTS

Entry into stationary phase upregulates known *H. ducreyi* virulence determinants and a homolog of *hfq*. To identify growth-phase-regulated genes in *H. ducreyi*, here we compared the transcriptomes of 35000HP at different growth phases using the previously reported RNA-Seq data (23). Four biological replicates were included for each growth phase, summing to a total of 12 samples. To identify genes differentially expressed at different growth phases, we calculated the fold change in the expression of genes in stationary phase compared to mid-log phase, stationary phase compared to transition phase, and transition phase compared to mid-log phase. We used a false-discovery rate (FDR) value of ≤0.1 and a 2-fold change as criteria for differential transcript expression as described previously (23). Comparison of transcriptomes in stationary phase to those in mid-log phase and of those in stationary phase to those in transition phase yielded 288 and 241 differentially expressed genes, respectively; approximately equal numbers of genes were up- and downregulated (Fig. 1). In general, the genes whose expression decreased in stationary phase relative to the mid-log and transition phases encode proteins involved in energy metabolism, biosynthesis, cell envelope homeostasis, transcription, and transport and binding (see Table S1 in the supplemental material). The genes whose expression increased in stationary phase relative to the mid-log and transition phases encode virulence determinants such as those in the

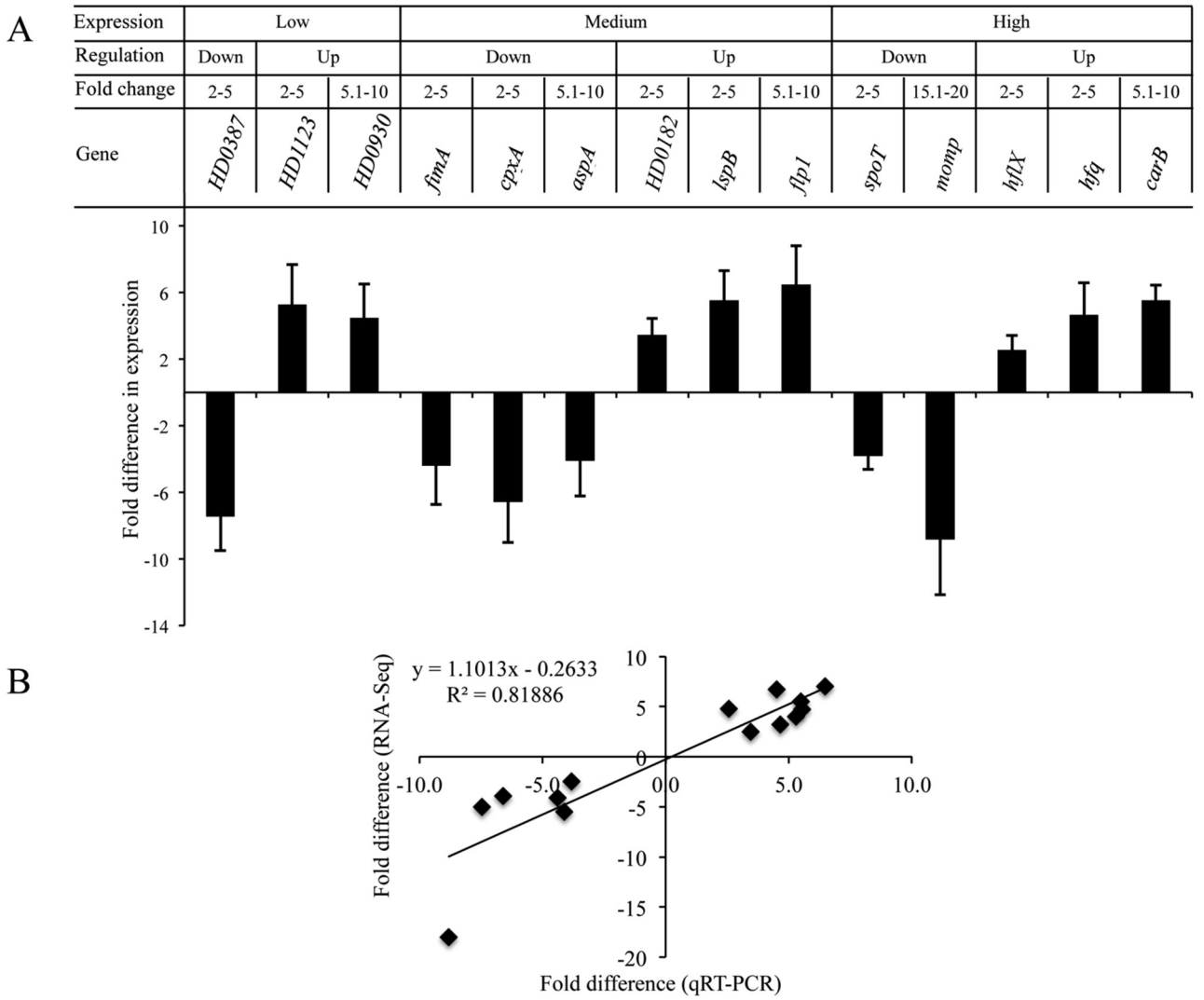

FIG 2 qRT-PCR validation of growth-phase-dependent differences in gene expression derived from RNA-Seq. (A) The fold change in the expression of target genes in stationary phase relative to mid-log phase. The expression levels of target genes were normalized to that of *dnaE*. The data represent the means ± SD of the results of four independent experiments. (B) Correlation between the fold changes obtained from qRT-PCR and RNA-Seq analysis. The diagonal line represents the power trendline ($R^2 = 0.82$).

flp-tad and *lspB-lspA2* operons, a large number of hypothetical proteins (~64% of the upregulated genes), and a homolog of the RNA binding chaperone Hfq (see Table S2). There were only 7 genes differentially expressed between the mid-log and transition phases, suggesting that these two growth phases have similar gene expression patterns.

Since the most profound differential regulation was noted in stationary phase relative to mid-log phase, we used this data set for quantitative reverse transcriptase PCR (qRT-PCR) validation. As described previously (23), the differentially expressed genes were grouped into 3 categories based on their expression levels (low, medium, and high); genes in each expression level were grouped into up- and downregulated targets, which were then further subgrouped based on their fold change ranges (2.0-fold to 5.0-fold, 5.1-fold to 10.0-fold, 10.1-fold to 15.0-fold, and 15.1-fold to 20.0-fold). Representative genes were selected arbitrarily from each category; a total of 14 genes were selected for qRT-PCR validation. qRT-PCR analysis confirmed the differential expression of 14/14 genes identified by RNA-Seq (Fig. 2). In general, the fold changes derived from RNA-Seq were in good agreement with those obtained from qRT-PCR (Fig. 2) ($R^2 = 0.82$). Thus, qRT-PCR analysis confirmed that genes encoding known virulence determinants such as those in the *flp-tad* and *lspB-lspA2* operons and *hfq* were upregulated in stationary phase.

