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DksA and (p)ppGpp Have Unique and Overlapping Contributions to *Haemophilus ducreyi* Pathogenesis in Humans

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The (p)ppGpp-mediated stringent response is important for bacterial survival in nutrient limiting conditions. For maximal effect, (p)ppGpp interacts with the cofactor DksA, which stabilizes (p)ppGpp's interaction with RNA polymerase. We previously demonstrated that (p)ppGpp was required for the virulence of *Haemophilus ducreyi* in humans. Here, we constructed an *H. ducreyi dksA* mutant and showed it was also partially attenuated for pustule formation in human volunteers. To understand the roles of (p)ppGpp and DksA in gene regulation in *H. ducreyi*, we defined genes potentially altered by (p)ppGpp and DksA deficiency using transcriptome sequencing (RNA-seq). In bacteria collected at stationary phase, lack of (p)ppGpp and DksA altered expression of 28% and 17% of *H. ducreyi* open reading frames, respectively, including genes involved in transcription, translation, and metabolism. There was significant overlap in genes differentially expressed in the (p)ppGpp mutant relative to the *dksA* mutant. Loss of (p)ppGpp or DksA resulted in the dysregulation of several known virulence determinants. Deletion of *dksA* downregulated *lspB* and rendered the organism less resistant to phagocytosis and increased its sensitivity to oxidative stress. Both mutants had reduced ability to attach to human foreskin fibroblasts; the defect correlated with reduced expression of the Flp adhesin proteins in the (p)ppGpp mutant but not in the *dksA* mutant, suggesting that DksA regulates the expression of an unknown cofactor(s) required for Flp-mediated adherence. We conclude that both (p)ppGpp and DksA serve as major regulators of *H. ducreyi* gene expression in stationary phase and have both overlapping and unique contributions to pathogenesis.

Haemophilus ducreyi is the causative agent of chancroid, a sexually transmitted genital ulcer disease that facilitates both the transmission and acquisition of HIV-1 (1). Chancroid has a short duration of infectivity and is maintained only in populations with high sex partner change rates, such as commercial sex workers (2). Due to widespread implementation of syndromic management of genital ulcers, the epidemiology of chancroid is poorly defined, but its prevalence has declined in many areas where chancroid formerly was endemic (2). However, reports of chancroid persist from several countries in Africa and Asia, implying that these regions have clinical reservoirs of infected sex workers (3–8).

Although non-sexually-transmitted cutaneous ulcers in children in the South Pacific islands and equatorial Africa are usually attributed to *Treponema pallidum* subsp. *pertenue*, recent studies performed as part of a World Health Organization-directed yaws eradication campaign suggest that *H. ducreyi* is a major cause of this syndrome. In three large cross-sectional community surveys, the proportion of ulcers in which *H. ducreyi* DNA was detected greatly exceeded that of *T. pallidum* DNA (9–11). Considering the global prevalence of yaws, infections due to *H. ducreyi* may be much more common than was previously recognized. Thus, understanding the pathogenesis of *H. ducreyi* remains important from the point of view of public health.

In order to study *H. ducreyi* pathogenesis, we developed a human inoculation model in which healthy adult volunteers are infected on the skin of the upper arm with the strain 35000HP, which was originally isolated from a patient with chancroid (12, 13). Phylogenetic analysis of cutaneous ulcer strains show that they are highly related to strain 35000HP, suggesting that the human inoculation model is relevant to both chancroid and cutaneous ulcers (14). In natural chancroid and experimental infection, *H. ducreyi* is found within abscesses surrounded by macrophages and polymorphonuclear leukocytes and is likely subject to a variety of stresses, including antimicrobial peptides, reactive oxygen species, and nutrient limitation (15, 16).

One mechanism used by bacteria to adapt to stress is the stringent response mediated by GTP 3′-diphosphate (pppGpp) and GDP 3′-diphosphate (ppGpp), collectively referred to here as (p)ppGpp. During the stringent response, (p)ppGpp interacts with a cofactor known as the DnaK suppressor protein or DksA. DksA helps to stabilize the interaction between (p)ppGpp and the RNA polymerase (RNAP), thus enhancing (p)ppGpp-controlled transcription (17). In *Escherichia coli*, DksA overexpression can compensate for the loss of (p)ppGpp synthesis (18, 19). The com-

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pensated phenotypes include restoration of defects in amino acid auxotrophy and cell-cell aggregation.

In several pathogenic bacterial species, DksA regulates the transcription of genes involved in virulence. DksA regulates motility, expression of the hemagglutinin protease, and production of cholera toxin in *Vibrio cholerae*, all processes related to virulence (20). DksA is also involved in the posttranscriptional control of rhamnolipids and LasB elastase, which are involved in quorum sensing in *Pseudomonas aeruginosa* (21). In *Shigella flexneri*, DksA is essential for regulation of *hfq*, which is an important regulator of *S. flexneri* virulence genes (22). However, the role of DksA in bacterial pathogenesis has never been directly studied in humans.

An *H. ducreyi* Δ*relA* Δ*spoT* mutant, which is unable to synthesize (p)ppGpp, is partially attenuated for pustule formation in human volunteers (23). The (p)ppGpp null [(p)ppGpp°] mutant has increased expression of *dksA* transcripts, which may have compensated for the loss of (p)ppGpp and contributed to its partial attenuation (23). To investigate the potential role of DksA in *H. ducreyi* pathogenesis, here we constructed a *dksA* deletion mutant and compared its virulence to that of the parent strain in human inoculation experiments. We also defined genes whose expression is altered by (p)ppGpp and DksA deficiency in *H. ducreyi* by using transcriptome sequencing (RNA-seq). We used the results of the transcriptome analysis to direct further characterization of the *dksA* and (p)ppGpp° mutants in assays relevant to *H. ducreyi* pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *H. ducreyi* strains were grown on chocolate agar plates supplemented with 1% IsoVitaleX at 33°C with 5% CO_2 or in gonococcal (GC) broth supplemented with 5% fetal bovine serum, 1% IsoVitaleX, and 50 μg/ml hemin (Aldrich Chemical Co.) at 33°C. *H. ducreyi* 35000HP was grown to mid-log phase (optical density at 660 nm [OD_{660}] = 0.2), transition (OD_{660} = 0.35), or stationary phase (OD_{660} = 0.5); mutant strains were harvested when the OD_{660} of strain 35000HP was in the appropriate range for each phase of growth. *E. coli* strains were grown in Luria-Bertani medium at 37°C with the exception of strain DY380, which was maintained in low-salt broth or agar at 32°C and grown at 42°C for induction of the lambda red recombinase. When necessary, medium was supplemented with kanamycin (20 μg/ml for *H. ducreyi*; 50 μg/ml for *E. coli*) or spectinomycin (200 μg/ml for *H.* ducreyi; 50 μg/ml for *E. coli*). For *H. ducreyi* strains containing a pACYC177 backbone, medium was supplemented with 30 μg/ml kanamycin.

Characterization of the *dksA* gene. The Basic Local Alignment Search Tool (BLAST) was used to identify putative homologues of *dksA* in *H. ducreyi* (GenBank accession no. AE017143). Reverse transcriptase PCR (RT-PCR) was conducted to determine whether *dksA* was in an operon with its surrounding genes using primers P1-P6 (see Table S2 in the supplemental material).

Construction and complementation of an unmarked, in-frame *dksA* deletion mutant. An unmarked, in-frame *dksA* deletion mutant was constructed using the lambda red and FLP recombinase method described previously using primers P7-P14 (24, 25). Quantitative RT-PCR (qRT-PCR) determined that deletion of *dksA* did not affect transcription of the downstream gene *pcnB* (data not shown).

