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A (p)ppGpp-Null Mutant of *Haemophilus ducreyi* Is Partially Attenuated in Humans Due to Multiple Conflicting Phenotypes

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(p)ppGpp responds to nutrient limitation through a global change in gene regulation patterns to increase survival. The stringent response has been implicated in the virulence of several pathogenic bacterial species. *Haemophilus ducreyi*, the causative agent of chancroid, has homologs of both *relA* and *spoT*, which primarily synthesize and hydrolyze (p)ppGpp in *Escherichia coli*. We constructed *relA* and *relA spoT* deletion mutants to assess the contribution of (p)ppGpp to *H. ducreyi* pathogenesis. Both the *relA* single mutant and the *relA spoT* double mutant failed to synthesize (p)ppGpp, suggesting that *relA* is the primary synthetase of (p)ppGpp in *H. ducreyi*. Compared to the parent strain, the double mutant was partially attenuated for pustule formation in human volunteers. The double mutant had several phenotypes that favored attenuation, including increased sensitivity to oxidative stress. The increased sensitivity to oxidative stress could be complemented in *trans*. However, the double mutant also exhibited phenotypes that favored virulence. When grown to the mid-log phase, the double mutant was significantly more resistant than its parent to being taken up by human macrophages and exhibited increased transcription of *lspB*, which is involved in resistance to phagocytosis. Additionally, compared to the parent, the double mutant also exhibited prolonged survival in the stationary phase. In *E. coli*, overexpression of DksA compensates for the loss of (p)ppGpp; the *H. ducreyi* double mutant expressed higher transcript levels of *dksA* than the parent strain. These data suggest that the partial attenuation of the double mutant is likely the net result of multiple conflicting phenotypes.

Haemophilus ducreyi is the causative agent of chancroid, a sexually transmitted genital ulcer disease that is endemic in the developing world (1). Like other genital ulcer diseases, chancroid facilitates both the transmission and acquisition of HIV-1 (1). Due to a short duration of infectivity, *H. ducreyi* can be maintained only in populations with high sex partner change rates, such as commercial sex workers. As a result of syndromic management of genital ulcers, chancroid prevalence has declined in many areas of endemicity and the overall global prevalence of chancroid is now undefined (2). However, reports of chancroid persist from Africa and Asia, implying that these regions have reservoirs of infected sex workers (3–8).

In the South Pacific, *H. ducreyi* also causes a non-sexually transmitted skin ulceration syndrome that clinically resembles yaws due to *Treponema pallidum* subspecies *pertenue* (9–13). In a recent prospective cohort study conducted in an area of yaws endemicity of Papua New Guinea, the prevalence of this syndrome is 3.2 cases per 100 persons; *H. ducreyi* DNA is detected in 60% of the ulcers and *Treponema pallidum* DNA is detected in 34%, suggesting that *H. ducreyi* is the major cause of this syndrome (9).

Although *H. ducreyi* is thought to be universally susceptible to third-generation cephalosporins, macrolides, and quinolones, reports of resistance to or treatment failures with these agents occurred in the early 1990s (14–16). Due to syndromic management, no new data on antimicrobial susceptibility have been available for nearly 20 years. Given the new epidemiological data and lack of information regarding its antimicrobial susceptibility, understanding the pathogenesis of *H. ducreyi* remains important for the development of alternative disease control strategies.

In order to study *H. ducreyi* pathogenesis, we developed a human inoculation model in which healthy adult volunteers are infected on the upper arm with *H. ducreyi* (17). The model mimics both the papular and pustular stages of natural infection. Importantly, in both natural and experimental infection, *H. ducreyi* is found within abscesses surrounded by macrophages and polymorphonuclear leukocytes (18). Within these abscesses, *H. ducreyi* encounters a variety of stresses, including antimicrobial peptides, an anaerobic environment, and reactive oxygen species (19).

When faced with stress, bacteria alter gene expression through the use of alternative sigma factors, two-component systems, and second messengers. Although these systems overlap, each system is optimized to respond to a particular stressor. The genome of *H. ducreyi* contains only two alternative sigma factors (RpoE and RpoH) and lacks RpoS, which usually plays a major role in stress tolerance and stationary-phase survival. Additionally, *H. ducreyi* has only one intact two-component system, CpxRA, which is dispensable for human infection (20). *H. ducreyi* contains homologs of *relA* and *spoT*, key components of the stringent response system; *relA* transcripts are upregulated during human infection (21).