Hfq is a major contributor of gene regulation in *H. ducreyi*. Since *hfq* transcripts were upregulated in stationary phase relative to mid-log phase and Hfq contributes to stationary-phase survival and posttranscriptional regulation of gene expression in other bacteria (10, 13), we compared the global expression profile of the 35000HP parent strain with that of the *hfq* insertional inactivation mutant (35000HP*hfq*::*cat*) using DNA microarray analysis. Inactivation of the *hfq* gene resulted in the differential regulation of 15.8% (289 open reading frames [ORFs]) of the predicted ORFs in the *H. ducreyi* genome. A total of 191 genes were significantly

TABLE 1 Inverse relationship in the expression patterns between the genes differentially expressed in 35000HP*hfq*::*cat* relative to 35000HP and the 35000HP genes differentially expressed in stationary phase relative to mid-log phase

Locus tag[a]	Gene	Description or homolog	Fold change	
			35000HP-Δ*hfq*::*cat*/35000HP[b]	35000HP-stationary/mid-log[c]
HD1435	*ompP2B*	Outer membrane protein P2 homolog	9.61	−5.0
HD1591		Conserved hypothetical protein	7.61	—[d]
HD1434		Hypothetical protein	6.99	−4.4
HD0384	*nqrF*	Na^+-translocating NADH-ubiquinone oxidoreductase, subunit F	6.74	−4.2
HD1590	*deaD*	Cold-shock DEAD box protein-A	6.50	−3.1
HD0382	*nqrD*	Na^+-translocating NADH-ubiquinone oxidoreductase, subunit D	6.41	−3.7
HD0647		Conserved hypothetical protein	6.27	−2.4
HD0386	*apbE*	Thiamine biosynthesis lipoprotein	6.22	−4.1
HD0357		Probable carbon starvation protein A	5.84	−4.2
HD0383	*nqrE*	Na^+-translocating NADH-ubiquinone oxidoreductase, subunit E	5.75	−3.9
HD1512		Acriflavine resistance protein	5.57	−3.4
HD0381	*nqrC*	Na^+-translocating NADH-ubiquinone oxidoreductase, subunit C	5.52	−4.6
HD0045	*momP*	Major outer membrane protein	5.23	−17.9
HD0710	*mutM*	Formamidopyrimidine-DNA glycosylase	5.22	−2.6
HD0648	*tnaB*	Tryptophan-specific transport protein	4.64	−2.5
HD1470	*cpxA*	Sensor kinase CpxA	4.48	−3.9
HD0564	*aspA*	Aspartate ammonia-lyase	4.45	−5.6
HD1109		Putative oxalate/formate antiporter	4.43	—
HD0387		Conserved hypothetical protein	4.41	—
HD1622		Conserved hypothetical protein	4.39	—
HD1163	*ribAB*	Riboflavin biosynthesis protein RibA	4.38	−3.5
HD0195		Hypothetical protein	4.35	−4.1
HD1343		Hypothetical protein	4.29	—
HD0380	*nqrB*	NADH dehydrogenase	4.26	−4.3
HD1162	*ribE*	Riboflavin synthase, alpha chain	4.11	−3.7
HD1357		Conserved possible translation initiation factor	4.11	−2.9
HD0282	*fimB*	Possible fimbrial structural subunit	3.77	—
HD0766	*manZ*	Mannose-specific phosphotransferase system IID component	3.66	−4.8
HD0646		Conserved hypothetical protein	3.59	—
HD0709	*brnQ*	Branched-chain amino acid carrier protein	3.59	−2.6
HD1624	*aceF*	Dihydrolipoamide acetyltransferase	3.56	−2.1
HD1471		Conserved hypothetical protein	3.54	−3.5
HD0889	*deoD*	Purine nucleoside phosphorylase	3.46	−3.3
HD0767	*manY*	Mannose-specific phosphotransferase system IIC component	3.40	−3.6
HD0876		Conserved probable RNase	3.37	—
HD1143	*sdaC*	Serine transporter	3.37	−2.5
HD1356	*pyrF*	Orotidine 5′-phosphate decarboxylase	3.37	−3.3
HD1312	*flp1*	*flp* operon protein Flp1	−3.70	7.0
HD0740	*hflX*	GTP-binding protein HflX	−3.70	4.8
HD1311	*flp2*	*flp* operon protein Flp2	−3.85	7.4
HD1503	*guaB*	Inosine-5-monophosphate dehydrogenase	−4.17	—
HD1310	*flp3*	*flp* operon protein Flp3	−4.35	7.4
HD0997		Hypothetical protein	−4.55	3.0
HD0805		Conserved hypothetical protein	−5.26	—
HD1433	*ompP2A*	Outer membrane protein P2 homolog	−7.14	2.2
HD0998	*uraA*	Uracil permease	−10.00	3.6
HD1985		Possible DNA transformation protein	−11.11	2.4
HD0232	*arcB1*	Ornithine carbamoyltransferase	−12.50	2.4
HD0233	*carB*	Carbamoyl-phosphate synthase, large subunit	−16.67	5.5
HD0235	*carA*	Carbamoyl-phosphate synthase, small subunit	−20.00	9.3

[a] This table includes only the top 50 genes differentially expressed in the *hfq* inactivation mutant relative to its parent and their inverse relationship in expression patterns to the genes differentially expressed in stationary phase relative to mid-log phase.
[b] 35000HPΔ*hfq*::*cat*/35000HP, fold change in transcript levels in 35000HPΔ*hfq*::*cat* relative to 35000HP.
[c] 35000HP-stationary/mid-log, fold change in transcript levels in stationary phase relative to mid-log phase.
[d] —, no difference in expression was found.

upregulated, and 98 genes were significantly downregulated ($P < 0.05$) (see Table S3 in the supplemental material). A list of the top 50 genes that were differentially regulated when *hfq* was inactivated, not including those ORFs annotated as encoding hypothetical proteins, is shown in Table 1. Genes encoding several different cellular processes were affected by inactivation of *hfq* (see Fig. S1

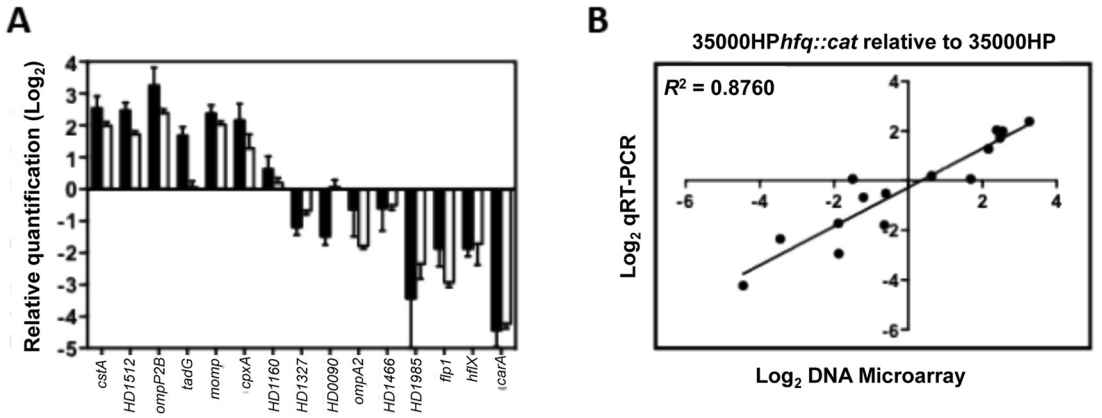

FIG 3 Relative expression levels of selected *H. ducreyi* genes in 35000HP*hfq*::cat. (A) Expression levels of 15 selected genes in 35000HP*hfq*::cat compared to 35000HP were measured by DNA microarray (black bars) or real-time RT-PCR (white bars) as described in Materials and Methods. These data are from a representative experiment. (B) Correlation between $\log_2$ values obtained by DNA microarray analysis and qRT-PCR analysis. The diagonal line represents the power trendline ($R^2 = 0.8760$).

in the supplemental material). Hfq positively affected the transcript levels of several virulence factors, including components of the *flp-tad* operon (24, 25) and the two-partner secretion system encoded by *lspB, lspA2*, and *lspA1* (26–28). qRT-PCR performed on a subset of 15 genes validated the DNA microarray data (Fig. 3) ($R^2 = 0.876$). Taken together, these data suggest that Hfq is a major contributor of *H. ducreyi* gene regulation.

Hfq is a major contributor of *H. ducreyi* stationary-phase gene regulation. Given that *H. ducreyi hfq* was upregulated in stationary phase relative to mid-log phase, we examined whether there was an overlap between the genes altered by inactivation of *hfq* and the genes differentially expressed in stationary phase relative to mid-log phase. Since transcripts encoding ribosomal proteins were depleted by RNA-Seq, 17 of the 289 genes directly or indirectly regulated by Hfq were excluded from this analysis; since the microarray analysis was done on the *hfq* inactivation mutant, *hfq* was excluded from the 288 genes affected by the transition from mid-log to stationary phase. Our results showed that there was a significant overlap ($n = 118$ genes) in the genes differentially expressed in the *hfq* inactivation mutant relative to its parent and the genes differentially expressed in stationary phase relative to mid-log phase (chi-square test, $P < 2.2e\text{-}16$) (Fig. 4A and Table 1; see also Table S4 in the supplemental material). Comparison of the fold changes of the overlapping genes showed that the fold changes of genes altered by inactivation of *hfq* inversely correlated with the fold changes of genes differentially expressed in stationary phase relative to mid-log phase ($R^2 = 0.8$) (Fig. 4B and Table 1; see also Table S4). There was also an overlap in the expression patterns of several genes encoding known virulence determinants, including the components of the *flp-tad* operon (*flp1*, *flp2*, *flp3*, and *tadA*) and *lspB-lspA2* operon (*lspB*) (Table 1; see also Table S4). These data support the hypothesis that Hfq serves as a major contributor of *H. ducreyi* stationary-phase and virulence gene regulation.