To complement the *dksA* deletion mutant, the gene was expressed under the control of a constitutive *cat* promoter from pACYC184 in the expression vector pACYC177 as described previously using primers P15-P18 (23). The final construct, designated pCH24, was confirmed by PCR and sequence analysis and electroporated into 35000HPΔ*dksA* mutant strain to yield 35000HPΔ*dksA*(pCH24). For controls, strains 35000HP and 35000HPΔ*dksA* were also electroporated with pACYC177, and the resulting strains were designated 35000HP(pACYC177) and 35000HPΔ*dksA*(pACYC177), respectively. Although all transformed strains grew well on antibiotic-supplemented plates, and strain 35000HP Δ*dksA*(pCH24) grew normally in broth containing kanamycin, the *H. ducreyi* strains containing pACYC177 grew poorly in antibiotic-supplemented broth. The strains transformed with pACYC177 could be grown in broth without antibiotics for only 6 h before losing the plasmid. Thus, use of the complemented strains was limited to those assays using cells that had been grown or maintained in broth without antibiotics for 6 h or those that had been grown on plates supplemented with antibiotics.

Human inoculation experiments. Human inoculation experiments were conducted according to the guidelines of the U.S. Department of Health and Human Services and the Institutional Review Board (IRB) of Indiana University. Seven adult volunteers (5 females and 2 males; 2 African Americans and 5 European Americans; mean age, 36 years) participated in the study. All volunteers gave written, informed consent for participation and HIV serology. The procedures for the human inoculation experiments, including calculation of the estimated delivered dose (EDD), are described in detail elsewhere (26). Papule and pustule formation rates for parent and mutant inoculation sites were compared using logistic regression with generalized estimating equations (GEE) as previously described (27). Ninety-five percent confidence intervals (95% CI) for papule and pustule formation rates were calculated using GEE-based sandwich standard errors.

To ensure that there was no cross contamination of samples, colony hybridization was performed on colonies derived from the inocula, surface cultures, and biopsy specimens. Probes specific for *dksA* and *dnaE* were designed, and the DIG DNA labeling kit (Roche Applied Sciences) was used to label the probes with digoxigenin. The DIG Easy Hyb protocol was performed according to the manufacturer's instructions (Roche Applied Sciences).

RNA isolation and quality assessment. Total RNA was extracted from strains 35000HP, 35000HPΔ*relA*Δ*spoT*, and 35000HPΔ*dksA* in the mid-log, transition, and stationary growth phases using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA isolation was performed on four independent bacterial cultures for each strain in each growth phase. RNA was treated twice with Turbo DNA-free DNase (Ambion). The integrity and concentration of RNA were determined using the Agilent 2100 bioanalyzer (Agilent Technologies) and the NanoDrop ND2000 spectrophotometer (Thermo Scientific), respectively. The efficacy of DNase treatment was confirmed by reverse transcriptase PCR analysis of *dnaE* with the primers P19-P20 (see Table S2 in the supplemental material).

RT-PCR and qRT-PCR. cDNA from strains 35000HP and 35000HP Δ*dksA* was synthesized from total RNA using Advantage RT-for-PCR kit (Clontech). Reverse transcriptase PCR was performed using the cDNA and FastStart PCR master mix (Roche).

Quantitative RT-PCR was performed using the QuantiTect SYBR green RT-PCR kit (Qiagen) and a Mastercycler ep realplex 4 (Eppendorf). All primer pairs (see Table S2 in the supplemental material) had greater than 95% amplification efficiency. Relative expression was calculated as $E_{\text{target}}^{\Delta\text{CTtarget}}/E_{\text{reference}}^{\Delta\text{CTreference}}$, where E is the amplification efficiency (10^{-1}/slope) and the ΔCT is the change in cycle threshold. *dnaE* was amplified to normalize the expression levels of target genes.

mRNA enrichment. The removal of 23S, 16S, and 5S rRNA from total RNA was performed with the Ribo-Zero Magnetic kit for Gram-negative bacteria (Epicentre Biotechnologies) by following the manufacturer's instructions. The Agilent 2100 bioanalyzer confirmed removal of rRNA from total RNA.

Preparation of RNA-seq libraries and sequencing. The TruSeq Stranded mRNA sample preparation kit (Illumina) was used to prepare stranded RNA-seq libraries by following the manufacturer's instructions. Briefly, approximately 400 ng of the enriched mRNA was fragmented and randomly primed for first-strand cDNA synthesis. Second-strand synthe-

sis incorporated dUTP in place of dTTP, which prevents second-strand synthesis during subsequent amplification and results in a stranded library. The cDNA was end repaired, adenylated, and ligated to adapters. The adapter-ligated cDNA library was then PCR enriched. Finally, the enriched RNA-seq library was validated with the Agilent 2100 bioanalyzer and qRT-PCR. Clusters were generated on the cBOT automated cluster-generating system with the TruSeq PE cluster kit (Illumina). Libraries were sequenced with the Illumina HiSeq 2500 sequencer with the TruSeq SBS kit (Illumina) for single-end sequencing with read lengths of 100 bp in the Biomedical Genomics Core facility at Nationwide Children's Hospital (Columbus, OH). Image analysis and base calling were performed with the HiSeq Control software and the Real Time Analysis software. Demultiplexing was performed with the Illumina CASAVA software.

Sequence mapping and quantification of transcript levels. The sequenced reads were mapped to the *H. ducreyi* 35000HP genome (GenBank accession no. AE017143) with the Burrows-Wheeler Alignment tool (28) allowing up to two base mismatches. Reads that failed to map to any gene in the chromosome and reads that mapped to multiple locations in the genome were removed before quantifying the transcript levels. The total number of reads corresponding to the coding region of each gene was determined with the NGSUtils suite (29). The percentage of total reads aligned with the reference genome from all samples ranged from 82.89 to 95.87% (see Table S3 in the supplemental material).

Identification of differentially expressed genes. Differential expression of genes across all three strains and growth phases was determined with edgeR software, a Bioconductor package, as previously described (30). To favor true identification of differentially expressed genes of biological significance, we used a prespecified falsediscovery rate of ≤0.1 and a 2-fold change as a threshold. The differentially expressed genes were functionally classified using the annotations and pathway information from the sequenced *H. ducreyi* genome (R. S. Munson, Jr., unpublished data), and KEGG (Kyoto Encyclopedia of Genes and Genomes) (31). We also determined whether any biological pathways were enriched among the genes differentially expressed in the (p)ppGpp° and *dksA* mutants compared to strain 35000HP by using pathway annotations from BioCyc (32). We used the functional annotation-clustering algorithm of the DAVID (Database for Annotation, Visualization, and Integrated Discovery) bioinformatics resources (http://david.abcc.ncifcrf.gov/) to identify the biological pathways enriched in the (p)ppGpp° and *dksA* mutants relative to the 35000HP strain (33). A pathway was considered enriched if the Fisher's exact *P* value for the cluster was less than 0.05, the enrichment score for the cluster was greater than 2, and the cluster involved greater than 5% of the genes on the submitted list (34).

Phenotypic comparisons. Lipooligosaccharides (LOS) and outer membrane proteins (OMP) were isolated from strains 35000HP and 35000HPΔ*dksA* and analyzed as described previously (35, 36). Serum bactericidal assays were performed on organisms grown on plates as described previously (37). To determine the expression of Flp1/2 and peptidoglycan-associated lipoprotein (PAL), Western blots were performed on whole-cell lysates and purified outer membranes and probed with antisera or monoclonal antibody specific to each protein. The antisera for Flp1/2 (kindly provided by E. Hansen, University of Texas Southwestern) and the PAL-specific monoclonal antibody 3B9 are described elsewhere (36, 50).

Phagocytosis assays. Human peripheral blood mononuclear cells (PBMCs) were isolated from blood obtained from five healthy adult volunteers. Informed consent was obtained in accordance with an IRB-approved protocol. PBMCs were isolated by Ficoll-Paque Plus purification, and $CD14^+$ cells were isolated (Miltenyi Biotec). The isolated $CD14^+$ cells were differentiated into monocyte-derived macrophages (MDMs) in X-vivo 15 medium (Lonza) supplemented with 1% human AB serum (Invitrogen) for 5 days. The phagocytosis assays were performed as previously described (23). The percentage of bacterial uptake was calculated as the percentage of bacteria within the lysed MDMs after 1 h coculture compared to the initial CFU. To determine intracellular bacterial survival, the cocultures were incubated in antibiotic-free medium containing 10% fetal bovine serum for an additional 5 h and quantitatively cultured. Survival was determined by calculating the ratio of recoverable bacteria after 6 h of coculture compared to the initial uptake.