The stringent response is usually induced by nutrient-limiting conditions. The response allows a global alteration in gene

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TABLE 1 Bacterial strains and plasmids used in this study

Strain(s) or plasmid	Description[a]	Source or reference
E. coli strains		
DH5α, Top10, and HB101	Strains used for general cloning procedures	Invitrogen
DY380	DH10B derivative containing a defective λ prophage in which the *red*, *bet*, and *gam* genes are controlled by the temp-sensitive λ*cI857* repressor	64
H. ducreyi strains		
35000HP	Human-passaged variant of strain 35000	65
35000HPΔ*relA*	Unmarked, in-frame *relA* deletion mutant	This study
35000HPΔ*relA*Δ*spoT*	Unmarked, in-frame *relA* and *spoT* double-deletion mutant	This study
FX517	35000HPΔ*dsrA*::*cat* insertion mutant	44
Plasmids		
pCH1	pCR-XL-TOPO containing the *relA*-coding region along with 0.5-kb flanking regions	This study
PCH11	pCR-XL-TOPO containing the *spoT*-coding region along with 0.5-kb flanking regions	This study
pRSM2832	Plasmid containing spectinomycin resistance cassette flanked by the FRT sites	66
pRSM2072	*H. ducreyi* suicide vector	33
pRSM2975	Plasmid containing the origin of replication and kanamycin resistance gene from pLS88, FLP recombinase gene from pFT-A, and a point mutation conferring a temp-sensitive phenotype in *H. ducreyi*	42
pACYC177	Cloning vector, Kan^r Amp^r for complementation	New England Biolabs
pACYC184	Cloning vector, Cm^r Tet^r	New England Biolabs
pCH30	pACYC177 containing the *cat* promoter from pACYC184 followed by the 35000HP *spoT* gene in front of the 35000HP *relA* gene	This study

[a] Kan^r, kanamycin resistance; Amp^r, ampicillin resistance; Cm^r and *cat*, chloramphenicol resistance and gene; Tet^r, tetracycline resistance.

expression through guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp), collectively referred to here as (p)ppGpp. In response to amino acid limitation, RelA catalyzes the synthesis of (p)ppGpp. (p)ppGpp directly interacts with the β-subunit of the RNA polymerase, altering its activity (22, 23). Through its interaction with RNA polymerase, (p)ppGpp upregulates genes that are necessary for survival and increases use of alternative sigma factors. Simultaneously, genes that are not crucial for survival, such as those involved in protein synthesis and growth, are downregulated. Upon removal of the stressor, SpoT degrades (p)ppGpp to quench the response. Under certain conditions such as iron limitation, SpoT's limited (p)ppGpp synthetic capabilities also serve to initiate the stringent response (24). A mutant with deletions in both *relA* and *spoT* is generally incapable of initiating a stringent response; such strains are called (p)ppGpp null [(p)ppGpp^0] mutants.

The stringent response is linked to the virulence of several pathogenic bacterial species. *Legionella pneumophila* (p)ppGpp^0 mutants are defective in transmission between macrophages, a critical component of *Legionella* pathogenesis (25). (p)ppGpp^{0}mutants of *Yersinia pestis*, *Salmonella enterica* serovar Typhimurium, and *Borrelia burgdorferi* are completely avirulent in animal models of infection (26–28). (p)ppGpp^0 mutants of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, and *Enterococcus faecalis* are less virulent than their wild-type parents in their respective animal models of infection (29–32). However, the role of the stringent response in a bacterial pathogen has never been directly studied in humans.