Hfq is required for pustule formation in humans. Inactivation of *hfq* altered the expression of several genes encoding virulence determinants, suggesting that Hfq might contribute to *H. ducreyi* infection in humans. Both by microarray and qRT-PCR analyses, the expression of *hflX*, the gene downstream of *hfq*, was reduced approximately 3.5-fold in the inactivation mutant. Given

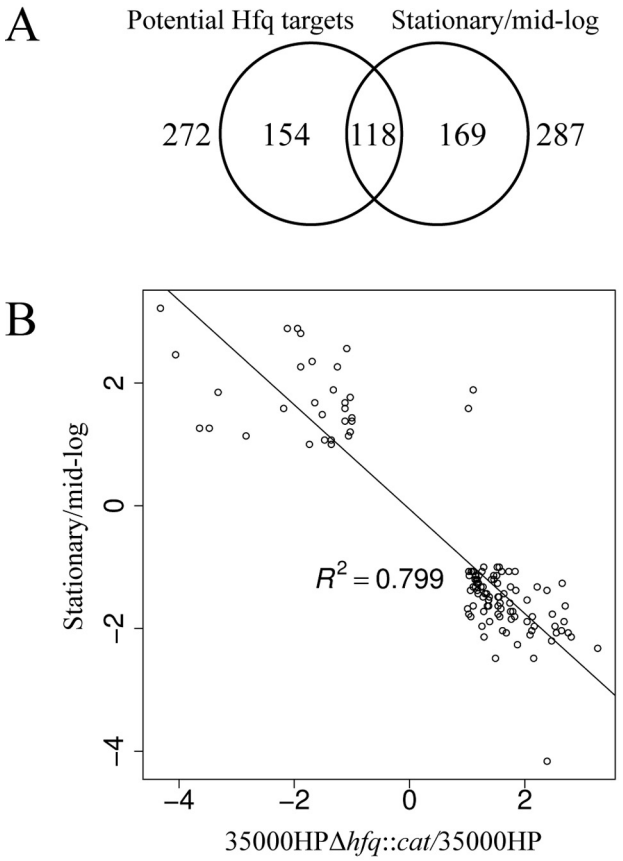

FIG 4 Comparison of the expression patterns of the genes altered by inactivation of *hfq* to those of the genes differentially expressed in stationary phase relative to mid-log phase. (A) Venn diagram showing the overlap in the genes altered by inactivation of *hfq* and the genes differentially expressed in stationary phase relative to mid-log phase. The total number of genes in each comparison is indicated in bold outside the Venn diagram. Potential Hfq targets, genes differentially expressed in 35000HPΔ*hfq*::*cat* relative to 35000HP; Stationary/mid-log, genes differentially expressed in stationary phase relative to mid-log phase. The significance of the overlap was tested using the chi-square test ($P < 2.2e\text{-}16$). (B) Inverse correlation between the fold changes of the genes altered by inactivation of *hfq* and those of the genes differentially expressed in stationary phase relative to mid-log phase. Only genes that overlapped in their expression patterns as shown in panel A were used for the correlation analysis.

TABLE 2 Response to inoculation with live *H. ducreyi* strains

Volunteer (sex)[a]	Observation period (days)	Strain[b]	Dose(s) (CFU)[c]	No. of initial papules	No. of pustules at endpoint
420 (M)	9	P	109	3	3
		M	29–116	2	0
423 (M)	13	P	109	3	1
		M	29–116	3	0
428 (M)	7	P	87	3	0
		M	52–209	2	0
429 (F)	7	P	87	3	3
		M	52–209	3	0
430 (M)	8	P	87	2	0
		M	52–209	3	0

[a] Volunteers 420 and 423 were inoculated in one group; volunteers 428, 429, and 430 were inoculated in another group. M, male; F, female.
[b] P, parent (35000HP); M, mutant (35000HPΔ*hfq*).
[c] Data represent the doses inoculated at 3 sites, except 29–116 (one dose each of 29, 58, and 116 CFU) and 52–209 (one dose each of 52, 104, and 209 CFU).

that the U.S. Food and Drug Administration prefers use of unmarked mutants for testing in humans and that we did not know if the downregulation of *hflX* was due to regulation by Hfq or to a polar effect of the insertion, we constructed an unmarked deletion mutant of *hfq* (35000HPΔ*hfq*) for mutant-parent trials in human volunteers in strain 35000HP using recombineering methodology (29). Sequence analysis confirmed the expected deletion with no secondary mutations in the flanking regions. By qRT-PCR analysis, there was no difference in the expression levels of *hflX* between the deletion mutant and the parent, suggesting that Hfq does not regulate *hflX* expression. 35000HP**Δ***hfq* grew at the same rate as 35000HP to mid-log phase but lagged slightly in the late log and stationary phases (see Fig. S2 in the supplemental material), suggesting that deletion of *hfq* affected the growth of *H. ducreyi* in broth culture only slightly. Except for downregulation of DsrA, the outer membrane proteins (OMPs) and the lipooligosaccharide profiles of the 2 strains were identical (data not shown). Thus, the unmarked *hfq* deletion mutant met the phenotypic criteria required by our protocol for mutant-parent trials in humans.

To examine whether Hfq is required for virulence in humans, we infected groups of volunteers with the *hfq* deletion mutant and its parent in escalating dose-ranging studies. In this model, papule formation signifies initiation of infection, while pustule formation signifies disease progression. In one group, two volunteers (420 and 423) formed pustules at 4 of 6 parent sites that were inoculated with 109 CFU of the parent and at 0 of 6 mutant sites that were inoculated with 29, 58, or 116 CFU of the mutant (Table 2). These results suggested that the *hfq* deletion mutant was attenuated for virulence. In another group, three volunteers (428, 429, and 430) were inoculated at 3 sites with 87 CFU of the parent and at 3 sites with 52, 104, and 209 CFU of the mutant. Pustules formed at 3 of 9 parent sites and at 0 of 9 mutant sites (Table 2). Cumulative results from the two groups of subjects showed that papule formation rate was 93.3% (95% confidence interval [CI], 81.7% to 99.9%) at 15 parent sites and 86.7% (95% CI, 72.4% to 99.9%) at 15 mutant sites ($P = 0.55$) (Table 2). After 24 h of infection, the mean parent papule size (16.5 ± 10.4 mm^2) was significantly larger than mutant papule size (6.8 ± 6.9 mm^2) ($P = 0.003$). The pustule formation rates were 46.7% (95% CI, 7.0% to 86.3%) at 15 parent sites and 0% (95% CI, 0.0% to 0.45%) at 15 mutant sites ($P = 0.011$) (Table 2). At least one positive surface culture, defined as a culture that yielded at least one colony of *H. ducreyi*, was obtained during follow-up visits from 6.7% of the parent-inoculated and 0% of the mutant-inoculated sites. Thus, the *hfq* deletion mutant was fully attenuated for virulence in humans (30).

Colonies from subcultures of the parent ($n = 71$) and mutant ($n = 72$) inocula used to infect volunteers were tested for the presence of *hfq* and *dnaE* sequences by colony hybridization. The *dnaE* probe hybridized to all colonies from both the parent and mutant inocula, while the *hfq* probe hybridized only to colonies from parent inocula. One biopsy specimen was cultured from a parent site; both probes hybridized to all colonies tested ($n = 35$). Thus, there was no evidence of cross-contamination between mutant and parent inocula for the five subjects included in the trial.