Oxidative stress assays. As previously described, bacteria were treated with hydrogen peroxide (H_2O_2) to mimic extracellular oxidative stress (23). Percent survival was calculated as the ratio of recovered bacteria to the input CFU. For assays using complemented strains, bacterial cells were grown for 6 h in broth without antibiotics, collected, and exposed to H_2O_2 as described previously.

Adherence assays. Adherence to human foreskin fibroblasts (HFF) was performed as described previously (40). Briefly, 24-well tissue culture plates (Costar) were seeded with 10^5 HFF cells and allowed to reach confluence. *H. ducreyi* strain 35000HP, 35000HPΔ*dksA* (35000HP strain with the *dksA* gene deleted), 35000HPΔ*relA*Δ*spoT* (35000HP strain with the *relA* and *spoT* genes deleted), and 35000HPΔ*flp1-3* (35000HP strain with the *flp-1* to *flp-3* genes deleted) were grown to mid-log and stationary phase, harvested, and added to confluent HFF cells at a multiplicity of infection (MOI) of 10:1 at 35°C for 2 h. After a wash, the HFF cells were lysed with 0.2% saponin (Sigma-Aldrich) and quantitatively cultured. Percent adherence of *H. ducreyi* to HFF cells was determined by calculating the ratio of HFF-adhered bacteria to the initial CFU.

Statistical analysis. All data are expressed as means ± standard deviations. Oxidative stress, serum bactericidal, attachment, and phagocytosis data were analyzed using a mixed-model analysis of variance (ANOVA) followed by Tukey's honestly significant difference test. qRT-PCR and densitometry data were analyzed using Student's *t* test. An adjusted two-sided *P* value of ≤0.05 was considered statistically significant.

RNA-seq data accession number. The data from these RNA-seq experiments were deposited at the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE67202.

RESULTS

Characterization of the *dksA* genomic locus. The *H. ducreyi* homologue of *dksA* (HD0603) is in a putative operon with the gene order *tbpA* → *dksA* → *pcnB* → *folK*. RT-PCR analysis indicated that *dksA* is cotranscribed with *tbpA*, *pcnB*, and *folK* (data not shown).

***dksA* is required for pustule formation in human volunteers.** To determine whether DksA contributes to virulence in humans, we constructed an unmarked, in-frame *dksA* deletion mutant. As required by our clinical protocol, we investigated whether deletion of *dksA* affected the expression of outer membrane components of *H. ducreyi*. LOS and OMP profiles isolated from the *dksA* mutant showed no qualitative differences compared to the parent strain. In bactericidal assays, the *dksA* mutant was as resistant to killing by 50% normal human serum as the parent was (data not shown).

Mutations in *dksA* can profoundly impair growth (41, 42). Relative to *H. ducreyi* 35000HP, deletion of *dksA* did not result in a growth defect in broth (see Fig. S1 in the supplemental material). Moreover, similar to the (p)ppGpp° mutant, the *dksA* mutant had increased survival at 24 h compared to the parent strain. Thus, the virulence of the *dksA* mutant should not be affected by impaired growth.

We inoculated volunteers in three iterations with strain 35000HP and with strain 35000HPΔ*dksA*. In the first iteration, three volunteers were inoculated at three sites with an EDD of 82 CFU of strain 35000HP and at one site each with an EDD of 52, 104, and 207 CFU of strain 35000HPΔ*dksA*. In the second iteration, two volunteers were each inoculated at three sites with an EDD of 150 CFU of strain 35000HP and with the Δ*dksA* mutant at

TABLE 1 Response to inoculation of live *H. ducreyi* strains

Volunteer (gender)[a]	Observation period (no. of days)	Strain[b]	Dose(s) (CFU)[c]	No. of initial papules	Final outcome of papules	
					No. of pustules	No. of papules that resolved
454 (F)	7	P	82	2	0	2
		M	52–207	2	0	2
455 (M)	9	P	82	3	1	2
		M	52–207	3	1	2
456 (F)	5	P	82	3	0	3
		M	52–207	2	0	2
457 (F)	7	P	150	3	3	0
		M	87–348	3	1	2
458 (F)	5	P	150	2	0	2
		M	87–348	1	0	1
459 (M)	8	P	77	3	1	2
		M	137	3	1	2
460 (F)	12	P	77	3	3	0
		M	137	3	0	3

[a] Volunteers 454, 455, and 456 were inoculated in the first iteration. Volunteers 457 and 458 were inoculated in the second iteration. Volunteers 459 and 460 were inoculated in the third iteration. F, female; M, male.
[b] P, parent strain 35000HP; M, mutant strain 35000HPΔ*dksA*.
[c] Doses (single doses) inoculated at three sites. 52–207, one dose each of 52, 104, and 207 CFU; 87–348, one dose each of 87, 174, and 348 CFU.

three sites with EDDs of 87, 174, and 348 CFU. In the third iteration, two volunteers were inoculated with an EDD of 77 CFU of strain 35000HP at three sites and an EDD of 137 CFU of the Δ*dksA* mutant at three sites. Overall, papules formed at 90.5% (95% CI, 79.3 to 99.9%) of the parent-inoculated sites and at 81.0% (95% CI, 63.0 to 98.9%) of the mutant-inoculated sites ($P = 0.094$) (Table 1). After 24 h of infection, the mean surface area of the papules was 9.1 ± 9.2 mm^2 at parent sites and 4.6 ± 6.9 mm^2 at mutant sites ($P = 0.012$). Pustules formed at 42.9% (95% CI, 14.2 to 71.5%) of parent sites and 14.3% (95% CI, 2.1 to 26.5%) of mutant sites ($P = 0.043$). Thus, the *dksA* mutant was partially attenuated for pustule formation in humans.

Three volunteers (455, 457, and 459) developed pustules at both mutant and parent sites. For each subject, one parent site and one mutant site were biopsied, and the biopsy specimen was divided in half and semiquantitatively cultured or stained with hematoxylin-eosin and anti-CD3 antibodies as previously described (43). All samples contained epidermal micropustules and dermal CD3 cells (data not shown). Histopathologically, pustules formed at mutant sites were indistinguishable from pustules formed at parent sites.

All colonies recovered from the inocula, surface cultures, and biopsy specimens were tested for the presence of *dksA* and *dnaE* sequences by colony hybridization. The *dnaE* probe hybridized to all the colonies tested from both parent ($n = 108$) and mutant ($n = 107$) inocula, while the *dksA* probe hybridized only to colonies from the parent inocula. At least one positive surface culture for *H. ducreyi* was obtained during follow-up visits from 19.5% of the parent inoculation sites and 4.8% of the mutant inoculation sites. The *dnaE* probe hybridized to colonies from both parent ($n = 106$)- and mutant ($n = 29$)-inoculated sites, while the *dksA* probe bound only to colonies from the parent sites. All three paired biopsy specimens of mutant and parent pustules yielded *H. ducreyi*. The *dnaE* probe hybridized to all of the colonies, both parent ($n = 107$) and mutant ($n = 69$), obtained from the biopsy specimens, while the *dksA* probe hybridized only to colonies obtained from the parent biopsy specimens. Thus, there was no evidence of cross contamination between the inocula and mutant and parent inoculation sites.