To investigate the role of the stringent response in *H. ducreyi* pathogenesis, we constructed *H. ducreyi* Δ*relA* and Δ*relA*Δ*spoT* mutants, the latter of which should be (p)ppGpp^0. We compared the virulence of the double mutant to that of the parent strain in human inoculation experiments. We also compared both mutants to the parent strain in phagocytosis, serum killing, and oxidative stress tolerance assays. We found that the (p)ppGpp mutants had increased sensitivity to oxidative stress. Importantly, *relA* and *spoT* contributed to disease progression in human volunteers.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *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 (HyClone), 1% IsoVitalex, and 50 μg/ml hemin (Aldrich Chemical Co.) at 33°C. *H. ducreyi* 35000HP was grown to the mid-log phase (optical density at 660 nm [OD_{660}] = 0.2), transition phase (OD_{660} = 0.3 to 0.35), and stationary phase (OD_{660} = 0.45 to 0.55); mutant strains were harvested when the OD_{660} of 35000HP was in the appropriate range for each phase of growth. *Escherichia coli* strains were grown in Luria-Bertani media 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, media were 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, media were supplemented with 30 μg/ml kanamycin.

Reverse transcriptase PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was treated with Turbo DNA-free DNase (Ambion) and purified using an RNeasy minikit (Qiagen). The integrity and concentration of the RNA were determined using an Agilent Bioanalyzer (Agilent Technologies) and a NanoDrop ND2000 (Thermo Scientific), respectively. cDNA was synthesized from total RNA using a Super Smart cDNA synthesis kit (Clontech).

RT-PCR and qRT-PCR were performed using a QuantiTect SYBR green RT-PCR kit (Qiagen) in an ABI Prism 7000 sequence detector (Applied Biosystems). All primer pairs (see Table S1 in the supplemental material) had greater than 95% amplification efficiency. Relative expres-

sion was calculated as $[(E_{target}{}^{\Delta CT target})/(E_{referencce}{}^{\Delta CT reference})]$, where E is the amplification efficiency, $(10^{-1}/\text{slope})$, and ΔCT is the change in the cycle threshold. For normalization of expression levels of target genes, *dnaE* was amplified using primer pair P1 and P2.

Characterization of the *relA* and *spoT* genes. The Basic Local Alignment Search Tool (BLAST) was used to identify putative homologues of *relA* and *spoT* in *H. ducreyi* (GenBank accession no. AE017143). RT-PCR was conducted to determine if *relA* or *spoT* was in an operon with surrounding genes. The *relA*-to-*dxr* intergenic region was amplified using primers P3 and P4. The intergenic regions around *spoT* were amplified using primers P5 and P6 for *recG* to *rpoZ*, P7 and P8 for *rpoZ* to *spoT*, and P9 and P10 for *spoT* to *menB*.

Construction and complementation of unmarked, in-frame *relA* single-deletion and *relA spoT* double-deletion mutants. An unmarked, in-frame *relA* deletion mutant was constructed using the λ red and FLP recombinase method described previously (33, 34). To construct a *relA* deletion mutant, primers P11 and P12 were used to amplify the spectinomycin (*spec*) resistance cassette flanked by flippase recognition target (FRT) sites from pRSM2832. P11 included 47 bp upstream of *relA* and the ATG start codon. P12 included 21 bp at the 3′ end of *relA*, the stop codon, and 29 bp downstream of *relA*.

The coding region of *relA*, along with a 0.5-kb flanking region on either side of the gene, was amplified using primers P13 and P14. The product was cloned into pCR-XL-TOPO. The construct, designated pCH1, was then electroporated into *E. coli* DY380, which contains a temperature-sensitive λ red recombinase. The product from primers P11 and P12 was electroporated into the DY380 strain generated as described above. Following induction of the recombinase, the *relA* gene was replaced with the *spec* cassette with the exception of the start codon and the last 21 bp of the *relA* gene. The *spec* cassette containing the flanking regions was digested with SpeI and cloned into the pRSM2072 suicide vector. This construct was electroporated into strain 35000HP. Chocolate agar containing spectinomycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was used to detect clones in which allelic exchange had occurred. PCR and sequence analysis confirmed the replacement of the *relA* gene with the *spec* cassette.

FLP recombinase was used to remove the *spec* cassette as described previously (33). PCR and sequence analysis confirmed deletion of *relA*. The final mutant was designated 35000HPΔ*relA*. Primers P15 and P16 were used to determine if *relA* affected expression of the downstream *dxr* gene using qRT-PCR; deletion of *relA* did not affect transcription of *dxr* (data not shown).