Hfq contributes to positive regulation of important *H. ducreyi* virulence determinants. Given that the *hfq* deletion mutant was compromised for virulence *in vivo* (Table 2), we sought to determine if Hfq contributes to the regulation of known *H. ducreyi* virulence determinants. Inactivation of the *hfq* gene in *H. ducreyi* 35000HP resulted in decreased synthesis of LspB, LspA2, DsrA, and Flp1 but not of LspA1 (Fig. 5, lanes 1 and 2); LspA2, DsrA, and Flp1 are absolutely required for pustule formation in humans (30, 31). LspB is involved in the secretion of LspA1 and LspA2 proteins that allow *H. ducreyi* to avoid phagocytosis (32), whereas DsrA is an autotransporter protein involved in resistance to serum killing (33). The Flp proteins are necessary for *H. ducreyi* microcolony formation *in vitro*; the ability of *H. ducreyi* to form microcolonies *in vitro* is correlated with virulence in humans (24, 25, 31). The decreased synthesis of these proteins in the *hfq* inactivation mutant was restored to parental levels by complementation with the *hfq* gene *in trans* (Fig. 5, lane 3). These data suggest that Hfq contributes to the positive regulation of several known *H. ducreyi* virulence determinants.

Hfq contributes to *H. ducreyi* microcolony formation. Western blot analysis (Fig. 5) indicated that inactivation of *hfq* downregulated the synthesis of Flp proteins, suggesting that the *H. ducreyi hfq* inactivation mutant might be deficient in microcolony formation. The *hfq* inactivation mutant formed bacterial cell aggregates, but these clusters of bacterial cells were much less compact than those formed by the parent strain (Fig. 6). This deficiency in microcolony formation by the *hfq* inactivation mutant could be corrected by complementation (Fig. 6). These data suggest that Hfq is important for *H. ducreyi* microcolony formation.

Deletion of *hfq* results in an intermediate serum resistance phenotype. Similarly to the inactivation mutant, the *hfq* deletion mutant synthesized less DsrA than 35000HP as assessed by West-

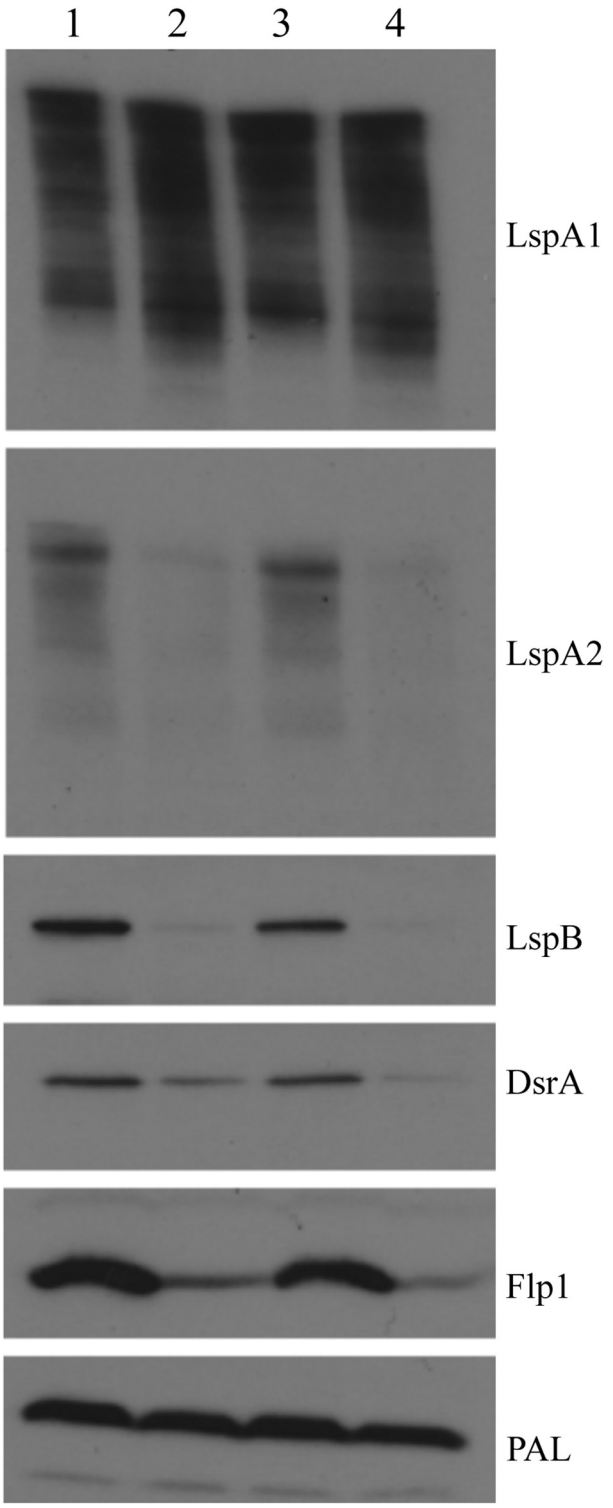

FIG 5 Inactivation of the *H. ducreyi hfq* gene has a positive effect on the synthesis of known virulence determinants. Data represent the results of Western blot analysis of whole-cell lysates from 35000HP (lane 1), 35000HP*hfq*::cat (lane 2), 35000HP*hfq*::*cat*(pML129) (lane 3), and 35000HP*hfq*::*cat*(pACYC177) (lane 4) probed with LspA1 MAb 40A4, LspA2 MAb 1H9, an LspB polyclonal antibody, a DsrA polyclonal antibody, or a Flp1 polyclonal antibody. The LspA1 and LspA2 proteins do not have discrete banding patterns in Western blots but instead form smears (27). PAL MAb 3B9 was used to confirm equivalent loading among lanes.

ern blot analysis (Fig. 5) and by OMP profiles (data not shown). Thus, we compared the survival rates of strain 35000HP, strain 35000HP**Δ***hfq*, and a previously constructed isogenic *dsrA* mutant (33) in 50% normal human serum (NHS). In these assays, the *hfq* deletion mutant survived at significantly lower levels (mean survival ± standard deviation [SD], 46% ± 17%) than did the parent (87% ± 19%) and at significantly higher levels than did the *dsrA* mutant (7% ± 4%) (Fig. 7A). We next compared the survival rates of strains 35000HP(pACYC177), 35000HP**Δ***hfq*(pACYC177), 35000HP**Δ***hfq*(pML129), and 35000HP**Δ***dsrA*(pACYC177) in 50% NHS. In these experiments, the mean percentages of survival ± the SD were 54% ± 8% for the parent, 40% ± 19% for the *hfq* deletion mutant, 80% ± 9% for the complemented strain, and 6% ± 2% for the *dsrA* mutant (Fig. 7B). In these experiments, the complemented strain survived at significantly higher levels than the *hfq* deletion mutant. All together, these data suggest that Hfq contributes to *H. ducreyi* serum resistance.

Structural biology of the *H. ducreyi* Hfq protein. Like Hfq from other organisms (10), *H. ducreyi* Hfq contributed to gene regulation, including the regulation of the genes that encode virulence determinants. Therefore, we sought to determine if *H. ducreyi* Hfq binds to RNA similarly to Hfq in other organisms. To this end, we performed structural analysis of the *H. ducreyi* Hfq protein. To gain insights into the structural properties of the *H. ducreyi* Hfq protein, the Protein Data Bank (PDB) was queried for similar sequences using a hidden-Markov approach (34). Several bacterial proteins with the Sm fold, a highly conserved bipartite sequence motif that mediates RNA binding and protein-protein interactions, were returned; the best match was to the Hfq protein from *Herbaspirillum seropedicae* (35). This protein shared about 70% amino acid sequence identity with *H. ducreyi* Hfq, and the reported probability of the match was 100%. These indicators of sequence similarity suggested that the structure of the *H. ducreyi* Hfq protein would be very similar to that of the *H. seropedicae* molecule. Consequently, using *H. seropedicae* Hfq as a template, MODELLER (36) was used to build a hypothetical model of the tertiary structure of the *H. ducreyi* Hfq protein (see Fig. S3 in the supplemental material). The model predicted that *H. ducreyi* Hfq likely adopts the classical Hfq fold (5), with an amino-terminal α-helix followed by 5 β-strands. These strands form two β-sheets that are arranged in a "squat barrel." The second β-strand in such structures adopts a highly curved configuration that allows it to participate in both sheets. The C termini of Hfq proteins tend to vary; therefore, this portion of the protein was removed from the model. The quaternary structure of other Hfq proteins is homohexameric (37), and the *H. ducreyi* protein very likely conforms to this expectation. This discoid structure is described as having two faces: the "proximal" face, which is close to the amino termini of the monomers, and the "distal" face, which is opposite the proximal face.