DksA- and (p)ppGpp-deficient transcriptomes significantly overlap at stationary phase. Loss of *dksA* and (p)ppGpp can have unique, overlapping, and pleiotropic effects on transcription. To better understand their respective contributions to pathogenesis, we next determined the effects of DksA and (p)ppGpp deficiency on gene expression. We compared the transcriptomes of strains 35000HP, 35000HPΔ*dksA*, and 35000HPΔ*relA*Δ*spoT*, which is (p)ppGpp°. Four biological replicates were included for each strain harvested at the mid-log, transition, and stationary phases of growth (see Fig. S1 in the supplemental material), for a total of 36 samples. We calculated the fold change in the expression of genes in the 35000HPΔ*dksA* mutant or 35000HPΔ*relA*Δ*spoT* mutant compared to strain 35000HP. As described previously, we used a false discovery rate (FDR) of ≤0.1 and a twofold change as criteria for differential transcript expression (44). A positive fold change indicates that expression of the gene is higher in the mutant strain, while a negative fold change signifies that the expression is higher in the parent strain (see Table S4 in the supplemental material).

Comparison of the transcriptomes of the (p)ppGpp° mutant to the parent in mid-log, transition, and stationary phase yielded 149, 107, and 494 differentially expressed genes, respectively; approximately equal numbers of genes were up- and downregulated (Fig. 1). Comparison of the transcriptomes of the *dksA* mutant to the parent in mid-log, transition, and stationary phase yielded 58, 184, and 304 differentially expressed genes, respectively; the majority of genes were upregulated (Fig. 1).

To identify the overlap of the genes that were differentially regulated by the loss of (p)ppGpp or DksA, we plotted the $\log_{10}$-transformed fold changes in the mutant 35000HPΔ*relA*Δ*spoT*/35000HP strain against mutant 35000HPΔ*dksA*/35000HP strain. Comparison of the genes differently regulated by loss of DksA to those differentially regulated by loss of (p)ppGpp in cells harvested from mid-log, transition, and stationary phase yielded 11, 11, and 222 overlapping genes, respectively. At mid-log and tran-

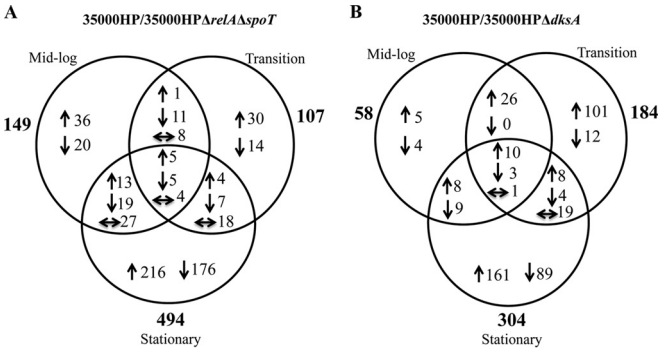

FIG 1 Venn diagrams showing the number of genes differentially regulated by (p)ppGpp or DksA deficiency at different phases of growth. (A) *H. ducreyi* 35000HPΔ*relA*Δ*spoT* mutant compared to strain 35000HP and (B) 35000HPΔ*dksA* mutant compared to strain 35000HP. The total number of genes or operons differentially regulated in different phases of growth is indicated in bold type outside the circles of the Venn diagram. Upregulated (↑) and downregulated (↓) genes and genes regulated differently in different growth phases (← and →) are indicated.

sition phase, (p)ppGpp and *dksA* deficiency primarily altered unique sets of genes, while at stationary phase, the differentially expressed genes significantly overlapped and were coordinately regulated (chi-square = 367.1903; $P < 0.001$) (Fig. 2). These data showed that in stationary phase, (p)ppGpp and DksA deficiencies primarily alter expression of similar targets. As (p)ppGpp and DksA primarily respond to nutrient stress, we focused our analysis on the stationary-phase transcriptomes.

We validated selected differentially regulated genes using qRT-PCR. We focused on the (p)ppGpp° mutant transcriptome, as it had the largest number of differentially expressed genes. The genes were grouped into three categories based on their expression levels (low, medium, and high), grouped into up- and downregulated targets, and further subgrouped based on their fold change ranges (2.0-fold to 5.0-fold, 5.1-fold to 10.0-fold, and 10.1-fold to 50.0-fold). Representative genes were selected from each category; a total of 15 genes were selected for qRT-PCR validation using primers P23-P52 (see Table S2 in the supplemental material). qRT-PCR analysis confirmed the differential expression of 14/15 genes identified by RNA-seq (Fig. 3). However, *hfq* expression was 11.67-fold upregulated by RNA-seq but unchanged (0.97) by qRT-PCR (Fig. 3B); the reason for this discrepancy is unclear. In general, the fold changes derived from RNA-seq were in good agreement with those obtained from qRT-PCR ($R^2 = 0.902$).

Functional classification of genes altered by deficiency of (p)ppGpp° or *dksA*. Using annotations and pathway information from the sequenced 35000HP genome (Munson, unpublished) and KEGG, the identified differentially expressed genes were classified into multiple functional categories, including energy metabolism, biosynthesis, transcription, translation, cell membrane, and substrate binding (Table 2). In both mutants, pathway enrichment analysis with annotations from both BioCyc and DAVID bioinformatics resources showed that genes encoding proteins involved in pilus formation, ion transport, oxidative reduction/phosphorylation, carbohydrate transport, and cytochrome complex assembly were enriched (data not shown). The (p)ppGpp° mutant also showed enrichment in genes encoding proteins involved in regulation of transcription and translation.

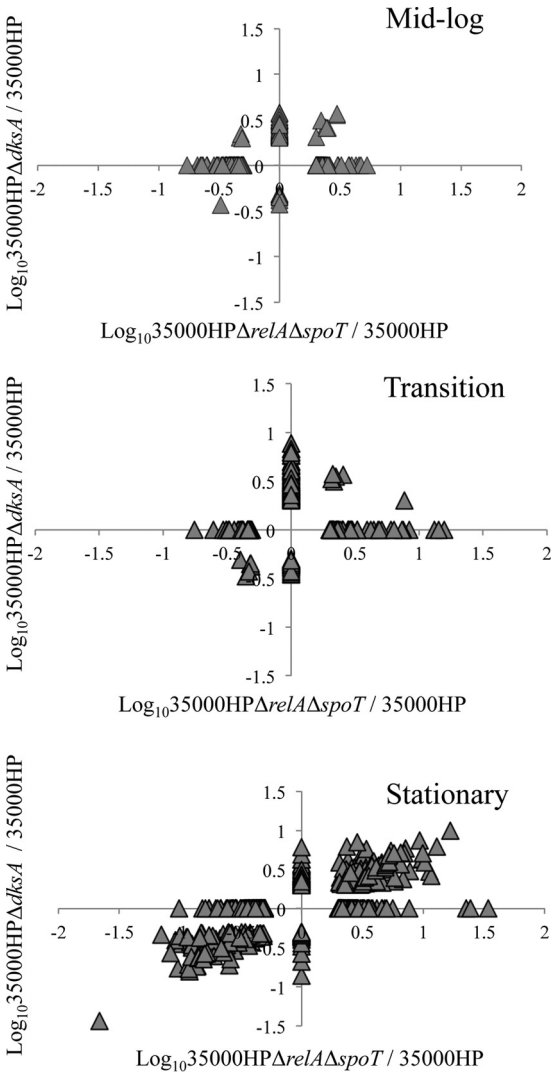

FIG 2 Scatter plots showing fold changes in the expression of genes differentially expressed in 35000HPΔ*relA*Δ*spoT* and 35000HPΔ*dksA* mutant strains compared to 35000HP strain. The scatter plots were generated by plotting the $\log_{10}$-transformed fold changes in 35000HPΔ*relA*Δ*spoT* mutant versus 35000HP strain against 35000HPΔ*dksA* mutant versus 35000HP strain at different growth phases. Each triangle in the graph indicates a single gene.

(p)ppGpp° and *dksA* deficiency leads to dysregulation of virulence determinants required for human infection. Since both the *dksA* and (p)ppGpp° mutants were partially attenuated in humans, we determined the effects of their deficiencies on the expression of genes required for human infection. (p)ppGpp deficiency in strain 35000HP resulted in decreased expression of genes in the *flp-tad* operon, *lspB-lspA2* operon, and the *lspA1*, *hgbA*, and *csrA* genes, which are all required for pustule formation in humans (Table 3) (26, 40, 45). Loss of (p)ppGpp increased the expression of *fgbA*, which is required for virulence (26). These data are consistent with the partial attenuation and some phenotypes of the (p)ppGpp° mutant reported previously (23).