Using these methods, a *spoT* mutant was not recovered. As has been observed for other species, a *spoT* deletion mutant may be lethal in *H. ducreyi* due to unopposed ppGpp synthesis by *relA* (35). Therefore, we constructed a *relA spoT* double-deletion mutant by deleting *spoT* in the *relA* mutant exactly as described above, except that primers P17 and P18 were used to amplify the *spec* cassette and primers P19 and P20 were used to amplify the *spoT* coding and flanking regions, resulting in pCH11 (Table 1). After allele exchange and excision of the *spec* cassette, PCR and sequence analysis confirmed deletion of the *spoT* gene. The double mutant was designated 35000HPΔ*relA*Δ*spoT*. To determine if deletion of *spoT* affected the expression of its *menB* downstream gene, qRT-PCR was performed using primers P21 and P22; deletion of *spoT* did not affect transcription of *menB* (data not shown).

To complement the *relA spoT* deletion mutant, the *relA* and *spoT* genes were expressed under the control of a constitutive chloramphenicol promoter from pACYC184 in the pACYC177 expression vector. Briefly, the *spoT* open reading frame (ORF), along with 21 bp of the upstream region and 22 bp of the downstream region, was amplified using primers P23 and P24. The *relA* open reading frame, along with 21 bp of the upstream region and a 45-bp downstream region, which includes the native transcription terminators, was amplified using primers P25 and P26. The chloramphenicol promoter was amplified from pACYC184 using primers P27 and P28. The PCR fragments were combined with BamHI-digested pACYC177 in a Gibson assembly reaction (36). Briefly, the three fragments were incubated at 50°C for 1 h in a reaction mixture (kindly provided by M. Goebl, Indiana University) that contained T5 exonuclease (Epicentre), Phusion *Taq* (New England BioLabs), and *Taq* DNA ligase (New England BioLabs). The final construct, designated pCH30, was confirmed by PCR and sequence analysis and electroporated into *H. ducreyi* 35000HPΔ*relA*Δ*spoT* to yield *H. ducreyi* 35000HPΔ*relA*Δ*spoT*(pCH30). As controls, *H. ducreyi* 35000HP and 35000HPΔ*relA*Δ*spoT* were also electroporated with pACYC177, and the resulting strains were designated *H. ducreyi* 35000HP(pACYC177) and 35000HPΔ*relA*Δ*spoT*(pACYC177), respectively.

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. Healthy adult volunteers (6 females and 4 males; 7 African Americans, 1 Asian, and 2 European Americans; mean age ± standard deviation, 48 ± 9.17 years) over 21 years of age initially enrolled in the study. All volunteers gave written, informed consent for participation and HIV serology. Two volunteers were excluded due to underlying medical conditions, and two withdrew consent prior to the start of the trial. The procedures for the human inoculation experiments, including calculation of the estimated delivered dose (EDD), are described in detail elsewhere (17). Papule and pustule formation rates for parent and mutant inoculation sites were compared using logistic regression with generalized estimating equations (GEE) as previously described (37). Ninety-five-percent confidence intervals (95% CI) for papule and pustule formation rates were calculated using GEE-based sandwich standard errors.

To ensure that the inocula contained the correct strains and 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 *relA*, *spoT*, and *dnaE* were designed using primer pair P29 and P30, primer pair P31 and P32, and primer pair P1 and P2, respectively. A digoxigenin (DIG) DNA labeling kit (Roche Applied Sciences) was used to label the probes with digoxigenin, and detection was performed using the DIG Easy Hyb protocol from the manufacturer's instructions.

(p)ppGpp synthesis assay. For detection of (p)ppGpp synthesis in *H. ducreyi*, we modified two published assays (38, 39). Briefly, *H. ducreyi* strains were grown in GC broth. At different phases of growth, the bacteria were harvested by centrifugation, washed in phosphate-limited RPMI medium (US Biological Life Sciences), and suspended (1×10^5 cells/ml) in phosphate-limited RPMI medium. Pilot experiments showed that there was no reduction in bacterial viability for up to 4 h in this media. For the labeling experiments, the bacteria were suspended in phosphate-limited RPMI media supplemented with 5% fetal bovine serum (FBS) and $KH_2{}^{32}PO_4$ (PerkinElmer) (specific activity, 900 to 1,100 mCi/mmol) at 100 μCi/ml. The bacteria were incubated for 3 h, washed, and incubated for an additional 15 min in phosphate-limited RPMI medium. For extraction of (p)ppGpp, cells were treated with 40 mg/ml lysozyme–10 mM Tris HCl (pH 8.0) for 20 min and lysed on ice using 1% SDS (wt/vol) for 20 min. Finally, an equal volume of 13 M formic acid was added to the lysate, which was incubated on ice for 15 min. Cell debris was removed by centrifugation. A sample of the supernatant containing the extracted (p)ppGpp was spotted onto polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) plates (Sigma-Aldrich). (p)ppGpp was separated in 1.5 M H_3PO_4 (pH 3.4) buffer and detected using autoradiography. CTP, ATP, and GTP standards (Sigma-Aldrich) were detected using iodine staining.