Several structures are known for other Hfq proteins bound to RNAs. For the purpose of examining the likely modes of RNA binding to *H. ducreyi* Hfq, we focused on two RNA-containing structures: *E. coli* Hfq bound to poly(A) (38) and Hfq bound to AU_6A (39). In the *E. coli* Hfq/poly(A) structure, the RNA is bound to the distal face of the hexamer, with some bases oriented away from the protein and some penetrating into invaginations in its surface. The *E. coli* Hfq/AU6A structure features RNA bound to the proximal face, with some bases making contacts with amino acid side chains and others facing away from the protein. When

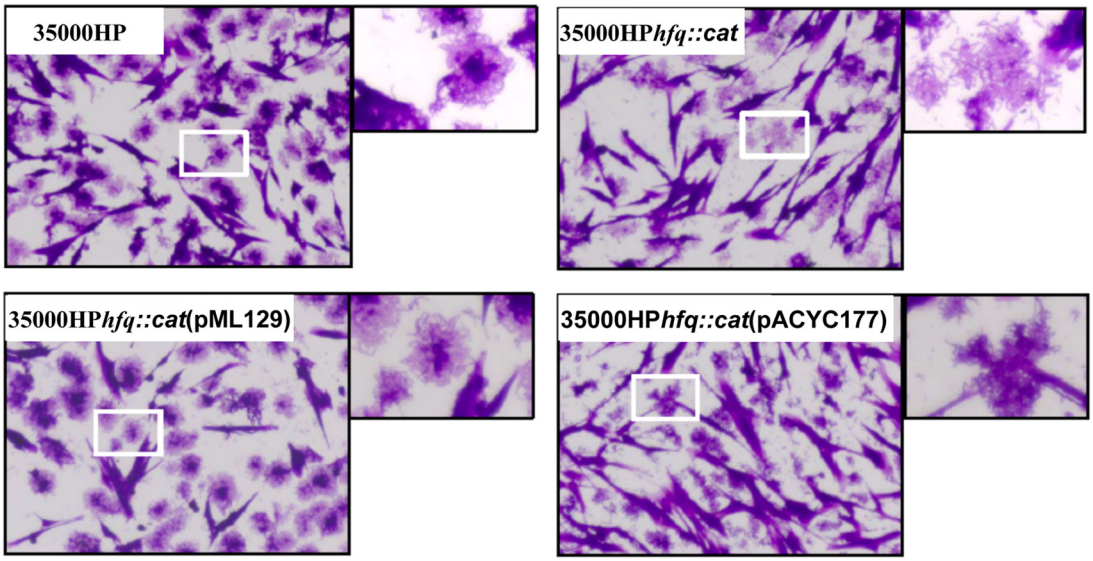

FIG 6 *H. ducreyi* microcolony formation assay. The relative abilities of 35000HP, 35000HP*hfq*::*cat*, 35000HP*hfq*::*cat*(pML129), and 35000HP*hfq*::*cat*(pACYC177) to form microcolonies was tested by incubating these strains with Hs27 fibroblasts. The larger pictures were taken at a ×14 magnification. The contents of the white boxes are shown to the right of each picture at a ×40 magnification. Results of a representative experiment are shown. Note that the *hfq* mutant formed bacterial cell aggregates but that these clusters of bacterial cells were much less compact than those formed by the parent and complemented strains.

three-dimensional alignments of these structures with the model of the *H. ducreyi* Hfq protein were examined, no conflicts were found that would preclude either of these RNA-binding modes. Thus, the *H. ducreyi* Hfq protein is likely to bind RNA similarly to *E. coli* Hfq.

DISCUSSION

To survive in the host environment, *H. ducreyi* likely senses and responds to stresses by altering its gene expression. Here, we sought to define the global gene expression patterns in *H. ducreyi* at distinct growth phases, with the goal of understanding how this organism adapts to stationary phase in the absence of RpoS and regulates its virulence determinants in response to alterations in growth. We showed that cells in stationary phase had a broad transcriptional response and upregulation of several virulence determinants and a homolog of *hfq*. We provided evidence that Hfq likely contributes to regulation of gene expression in stationary phase. We also showed that an *hfq* mutant exhibits reduced expression of genes encoding several virulence determinants and is attenuated for virulence in human volunteers. Taken together, these data suggest an important role for Hfq in controlling *H. ducreyi* stationary-phase gene expression and virulence.

In *E. coli*, entry into stationary phase is accompanied by distinct changes in gene expression that result in altered growth rate and increased resistance to a variety of stresses (1). In general, entry into stationary phase downregulates many genes involved in transcription, translation, biosynthesis of macromolecules, and energy metabolism; this is consistent with the idea that stationary-phase organisms exhibit metabolic downshift that is accompanied by reduced cell division and no net bacterial growth. A large number of genes involved in stress adaptation are induced upon entry into stationary phase; the majority of these genes are regulated by the alternative sigma factor RpoS (40). Stationary-phase *H. ducreyi* had a distinct transcriptional profile compared to organisms grown to mid-log and transition phases. Similarly to organisms that contain RpoS (41), cells harvested from stationary phase had decreased expression of homologs of genes involved in transcription, translation, biosynthesis of macromolecules, and energy metabolism. Stationary-phase cells had increased expression of homologs of genes involved in arginine biosynthesis, cell envelope homeostasis, and regulation and a large number of genes encoding hypothetical proteins. Thus, *H. ducreyi* regulates its gene expression in stationary phase similarly to organisms that contain RpoS.

In many Gram negative pathogens, entry into stationary phase is accompanied by upregulation of genes encoding virulence determinants (3, 4). Stationary-phase *H. ducreyi* had increased expression of genes encoding components of the *flp-tad* operon and a component of the two-partner secretion system, *lspB*. The Flp proteins are necessary for *H. ducreyi* microcolony formation; mutants that do not synthesize Flp proteins are fully attenuated for virulence in humans (24, 25, 31). LspB is required for secretion of LspA1 and LspA2 proteins, which are involved in the inhibition of phagocytosis; an *lspA1 lspA2* double mutant is also fully attenuated for virulence in humans (28, 32). Thus, stationary-phase cells had increased expression of genes encoding several known *H. ducreyi* virulence determinants.