DksA deficiency resulted in decreased expression of *flp-3*, *hgbA*, and *lspB*, which would favor decreased virulence (26). The *dksA* mutant also had increased expression of *hfq*, *dltA*, and *spoT*, which would favor increased virulence (23, 30). These results sug-

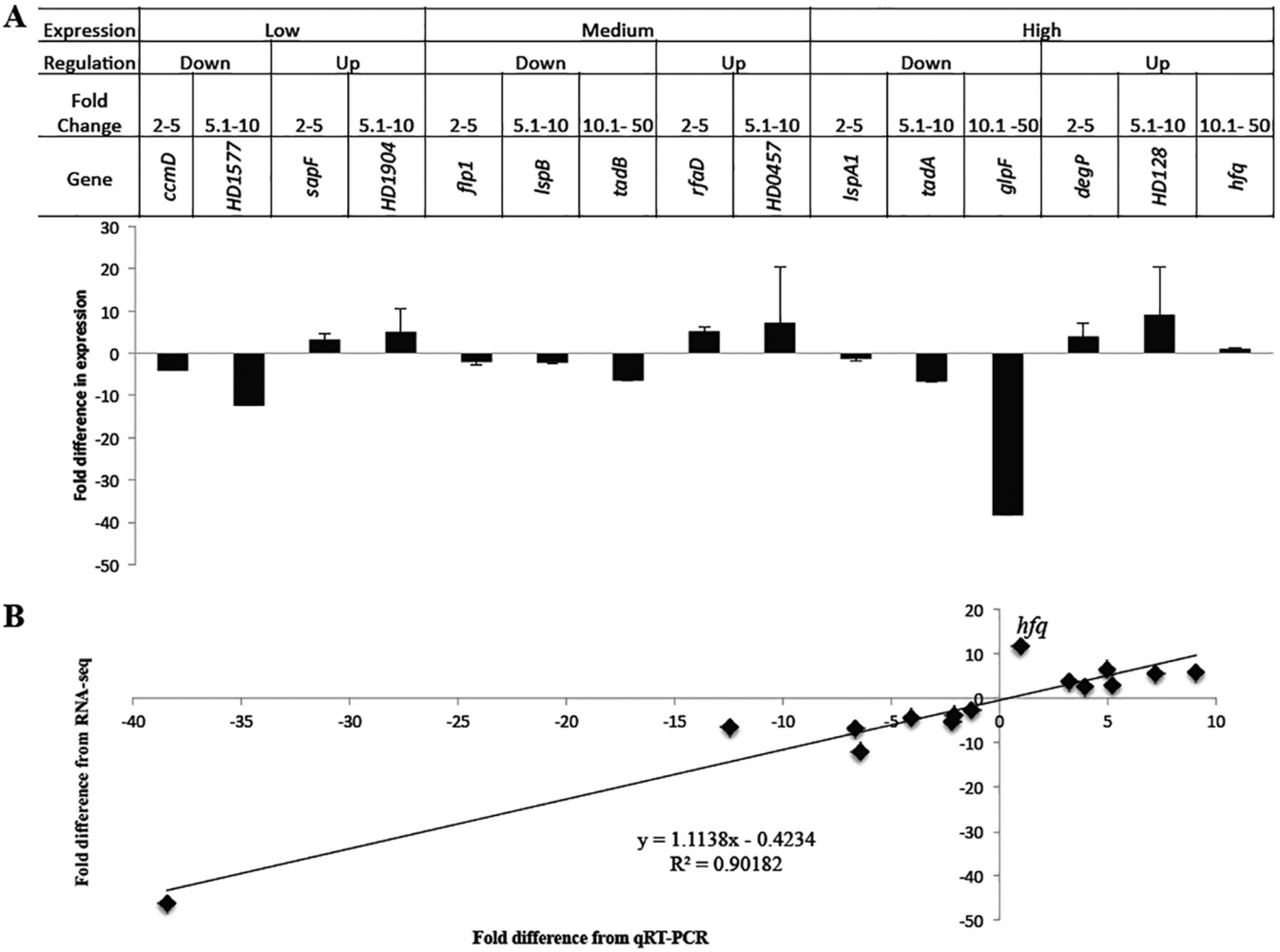

FIG 3 qRT-PCR validation of the RNA-seq data. (A) Fold change in the expression of target genes in 35000HPΔ*relA*Δ*spoT* mutant relative to 35000HP strain in stationary phase. The criteria used for selecting the targets for qRT-PCR validation are outlined in the figure. The expression levels of target genes were normalized to that of *dnaE*. The data represent the means plus standard deviations (SD) (error bars) from four independent experiments. (B) Correlation between the fold changes derived from qRT-PCR and RNA-seq.

gest that the partial attenuation of the *dksA* mutant might be due to conflicting phenotypes.

The set of transcripts altered by (p)ppGpp deficiency significantly overlaps with the sets of transcripts controlled by Hfq and CpxRA. Hfq is a major regulator of *H. ducreyi* stationary-phase gene expression and contributes to the positive regulation of proteins required for virulence such as LspB, DsrA, and Flp1 (30). Given that both mutants upregulated *hfq* (Table 3) and given the discrepancy in the *hfq* expression levels determined by different methods for the (p)ppGpp° mutant, we examined whether the transcriptomes of the (p)ppGpp° and DksA mutants significantly overlapped with that of the *hfq* mutant (30). The transcriptomes of the (p)ppGpp° and *hfq* mutants were negatively correlated and overlapped significantly (chi-square = 38.172; $P < 0.001$), while those of the *dksA* and *hfq* mutants did not. Thus, it is likely that (p)ppGpp deficiency results in upregulation of *hfq* transcription.

(p)ppGpp and DksA deficiencies resulted in 7.19- and 5.89-fold upregulation of *cpxR*, respectively (see Table S4 in the supplemental material). We therefore compared the effects of *dksA* and (p)ppGpp deficiencies on transcription to those produced by activation of CpxRA, defined as the transcription effects of a CpxR-activating mutant compared to a *cpxR* deficient mutant in stationary phase (44). The differentially expressed genes in the *dksA* mutant did not overlap with those of CpxRA. The effects of (p)ppGpp deficiency positively correlated and significantly overlapped with the effects of activation of CpxRA (chi-square = 17.070; $P < 0.001$). Since activation of the CpxRA system is associated with loss of virulence, these data are consistent with the partial attenuation of the (p)ppGpp° mutant (23).

Deletion of *dksA* increased uptake and reduced survival within macrophages of *H. ducreyi* grown to stationary phase. The (p)ppGpp° mutant had several *in vitro* phenotypes that favored attenuation, such as decreased survival in macrophages and increased sensitivity to oxidative stress (23). However, the (p)ppGpp° mutant also exhibited phenotypes that favored virulence, such as increased resistance to uptake by human macrophages and prolonged survival in stationary phase (23). Since the genes affected by DksA and (p)ppGpp deficiency overlapped, we focused on characterizing virulence-associated phenotypes of the *dksA* mutant in similar assays.