Phenotypic comparisons. Lipooligosaccharides (LOS) and outer membrane proteins (OMP) were isolated from *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* and analyzed as described previously (40, 41). Bactericidal assays were performed on plate-grown organisms as described previously (42). To determine the expression levels

of OmpP2B, DsrA, and the peptidoglycan-associated lipoprotein (PAL), Western blot analyses were performed using purified outer membranes and the blots were probed with antisera or monoclonal antibodies specific to each protein. The antisera for OmpP2B (kindly provided by A. Campagnari, State University of New York [SUNY]-Buffalo) and DsrA (kindly provided by C. Elkins, University of North Carolina) and the PAL monoclonal antibody were described elsewhere (41, 43–45).

To determine if the *relA* and *spoT* deletion affected the transcript levels of specific *H. ducreyi* genes, total RNA was extracted from mid-log- and stationary-phase *H. ducreyi* 35000HP and 35000HPΔ*relA*Δ*spoT*. qRT-PCR was performed using primer pair P33 and P34 to amplify *cpxA*, primer pair P35 and P36 to amplify *lspB*, primer pair P37 and P38 to amplify *oxyR*, primer pair P39 and P40 to amplify *sodC*, and primer pair P41 and P42 to amplify *dksA*.

Macrophage uptake assays. Human peripheral blood mononuclear cells (PBMCs) were isolated from leukopacks obtained from anonymous donors from the Central Indiana Regional Blood Center or from blood obtained from healthy volunteers. Informed consent was obtained in accordance with an IRB-approved protocol. PBMCs were isolated by Ficoll-Pacque Plus purification, and $CD14^+$ cells were isolated (Miltenyi Biotech). The $CD14^+$ 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 MDMs were harvested by centrifugation and seeded into 24-well tissue culture plates at 4×10^5 cells per well and grown for 24 h. *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* were grown to the mid-log and stationary phases and opsonized in 100% complement-replete normal human serum for 20 min at room temperature. After washes with Hanks' balanced salt solution (HBSS) were performed, the MDMs were infected with bacteria at a multiplicity of infection (MOI) of 10:1. The plates were centrifuged at 1,800 × *g* for 5 min to synchronize the infections and were incubated for 30 min at 35°C in 5% CO_2. To kill extracellular bacteria, gentamicin (100 μg/ml) was added, and the MDMs were incubated for 30 min and washed with HBSS. The MDMs were lysed with 0.2% saponin at room temperature for 10 min, and the lysates were quantitatively cultured. The percentage of bacterial uptake was calculated as the ratio of bacteria within the lysed MDMs compared to the initial CFU.

Oxidative stress and heat shock assays. Bacteria were treated either with hydrogen peroxide (H_2O_2) to mimic extracellular oxidative stress or with paraquat to mimic intracellular oxidative stress, as described previously (46). Briefly, *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* were grown to the mid-log or stationary phase and exposed to 0.2 mM and 2 mM H_2O_2 (Sigma-Aldrich) or 0.2 mM and 2 mM paraquat (Sigma-Aldrich) with PBS at 33°C for 1 h and quantitatively cultured. To determine the effect of deletion of *relA* and *spoT* on the ability to survive heat shock, cells collected from the mid-log and stationary phases were incubated at 37°C for 1 h in PBS and quantitatively cultured. Percent survival was calculated as the ratio of recovered bacteria to the input CFU. An untreated control was utilized as a measure of viability. Due to growth issues with *H. ducreyi* strains containing the pACYC177 vector, for complementation assays, *H. ducreyi* 35000HP(pACYC177), 35000HPΔ*relA*Δ*spoT*(pACYC177), and 35000HPΔ*relA*Δ*spoT*(pCH30) were grown only to the mid-log phase and harvested, and the assay was performed as described above.