H. ducreyi harvested from stationary phase also had increased expression of a homolog of the RNA-binding chaperone Hfq. The fact that *hfq* was upregulated in stationary phase in *H. ducreyi* led us to explore the potential contribution of Hfq to regulation of gene expression in *H. ducreyi*. To this end, we constructed an insertional inactivation mutant of *hfq*; sequence analysis indicated the presence of an in-frame insertion in the *hfq* ORF. Microarray analysis showed that inactivation of *hfq* led to differential expression of ~16% of the *H. ducreyi* open reading frames. Despite the presence of an in-frame insertion in the *hfq* ORF, both microarray and qRT-PCR analyses showed that the downstream gene, *hflX*, was downregulated in the *hfq* inactivation mutant. HflX is a GTPase that coprecipitates with the 50S ribosomal subunit and is

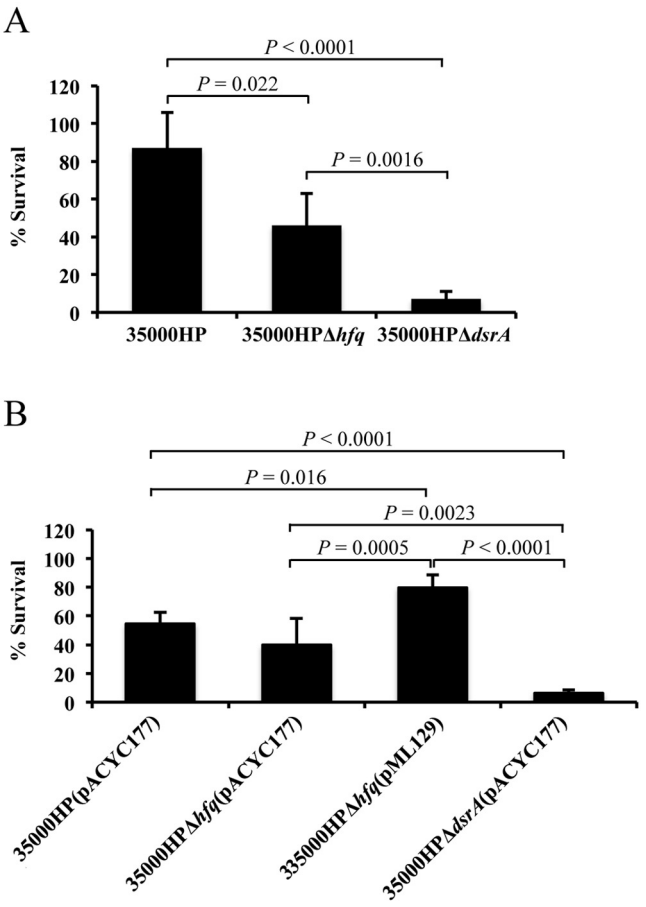

FIG 7 Serum bactericidal assays. (A) Percent survival of 35000HP, 35000HPΔ*hfq*, and the *dsrA* mutant in 50% NHS, calculated as follows: (geometric mean CFU in NHS/geometric mean CFU in heat-inactivated NHS) × 100. (B) Percent survival of 35000HP(pACYC177), 35000HPΔ*hfq*(pACYC177), 35000HPΔ*hfq*(pML129), and 35000HPΔ*dsrA*(pACYC177) in 50% NHS, calculated as follows: (geometric mean CFU in NHS/geometric mean CFU in heat-inactivated NHS) × 100. Values are means ± SD of the results of 5 independent experiments.

hypothesized to have a role in translation (42). Given that the function of HflX is unknown, whether downregulation of *hflX* in the *hfq* inactivation mutant contributes to altered gene expression in this mutant is unclear. However, the altered synthesis of virulence determinants and their associated phenotypes in the *hfq* inactivation mutant were restored to parental levels by complementation, suggesting that these phenotypes were due to inactivation of *hfq*.

Microarray analysis also showed that inactivation of *hfq* altered the expression of genes encoding several virulence determinants (30, 31). Therefore, we hypothesized that Hfq would be required for virulence in humans. Given that inactivation of *hfq* decreased the expression of *hflX* and that the FDA prefers the use of unmarked mutants in human volunteers, we generated an unmarked deletion mutant of *hfq* for the human inoculation experiments; *hflX* expression was unchanged in the deletion mutant relative to the parent. The *hfq* deletion mutant caused papules that were significantly smaller than those caused by its parent and formed no pustules. Similarly to the insertion mutant, the deletion mutant had decreased synthesis of DsrA; deletion of *hfq* decreased the ability of *H. ducreyi* to resist human serum. Inactivation of *hfq* reduced the ability of *H. ducreyi* to form microcolonies *in vitro*; Flp1, a determinant of microcolony formation, was also decreased in the *hfq* inactivation mutant. In addition, inactivation of *hfq* decreased the synthesis of LspB and LspA2, which are involved in resisting phagocytosis. Although we did not evaluate either *hfq* mutant for resistance to phagocytosis, it is highly likely that both mutants would also be impaired in this regard. Thus, the attenuation of the *hfq* deletion mutant in the human challenge model is likely due to downregulation of several virulence determinants.

Inactivation of *hfq* affected the expression levels of several genes that are involved in gene regulation. For example, the transcript levels of *cpxRA* were upregulated in the *hfq* inactivation mutant. Activation of the CpxRA system is dependent on the levels of phosphorylated CpxR, and activation of CpxR renders *H. ducreyi* totally avirulent in humans (29). Inactivation of *hfq* also increased the expression of *pta*, which is involved in the synthesis of acetyl phosphate (AcP), but did not affect the expression of *ackA*, which is involved in the degradation of AcP. CpxR can accept phosphoryl groups from AcP, causing the Cpx system to be activated (43). Whether the increase in *cpxRA* and *pta* transcript levels results in accumulation of phosphorylated CpxR in the *hfq* mutant is unknown. However, comparing the genes altered by inactivation of *hfq* to those regulated by activation of CpxR in the *cpxA* deletion mutant (23), approximately 20% of the genes altered by inactivation of *hfq* overlapped and were positively correlated with the genes regulated by activated CpxR (data not shown; chi-square test, $P = 9.6e\text{-}08$). Given that activation of the Cpx system attenuates the virulence of *H. ducreyi* in humans, these data suggest that the Cpx system may be activated in the *hfq* mutant and that such activation may in part be responsible for the attenuation of the *hfq* mutant.

In *E. coli* and many other Gram-negative bacteria, Hfq positively regulates RpoS; mutant strains that lack *hfq* are compromised in RpoS-dependent stationary-phase adaptation (44). However, several Gram-negative pathogens lack an obvious homolog of *rpoS* but contain a homolog of *hfq*, including *Brucella abortus*, *Neisseria gonorrhoeae*, *Francisella novicida*, *Actinobacillus pleuropneumoniae*, *Haemophilus influenzae*, *Pasteurella multocida*, *Leptospira interrogans*, *Bartonella bacilliformis*, and *Bordetella pertussis* (11, 13, 45–47). A *Brucella abortus hfq* mutant is compromised in its ability to survive under conditions of stresses in stationary phase, and a *Francisella novicida hfq* mutant shows increased cell density at the transition to stationary phase (11, 13). Similarly, the *H. ducreyi hfq* mutant slightly lagged in growth in the late log phase and stationary phase. There was a significant inverse relationship in the expression patterns of the genes altered by inactivation of *hfq* and the genes differentially expressed in stationary phase relative to mid-log phase. Thus, in the absence of an RpoS homolog, Hfq likely serves as a major contributor of stationary-phase gene regulation in *H. ducreyi*.

Hfq regulated only a subset (41%) of the *H. ducreyi* genes differentially expressed in stationary phase relative to mid-log phase. It is unknown how the remainder (59%) of the differentially expressed genes are regulated. Carbon storage regulator A (CsrA) and guanosine tetraphosphate (ppGpp) are important posttranscriptional and transcriptional regulators of stationary-phase gene expression, respectively (1, 48). An *H. ducreyi csrA* mutant is partially attenuated for virulence in humans and exhibits more pronounced stress survival phenotypes in stationary phase (49). Pre-

TABLE 3 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli DH5α + HB101	Strains used for general cloning procedures	Invitrogen
E. coli DY380	DH10B derivative containing a defective λ prophage in which the *red*, *bet*, and *gam* genes are controlled by the temperature-sensitive λ*cI*857 repressor	57
H. ducreyi 35000HP	Human passaged variant of strain 35000	58
H. ducreyi 35000HP*hfq*::*cat*	35000HP with a chloramphenicol resistance cassette inserted into the *hfq* gene	This study
H. ducreyi 35000HPΔ*hfq*	35000HP*hfq* unmarked, in-frame deletion mutant	This study
H. ducreyi FX517	35000*dsrA*::*cat* insertion mutant	33
Plasmids		
pCR2.1	Cloning vector	Invitrogen
pML104	pCR2.1 carrying the wild-type 35000HP *hfq* gene and flanking DNA	This study
pML121	pCR2.1 carrying the *cat* promoter from ΔE*cat* and the *cat* cassette from pSL1 flanked with SmaI sites	This study
pML120	pML104 with a chloramphenicol resistance cassette inserted into the *hfq* gene	This study
pKF10	pCR-XL-TOPO containing the *hfq*-coding region along with 0.5-kb flanking regions	This study
pRSM2832	Plasmid containing spectinomycin resistance cassette flanked by the FRT sites	29
pKF11	*hfq* replaced with spectinomycin resistance cassette in pCR-XL-TOPO	This study
pRSM2072	*H. ducreyi* suicide vector	59
pKF12	*hfq* replaced with spectinomycin resistance cassette in pRSM2072	This study
pACYC177	Cloning vector	New England Biolabs
pML129	pACYC177 carrying the wild-type 35000HP *hfq* gene	This study

liminary studies in our laboratory indicate that *H. ducreyi* synthesizes ppGpp, and a ppGpp null mutant is attenuated for virulence in humans (C. Holley, W. Li, K. R. Fortney, D. M. Janowicz, S. Ellinger, B. Zwickl, B. P. Katz, and S. M. Spinola, unpublished data). Thus, in addition to Hfq, CsrA and ppGpp likely play a role in stationary-phase gene regulation and survival in *H. ducreyi*.