TABLE 2 Functional classification of genes or operons differentially regulated by (p)ppGpp or DksA in *H. ducreyi*

Functional category	No. of genes differentially expressed			
	35000HPΔ*relA*Δ*spoT*/35000HP[a]		35000HPΔ*dksA*/35000HP[b]	
	Upregulated	Downregulated	Upregulated	Downregulated
Amino acid biosynthesis	2	4	5	1
Amino acid transport and metabolism	6	6	5	2
Amino sugar and nucleotide metabolism	4	12	2	7
Cellular carbohydrate biosynthetic process	7	5	3	1
Carbohydrate metabolism	1	2	3	0
Cell division	2	5	1	2
Cell membrane	11	15	9	7
Cellular homeostasis	4	2	2	1
Cellular response to stress	0	2	3	0
DNA binding	3	1	4	2
DNA metabolic process	8	4	8	1
Fatty acid biosynthesis and metabolism	7	3	4	2
Intracellular trafficking and secretion	1	11	1	5
Ion transport	3	7	2	4
Iron sulfur cluster binding	2	6	0	3
Lipid biosynthesis and metabolism	3	4	5	2
Metal ion binding	10	8	7	5
Nucleotide binding	8	19	4	11
Protein fate	7	9	5	5
RNA binding	4	0	1	0
RNA processing	9	8	5	3
Transcription	10	9	2	3
Translation	15	27	12	6
Transport of proteins and carbohydrates	7	11	2	6
Uncharacterized conserved protein	6	10	7	1
Hypothetical proteins	75	89	30	92

[a] Genes differentially expressed in 35000HPΔ*relA*Δ*spoT* mutant versus 35000HP strain at stationary phase. Fifty-two percent of the genes were downregulated genes, and 48% were upregulated genes.
[b] Genes differentially expressed in 35000HPΔ*dksA* mutant versus 35000HP strain at stationary phase. Sixty-two percent of the genes were downregulated genes, and 38% were upregulated genes.

During human infection, *H. ducreyi* associates with neutrophils and macrophages but remains extracellular (16, 46). The ability of *H. ducreyi* to evade phagocytosis is due to the secretion of the antiphagocytic proteins LspA1 and LspA2 by LspB (47, 48). Transcriptome analysis indicated that deletion of *dksA* downregulated expression of *lspB* at stationary phase, suggesting that the *dksA* mutant might be more readily phagocytosed than the parent strain. We compared the uptake and intracellular survival of *dksA* mutant cells harvested at mid-log and stationary phase by primary human macrophages. No differences were found in uptake of the *dksA* mutant harvested at mid-log phase compared to the parent (Fig. 4A). The *dksA* mutant tended toward being taken up at a higher rate than the parent when the bacteria were harvested at stationary phase ($P = 0.055$).

We next examined whether deletion of *dksA* affected *H. ducreyi* survival within phagocytes. No differences were found for the intracellular survival of the mutant compared to the parent harvested at mid-log phase (Fig. 4B). However, a significant reduction in the intracellular survival of the *dksA* mutant ($P = 0.023$) was shown for cells harvested at stationary phase. Thus, DksA plays a crucial role in resistance to phagocytic killing *in vitro*.

DksA is important for resistance to oxidative stress. The reduced survival of *H. ducreyi* within macrophages is linked to increased sensitivity to oxidative stress (23, 45). We compared the survival of *H. ducreyi* 35000HP and 35000HPΔ*dksA* after incubation with hydrogen peroxide. For cells grown to mid-log phase, the *dksA* mutant survived significantly less than the parent at 2 mM H_2O_2 ($P = 0.0005$) with a trend toward significance at 0.2 mM ($P = 0.052$) (Fig. 5A). When stationary-phase bacteria were incubated with 0.2 mM or 2 mM H_2O_2, the *dksA* mutant survived significantly less than the parent strain ($P = 0.023$ and $P = 0.0002$, respectively) (Fig. 5A). Complementation of the *dksA* mutant in *trans* with plasmid pCH24 partially restored resistance to 2 mM H_2O_2 ($P = <0.0001$) in cells harvested at mid-log phase (Fig. 5B). Deletion of *dksA* did not affect expression of *csrA* or *sodC*, both of which are associated with increased sensitivity to oxidative stress (see Table S4 in the supplemental material) (45, 49). Therefore, DksA contributes to *H. ducreyi* survival during *in vitro* oxidative stress through an uncharacterized mechanism.

DksA compensates for loss of (p)ppGpp in resistance to oxidative stress. In *H. ducreyi*, loss of (p)ppGpp coincided with an increase in transcript levels of *dksA* (23). The (p)ppGpp° mutant also exhibited increased sensitivity to oxidative stress. In *E. coli*, *dksA* overexpression can compensate for loss of (p)ppGpp (18, 19). We therefore determined whether overexpression of *dksA* could compensate for loss of (p)ppGpp. To this end, we generated 35000HPΔ*relA*Δ*spoT*(pCH24), which overexpresses *dksA* but does not synthesize (p)ppGpp. We used strain 35000HP Δ*relA*Δ*spoT*(pCH30), the Δ*relA* Δ*spoT* complemented strain as a control for the assay (23). The sensitivity of strain

TABLE 3 Regulation of genes required for virulence in humans by either (p)ppGpp or DksA

Gene(s)[a]	Function	35000HPΔ*relA*Δ*spoT*/35000HP[b,c]	35000HPΔ*dksA*/35000HP[b,d]
Fully attenuated			
flp-1–flp-2–flp-3	Adherence and microcolony formation	−3.72	−2.33[e]
tadA	Adherence and microcolony formation	−6.86	–
dsrA	Major role in serum resistance	–	–
lspA1, *lspA2*	Escape from phagocytosis	−2.75, −5.36[f]	−2.57[f]
ncaA	Collagen binding	–	–
hgbA	Heme and/or iron uptake	−2.54	−2.37
pal	Outer membrane stability	–	–
hfq	RNA binding chaperone	11.67	2.64
sapB-sapC	Resistance to antimicrobial peptides	–	–
cpxA	Two-component sensor kinase	–	–
Partially attenuated			
dltA	Partial role in serum resistance	–	4.98
wecA	Initiates synthesis of putative glycoconjugate	–	–
luxS	Quorum sensing	–	–
fgbA	Fibrinogen binding	2.04	–
sapA	Resistance to antimicrobial peptides	–	–
csrA	Posttranscriptional regulation	−2.34	–
dksA	DnaK repressor; (p)ppGpp cofactor	–	NA
relA, *spoT*	(p)ppGpp synthetase and hydrolase; stringent response	NA	2.09 (*spoT*)

[a] Strains with mutated genes that have been tested in human volunteers and classified as fully or partially attenuated.
[b] Fold change of the first differentially regulated gene in the operon. –, no change in expression; NA, not applicable.
[c] Fold change in 35000HPΔ*relA*Δ*spoT* mutant relative to 35000HP strain in stationary phase.
[d] Fold change in 35000HPΔ*dksA* mutant relative to 35000HP strain in stationary phase.
[e] Fold change of *flp-3* only.
[f] Fold change of the *lspB-lspA2* operon.

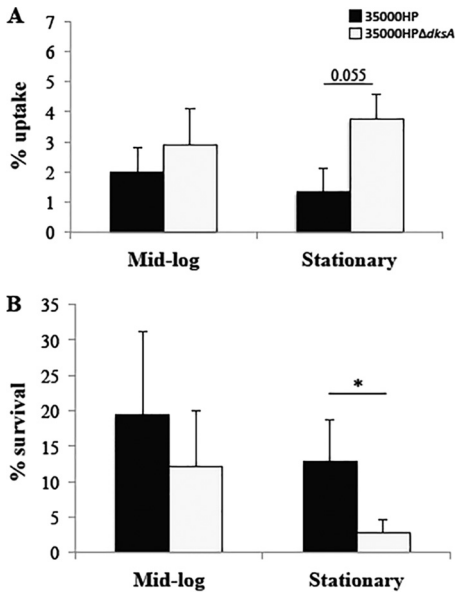

FIG 4 Uptake of *H. ducreyi* by and survival within human macrophages. (A) Percent uptake of 35000HP strain and 35000HPΔ*dksA* mutant strain by human monocyte-derived macrophages (MDMs). MDMs were infected with opsonized *H. ducreyi* at an MOI of 10:1. The percent uptake was calculated as follows: (geometric mean CFU of gentamicin-protected bacteria at 1 h/geometric mean CFU of input bacteria) × 100. (B) Percent survival of 35000HP strain and 35000HPΔ*dksA* mutant by MDMs. To determine survival, the MDMs were incubated an additional 5 h, and bacteria were collected. Survival was calculated as follows: (geometric mean CFU of bacteria collected at endpoint/geometric mean CFU of gentamicin-protected bacteria at 1 h) × 100]. Data are mean ± SD from 5 independent donors. Values that are significantly different ($P \leq 0.05$) are indicated by a bar and asterisk.

35000HPΔ*relA*Δ*spoT*(pACYC177) to 2 mM hydrogen peroxide was significantly restored in both 35000HPΔ*relA*Δ*spoT*(pCH30) ($P = 0.0002$) and 35000HPΔ*relA*Δ*spoT*(pCH24) ($P = 0.0029$) strains (Fig. 6). Therefore, DksA partially compensates for the loss of (p)ppGpp in resistance to oxidative stress *in vitro*.

DksA is important for adherence of *H. ducreyi* to HFF cells by a Flp-Tad-independent mechanism. The *flp-tad* operon is composed of a Tad secretion system that secretes three fimbria-like proteins, Flp1 to Flp3, which mediate adherence of the bacterium to HFF cells and are associated with virulence (40). The *flp-tad* operon was downregulated approximately 12-fold in the (p)ppGpp° mutant; only *flp-3* was downregulated in the *dksA* mutant (Table 3). We compared the adherence of strains 35000HP, 35000HPΔ*dksA*, 35000HPΔ*relA*Δ*spoT*, and 35000HPΔ*flp1-3* (strains harvested in stationary phase) to HFF cells. The 35000HPΔ*flp1-3* mutant served as a negative control for the assay. As expected, both the (p)ppGpp° (66.7% ± 9.5%) and 35000HPΔ*flp1-3* (4.6% ± 1.6%) mutants exhibited reduced attachment compared to the parent (115.8% ± 21.5%) ($P = 0.0002$ and $P = <0.0001$, respectively) (Fig. 7A). Surprisingly, the *dksA* mutant also attached to HFF cells at significantly lower levels than the parent (15.9% ± 5.2%; $P = <0.0001$) (Fig. 7A).

The decreased attachment of the *dksA* mutant could have resulted from decreased secretion of the Flp proteins (Flps) by the Tad secretion system, decreased expression of the Flp proteins, or decreased expression of a cofactor(s) required for Flp-dependent adherence (50). To determine whether the decreased adherence of the *dksA* mutant was a result of decreased expression or altered localization of the Flp proteins, we probed whole-cell lysates and outer membranes with Flp1/2 antiserum by Western blotting. The *tadA* mutant and the 35000HPΔ*flp1-3* mutant were used as con-

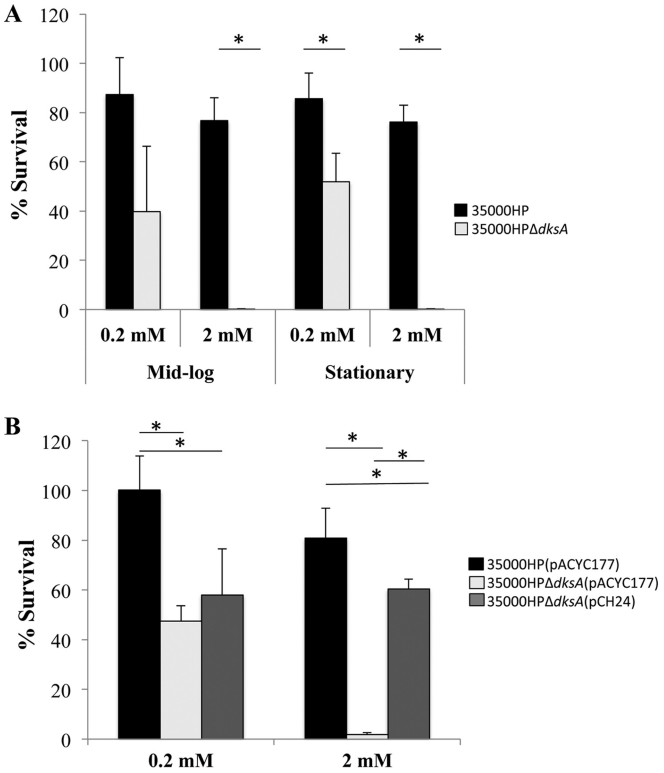

FIG 5 *H. ducreyi* survival after oxidative stress. (A) Percent survival of strains 35000HP and 35000HPΔ*dksA* following treatment with either 0.2 or 2 mM H_2O_2 at mid-log or stationary phase. (B) Percent survival of mid-log-phase strain 35000HP(pACYC177), strain 35000HPΔ*dksA*(pACYC177), and the complemented strain 35000HPΔ*dksA*(pCH24) following incubation with 0.2 mM or 2 mM H_2O_2 for 1 h. All values for percent survival were calculated as follows: (geometric mean CFU after treatment/geometric mean before treatment) × 100]. The data are means ± SD from five independent experiments. *, $P \leq 0.05$.

trols for the assay. As expected, the *tadA* mutant expressed the Flp proteins as seen in whole-cell lysates, but it did not secrete the proteins to the outer membrane (Fig. 7B and C). The (p)ppGpp° mutant expresses reduced levels of the Flp proteins (Fig. 7B). In contrast, both the wild type and the *dksA* mutant expressed the Flp proteins in whole cells and outer membranes (Fig. 7B and C). Thus, the adherence defect in the *dksA* mutant cannot be attributed to decreased expression or lack of localization of the Flp proteins to the outer membrane; these data suggest that DksA regulates the expression of unknown cofactor(s) that is required for Flp-mediated adherence *in vitro*.

DISCUSSION

The ability to respond to stress is a critical survival mechanism for bacterial pathogens. During human infection, *H. ducreyi* is found in the hostile environment of an abscess and utilizes several mechanisms to resist stress. We previously showed that (p)ppGpp, a molecule involved in the stress response to nutrient deprivation, was necessary for virulence of *H. ducreyi* in humans (23). In *E. coli*, DksA is a transcription factor that acts by binding directly to RNAP and amplifies the effects of (p)ppGpp to enhance the stringent response. In addition to its critical role in the stringent response, DksA can interact with RNAP and regulate gene transcription independently of (p)ppGpp. Here we showed that an *H. ducreyi dksA* mutant is also partially attenuated for virulence in human volunteers, indicating that DksA also plays an important role in *H. ducreyi* pathogenesis. We provided evidence that (p)ppGpp and DksA primarily contribute to the coordinated regulation of gene expression in stationary phase but also control some unique targets. Taken together, these data suggest an important role for (p)ppGpp and DksA in controlling *H. ducreyi* stationary phase and virulence gene expression.

In cells harvested from stationary phase, loss of (p)ppGpp and DksA led to differential expression of 28% and 17% of the *H. ducreyi* open reading frames, respectively, and significantly overlapped. We found that many of the differentially regulated genes

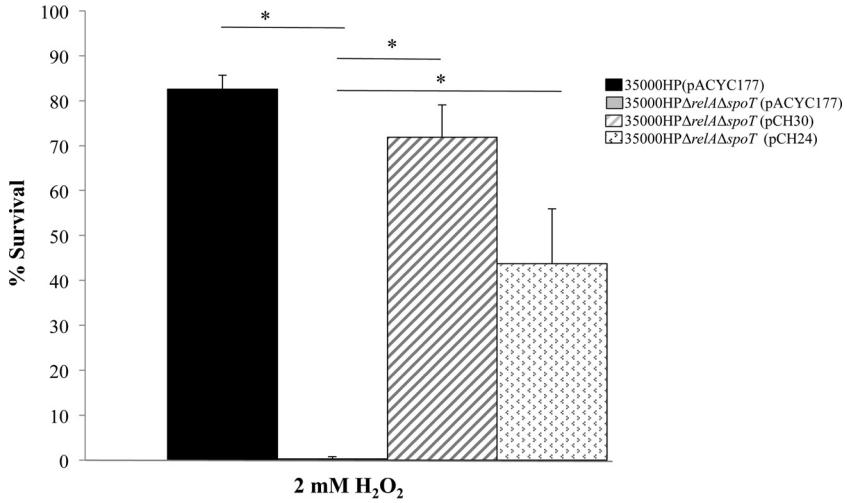

FIG 6 Complementation of the 35000HPΔ*relA*Δ*spoT* mutant strain by *dksA*. Percent survival of mid-log-phase strain 35000HP(pACYC177), strain 35000HPΔ*relA*Δ*spoT*(pACYC177), and strains 35000HPΔ*relA*Δ*spoT*(pCH30) (pCH30 is the *relA spoT* double complementation plasmid) and 35000HPΔ*relA*Δ*spoT*(pCH24) (pCH24 is the *dksA* complementation plasmid), following incubation with 0.2 mM or 2 mM H_2O_2 for 1 h. All values for percent survival were calculated as follows: (geometric mean CFU after treatment/geometric mean before treatment) × 100. The data are means plus SD from five independent experiments. *, $P \leq 0.05$.