In *E. coli* and other bacteria, Hfq serves as a chaperone that facilitates the interaction of sRNAs with their mRNA targets (5). Structure analysis of the *H. ducreyi* Hfq protein revealed that Hfq might bind RNA similarly to *E. coli* Hfq. By RNA-Seq analysis, we identified 10 putative sRNAs, 7 of which are homologous to sRNAs in other bacteria (transfer-mRNA [tmRNA], 6S RNA, GcvB, bacterial small RNA signal recognition particle [SRP], RNase P_bact_a, flavin mononucleotide [FMN] riboswitch, and a lysine riboswitch) and 3 of which appear to be unique to *H. ducreyi* (data not shown). However, the Hfq dependence of these putative sRNAs and their contribution to *H. ducreyi* pathogenesis are currently unknown.

By facilitating interaction of sRNAs with their mRNA targets, Hfq affects either the stability or translation initiation of its targets. Inactivation of *hfq* altered both the transcript and protein levels of genes encoded by the *flp-tad* and the *lspB-lspA2* operons; these data suggest that Hfq likely affects the stability of the transcripts of these genes. Despite the decreased expression of DsrA detected in Western blot analysis and OMP profiles and the decreased resistance to human serum, the transcript levels of *dsrA* were unchanged in the *hfq* mutant compared to its parent; these data suggest that the regulation of DsrA by Hfq is likely at the translational level. Thus, similarly to other organisms, *H. ducreyi* Hfq likely contributes to the regulation of its targets at both the transcript and translational levels.

In summary, we show that Hfq likely serves as a major contributor of virulence and stationary-phase gene regulation in *H. ducreyi*. Future studies will focus on identifying Hfq-dependent proteins and sRNAs and the potential contribution of Hfq-dependent sRNAs to *H. ducreyi* pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 3. *H. ducreyi* strains were grown on chocolate agar supplemented with 1% IsoVitalex at 33° C with 5% CO_2. Alternatively, *H. ducreyi* strains were grown in gonococcal (GC) broth or Columbia broth supplemented with 2.5% or 5% fetal bovine serum (Hyclone), respectively, 1% IsoVitalex, and 50 μg/ml of hemin (Aldrich Chemical Co.) at 33° C. For RNA-Seq experiments, strain 35000HP was grown to the mid-log phase (OD_{660} = 0.2), transition phase (OD_{660} = 0.31), or early stationary phase (referred to here as stationary phase; OD_{660} = 0.35) in supplemented GC broth. *E. coli* strains were grown in Luria-Bertani media at 37° C except for strain DY380, which was maintained in L-broth or agar and grown at 32° C or 42° C for induction of the λ red recombinase. Where necessary, the media was supplemented with kanamycin (20 μg/ml for *H. ducreyi*; 50 μg/ml for *E. coli*), spectinomycin (200 μg/ml for *H. ducreyi*; 50 μg/ml for *E. coli*), or chloramphenicol (1 μg/ml for *H. ducreyi*; 30 μg/ml for *E. coli*).

RNA-Seq analysis of growth-phase-dependent differences in gene expression. We previously reported a comparison of the transcriptomes of strain 35000HP, a *cpxA* deletion mutant, and a *cpxR* deletion mutant grown to the mid-log, transition, and stationary phases of growth (23). The growth-phase-dependent differences in gene expression were identified by comparing the transcriptomes of 35000HP at different growth phases using previously reported data (23). Given that transcripts encoding ribosomal proteins are depleted from the total transcripts, RNA-Seq experiments in general suffer from the caveat that genes encoding ribosomal proteins cannot be quantified using this technique. Therefore, as described in Results, genes encoding ribosomal proteins were not included in these analyses. As described previously (23), a prespecified FDR of ≤0.1 and a 2-fold change were used as criteria for differential transcript expression analysis. The differentially expressed genes were grouped into functional categories using the role classification available in the comprehensive microbial resource database (50).

Validation of RNA-Seq data by qRT-PCR. qRT-PCR was performed using a QuantiTect SYBR green RT-PCR kit (Qiagen) and an ABI Prism

7000 sequence detector (Applied Biosystems) as described previously (23). The primer pairs were designed to amplify internal gene-specific fragments ranging from 70 to 200 bp. The amplification efficiency was determined for each primer pair (P1 to P16; see Table S5 in the supplemental material); all primer pairs had >95% efficiency. The expression levels of target genes were normalized to that of *dnaE*, which was amplified using primer pair P17 and P18 (see Table S5).

DNA microarray analysis and qRT-PCR validation of microarray results. Total RNA was isolated from broth-grown *H. ducreyi* cultures as previously described (51). The *H. ducreyi* custom spotted DNA microarrays used in this study have been previously described (51). For each experiment, 5 μg of total RNA extracted from cells grown to mid-log phase (8 h) was used for first-strand cDNA synthesis as previously described (51). To avoid gene-specific dye bias, each sample was subjected to reverse labeling (dye swap). Differential expression was defined as a minimum of a 2-fold change in expression in the 35000HP*hfq*::*cat* strain relative to 35000HP. The data were further analyzed to include only expression profiles that had a $P \leq 0.05$ after a one-sample *t* test analysis. The differentially expressed genes that achieved statistical significance were grouped into functional categories based on the role classification available in the comprehensive microbial resource database (50).

Fifteen genes were randomly selected for further confirmation of their relative transcript levels by two-step qRT-PCR. Primers (P19 to P36) used in this study are listed in Table S5 in the supplemental material. The reverse transcriptase reaction was performed as described previously (51). Assays were performed on two independent biological replicates, using HD1643 (*gyrB*) to normalize the amount of cDNA per sample. The fold change of each gene was calculated using the $2^{-\Delta\Delta CT}$ method.

Construction and complementation of an *hfq* insertional inactivation mutant and an unmarked, nonpolar *hfq* deletion mutant. To construct an *hfq* insertional inactivation mutant, a ~3-kb fragment containing the *hfq* ORF as well as ~1 kb of both upstream and downstream flanking DNAs was introduced into pCR2.1 (Invitrogen) to obtain plasmid pML104. A *cat* cartridge from pSL1 (52), modified to contain its native promoter (51) and flanked with SmaI sites, was introduced into pCR2.1 to obtain plasmid pML121. The *hfq* ORF was interrupted by digesting pML104 with SwaI (native restriction site located 35 nucleotides [nt] inside the ORF) and inserting the *cat* cassette, which had been excised from pML121 using SmaI. The resultant construct was designated pML120. Primer pair P37 and P38 (see Table S5 in the supplemental material) was used to amplify a ~3-kb fragment from pML120 which was subsequently subjected to digestion with DpnI, gel purified, and used to electroporate *H. ducreyi* 35000HP as previously described (51). An *hfq* insertional inactivation mutant (35000HP*hfq*::*cat*) was selected on chocolate agar plates containing chloramphenicol; nucleotide sequence analysis confirmed the presence of the in-frame insertion within the *hfq* ORF.