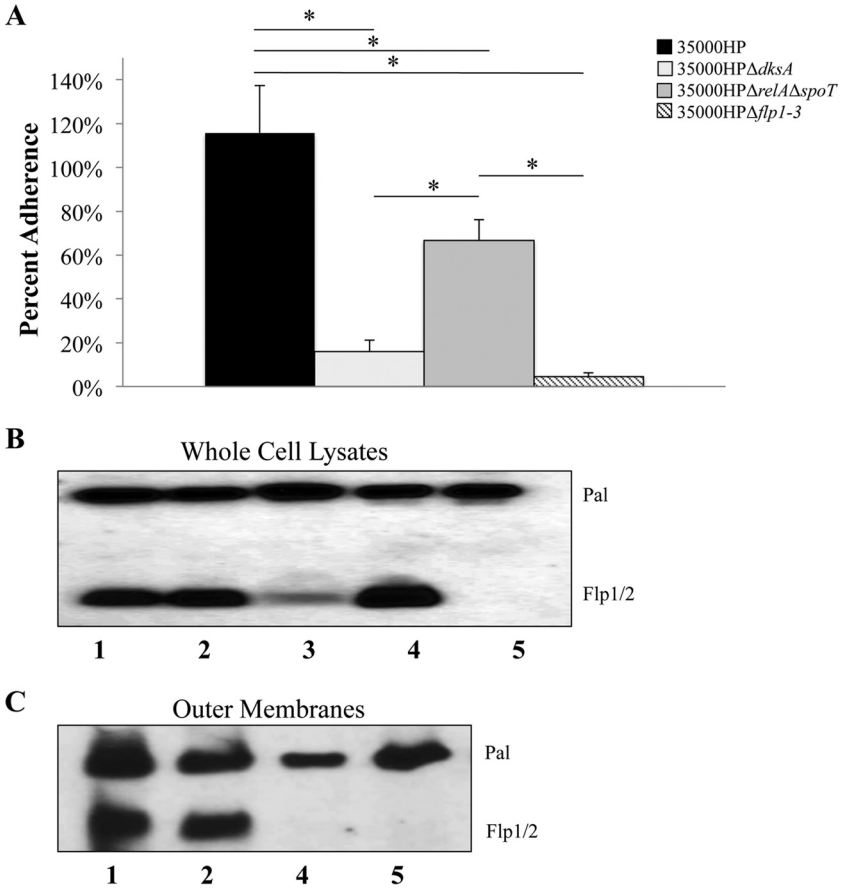

FIG 7 *H. ducreyi* adherence to HFF cells and Flp protein expression. (A) Percent adherence of *H. ducreyi* 35000HP, 35000HPΔ*dksA*, 35000HPΔ*relA*Δ*spoT*, and 35000HPΔ*flp1-3* to HFF cells calculated as follows: (geometric mean CFU of HFF-adherent bacteria/geometric mean CFU of initial bacteria added per well) × 100. The data are means plus SD from 5 independent experiments. *, $P \leq 0.05$. (B and C) Western blot analysis of whole-cell lysates (B) and purified outer membranes (C) analyzed by SDS-PAGE. Samples were probed with Flp1/2 antibody. MAb 3B9 was used to verify equivalent loading. Data are representative of four independent experiments. Lanes 1, 35000HP; lanes 2, 35000HPΔ*dksA*; lane 3, 35000HPΔ*rel*AΔ*spoT*; lanes 4, 35000HP.400, the *tadA* mutant; lanes 5, 35000HPΔ*flp1-3*, the *flp* mutant.

identified in our study have been previously associated with the stringent response (19, 51). (p)ppGpp and DksA deficiency in *H. ducreyi* resulted in altered expression of genes involved in transcription, translation, biosynthesis of macromolecules, and energy metabolism. This is consistent with findings in *E. coli* that the (p)ppGpp/DksA system regulates genes necessary for adaptation to nutritional stress (18, 19, 51). However, 27% of the genes regulated by DksA deficiency were independent of those regulated by (p)ppGpp deficiency. This suggests that *H. ducreyi* is similar to other organisms in that DksA functions both in concert with and independently of (p)ppGpp (18, 19).

Although the loss of DksA upregulated the expression of *hfq* transcripts, there was no significant overlap in the genes controlled by these regulators. In contrast, the loss of (p)ppGpp appeared to upregulate the expression of *hfq*, and the genes controlled by these factors significantly overlapped. (p)ppGpp deficiency also upregulated expression of *cpxR* transcripts and genes associated with CpxR activation. In *H. ducreyi*, Hfq positively regulates the expression of several virulence determinants, including *lspB-lspA2*, *flp1-3* (*flp-1* to *flp-3*), and *dsrA* (30). In contrast, activation of the CpxRA system downregulates the expression of *lspB*, *flp1-3*, and *dsrA* (44). (p)ppGpp deficiency resulted in decreased expression of *lspB-lspA2* and *flp1-3*, while *dsrA* expression was unchanged. These data suggest that upregulation of *cpxR* or another (p)ppGpp-dependent target overcomes the potential effects of overexpression of *hfq* on these virulence determinants in the (p)ppGpp-deficient mutant.

Both the *dksA* mutant and (p)ppGpp° mutant were partially attenuated for pustule formation in human volunteers. While the (p)ppGpp° mutant formed papules whose sizes were not significantly different than those of the parent, the *dksA* mutant caused significantly smaller papules than the parent did. Although we have not compared the two mutants directly in human volunteers, the data suggest that DksA and (p)ppGpp might have different effects on virulence. The *dksA* mutant was taken up more readily than the parent by macrophages, while the (p)ppGpp° mutant was phagocytized less readily than the parent was. Both the *dksA* mutant and (p)ppGpp° mutant exhibited increased sensitivity to oxidative stress. This increased sensitivity could be linked to the decreased survival within macrophages seen in both the *dksA* and (p)ppGpp° mutants (Fig. 4B and unpublished data). Both the *dksA* mutant and (p)ppGpp° mutant exhibited decreased attachment to HFF cells; however, the decreased attachment likely resulted from different mechanisms. Taken together, the data sug-

gest that DksA and (p)ppGpp have both distinct and overlapping functions.

The ability of *H. ducreyi* to attach to human foreskin fibroblasts has also been correlated with virulence, and the *dksA* mutant showed decreased attachment to HFF cells compared to the parent. Similarly, studies of pathogenic and nonpathogenic *E. coli* show that *dksA* mutants tend to have decreased attachment to eukaryotic cells as well as decreased expression of genes involved in the regulation of attachment (18, 19, 52). We found no evidence for decreased expression or altered localization of the Flp proteins in the *dksA* mutant. Taken together, these data suggest that DksA affects adherence to HFF cells through another mechanism, such as regulating one or more yet to be defined cofactors that are required for Flp-mediated adherence.

In summary, we show that DksA and (p)ppGpp likely serve as major contributors of virulence and stationary-phase gene regulation in *H. ducreyi*. We show that despite similar gene expression patterns, the *dksA* and (p)ppGpp° mutants are phenotypically distinct. Future studies will focus on identifying (p)ppGpp and/or DksA-dependent proteins to better understand the unique intersecting and diverging roles the two regulators play in *H. ducreyi* virulence.

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We declare that we have no conflicts of interest.

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