An unmarked, in-frame deletion mutant of *H. ducreyi hfq* was made using "recombineering" methodology exactly as described previously (29, 31, 49). All primer pairs used in the construction of the deletion mutant are listed in Table S5 in the supplemental material. Briefly, *hfq* and its flanking sequences were amplified using primer pair P39 and P40 and cloned into pCR-XL-TOPO to generate pKF10, which was electroporated into DY380, a strain of *E. coli that* expresses λ red recombinase. A pair of 70-bp primers was used to amplify a spectinomycin (spec) resistance cassette flanked by the flippase recognition target (FRT) sites employing pRSM2832 as the template (29). P41 included 47 bp upstream of *hfq*, its start codon, and 20 bp homologous to the 5′ end of the *spec* cassette; P42 included 21 bp at the 3′ end of *hfq*, 29 bp of the downstream region, and 20 bp corresponding to the 3′ end of the *spec* cassette. The mutagenic amplicon was electroporated into DY380(pKF10) for recombination, generating pKF11. A SpeI-digested fragment containing the *hfq* flanking regions and the *spec* cassette was cloned into the suicide vector pRSM2072, generating pKF12, which was electroporated into 35000HP. After allelic exchange was confirmed by PCR, FLP recombinase was used to excise the *spec* cassette exactly as previously described (29). This resulted in replacement of the *hfq* gene by a short ORF that includes the *hfq* start codon, 81 bp encoding a flippase (FLP) scar peptide, and the last 21 bp of *hfq*, including its stop codon. By employing this design, the downstream gene, *hflX*, should be transcribed and translated normally. The *hfq* deletion was confirmed by sequence analysis; the final mutant was designated 35000HPΔ*hfq*. Primer pair P43 and P44 (see Table S5), which binds to a region within the downstream gene *hflX*, was used to confirm that the deletion in 35000HPΔ*hfq* did not affect the expression of *hflX* by qRT-PCR.

To complement both *hfq* mutants, the wild-type 35000HP *hfq* gene, together with ~500 bp 5′ from the ATG translational start codon and ~200 bp 3′ from the translational stop codon, was amplified from chromosomal DNA using primer pair P45 and P46 (see Table S5 in the supplemental material). The amplicon was digested with SacII and ligated to SacII-digested pACYC177 (New England Biolabs) to obtain pML129. After the result was confirmed by PCR and sequence analysis, pML129 DNA was used to transform strains 35000HP*hfq*::*cat* and 35000HPΔ*hfq* to obtain kanamycin-resistant strains 35000HP*hfq*::*cat*(pML129) and 35000HPΔ*hfq*(pML129), respectively. The 35000HP wild-type, 35000HPΔ*hfq*, and 35000HP*hfq*::*cat* strains were also transformed with pACYC177, and the resulting strains were designated 35000HP(pACYC177), 35000HPΔ*hfq*(pACYC177), and 35000HP*hfq*::*cat*(pACYC177), respectively.

Phenotypic comparisons. Lipo-oligosaccharides and OMPs were isolated from 35000HP and 35000HPΔ*hfq* and analyzed by polyacrylamide gel electrophoresis as described previously (53–55).

Human inoculation experiments. Eleven healthy adult volunteers (7 men and 4 women; 3 white and 8 black; mean age ± SD, 48.9 ± 9.5 years) initially enrolled in the study. The volunteers gave informed consent for participation and for HIV serology, in compliance with the guidelines of the U.S. Department of Health and Human Services and the Institutional Review Board of Indiana University. One volunteer was excluded due to an underlying medical condition; 1 withdrew consent prior to inoculation; 9 participated in the study.

Stocks of 35000HP and 35000HPΔ*hfq* were prepared according to FDA guidelines under BB–IND 13064. Human inoculation experiments were performed exactly as described in detail elsewhere (30, 56). Comparisons of papule and pustule formation rates were performed using a logistic regression model with generalized estimating equations (GEE) (25); the GEE sandwich estimate for the standard errors was used to calculate 95% confidence intervals (95% CI) for the rates. For a rate of zero, the GEE estimate does not exist; in this instance, we calculated the exact binomial confidence intervals based on the number of subjects rather than sites, as described previously (25).

To confirm the identity of the bacteria in the inocula, surface cultures, and cultures of biopsies, colony hybridization was performed using probes specific for *dnaE* and the deleted region of *hfq* generated by primer pair P17 and P18 and primer pair P13 and P14 (see Table S5 in the supplemental material), respectively, using methods described previously (49).

Three groups of volunteers were inoculated in this study; however, the data from the second group of volunteers were excluded due to an experimental error. We intended to inoculate a group of four participants (424, 425, 426, and 427) in the second iteration with the parent and escalating doses of the mutant. Surprisingly, pustules formed at 4 of 9 parent sites and 12 of 12 mutant sites. However, colony PCR performed on colonies isolated from the inocula used to infect the subjects showed that the participants in the second group were mistakenly infected only with the parent (106 CFU at 3 sites on one arm; 244, 488, and 976 CFU at 3 sites on the other arm). All the participants in the second group achieved clinical endpoint (development of a painful pustule) in 6 or 7 days, which is typical for the model; the participants were not harmed by the error. After reporting a protocol violation, we amended our procedures so that PCR is performed on the broth cultures prior to inoculation of the volunteers to

confirm that the parent cultures contain the gene of interest and that the mutant cultures lack the gene of interest.

Western blot analysis. Whole-cell lysates (5×10^7 CFU/well) from broth-grown *H. ducreyi* strains were resolved by SDS-PAGE in 4% to 20% polyacrylamide gels and electroblotted onto polyvinylidene difluoride (PVDF) membranes as described previously (51). After transfer, membranes were incubated in StartingBlock (phosphate-buffered saline [PBS]) Blocking Buffer (Thermo Scientific) containing 5% normal goat serum for 1 h at room temperature or overnight at 4° C, followed by incubation in primary antibody (Ab) either for 4 h at room temperature or overnight at 4° C. Membranes were subsequently incubated for 1 h at room temperature in a 1:20,000 dilution of either goat anti-mouse IgG-horseradish peroxidase (IgG-HRP) or goat anti-rabbit IgG-HRP (Bio-Rad). The following primary antibodies used in this study have been previously described: LspA1-specific monoclonal antibody (MAb) 40A4 (26), LspA2-specific MAb 1H9 (26), mouse polyclonal LspB antibody (27), PAL-specific MAb 3B9 (53), mouse polyclonal DsrA antibody (33), and rabbit polyclonal Flp1 antibody (24).

Microcolony formation assay. Microcolony assays were performed as previously described (24). Briefly, 24-well tissue culture plates (Costar) were seeded with ~10^5 Hs27 human foreskin fibroblasts (CRL-1634; American Type Culture Collection) per well and incubated until they achieved confluence. *H. ducreyi* cells grown overnight in 5 ml of Columbia broth were collected by centrifugation and resuspended in tissue culture medium to an $OD_{600} = 0.1$. Portions (5 μl) of the bacterial suspension were added in triplicate to individual wells, and the bacterial cells were centrifuged onto the monolayers for 5 min at 1,000 $\times$ *g* at room temperature, after which the plates were incubated at 33° C and 5% CO_2. After incubation for 24 h, each well was washed three times with PBS (pH 7.4) and stained with crystal violet. Images were taken using an FSX100 Bio Imaging Navigator microscope system (Olympus) at $\times$14 and $\times$40 magnifications.

Serum bactericidal assays. Serum bactericidal assays were performed exactly as described previously (29).

Microarray data accession number. The data from these DNA microarray experiments were deposited at the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE44535.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01081-13/-/DCSupplemental.

Table S1, DOCX file, 0.2 MB.
Table S2, DOCX file, 0.2 MB.
Table S3, DOCX file, 0.2 MB.
Table S4, DOCX file, 0.3 MB.
Table S5, DOCX file, 0.2 MB.
Figure S1, TIF file, 3.1 MB.
Figure S2, TIF file, 3 MB.
Figure S3, TIF file, 6.4 MB.

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