Statistical analysis. Stress, bactericidal, and macrophage uptake data were analyzed using a mixed-model analysis of variance (ANOVA) followed by Tukey's honestly significant difference test. RT-PCR and densitometry data were analyzed using a paired *t* test followed by Tukey's adjustment. An adjusted two-sided *P* value of ≤0.05 was considered statistically significant. The data are expressed as means ± standard deviations. For growth curve analysis, the data were log transformed before analysis to correct for the large amount of skewness in the distribution. They were then analyzed using repeated-measurement (mixed-model) ANOVA followed by pairwise analysis of the 3 strains at each time point. The tests were adjusted for multiple comparisons using the Tukey method.

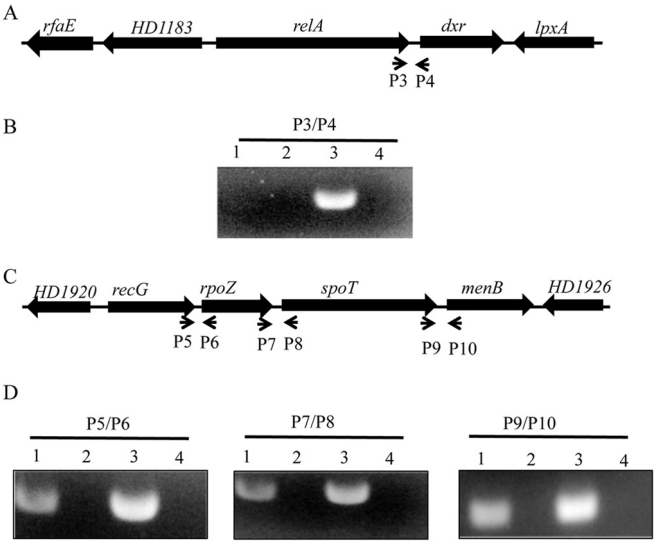

FIG 1 Chromosomal loci containing *relA* and *spoT* in *H. ducreyi*. (A) Genomic organization of *relA* locus in *H. ducreyi*. The small arrows indicate locations of intergenic primers used for RT-PCR analysis. (B) Agarose gel electrophoresis of the *relA*-to-*dxr* intergenic region amplified by RT-PCR. Lane 1, RNA sample with reverse transcriptase; lane 2, RNA sample without reverse transcriptase control; lane 3, genomic DNA control; lane 4, no-template control. (C) Genomic organization of *spoT* locus in *H. ducreyi*. (D) Agarose gel electrophoresis of products amplified by RT-PCR. The intergenic regions of *recG* to *rpoZ*, *rpoZ* to *spoT*, and *spoT* to *menB* were examined. Lanes are as described for panel B. Products were of the expected sizes: *relA* to *dxr*, 515 bp; *recG* to *rpoZ*, 541 bp; *rpoZ* to *spoT*, 497 bp; *spoT* to *menB*, 222 bp.

RESULTS

Characterization of the *relA* and *spoT* genomic loci. The *H. ducreyi* homolog of *relA* (HD1185) is in the same orientation and 77 bp upstream of the *dxr* coding region, which is predicted to encode a DXP reductoisomerase (Fig. 1A). To examine whether *relA* and *dxr* were in an operon, we conducted RT-PCR on the intergenic regions between *relA* and *dxr*. Although RT-PCR confirmed that both the *relA* and *dxr* transcripts were present in the cDNA utilized for analysis (data not shown), the data suggested that *relA* had not been cotranscribed with *dxr* (Fig. 1B). The *H. ducreyi spoT* homolog (HD1924) is in a putative operon with the gene order *recG* → *rpoZ* → *spoT* → *menB* (Fig. 1C). The *recG*, *rpoZ*, and *menB* genes are predicted to encode an ATP-dependent DNA helicase, RNA polymerase omega chain, and dihydroxynaphthoic acid synthase, respectively. RT-PCR suggested that *spoT* is cotranscribed with *rpoZ*, *menB*, and *recG* (Fig. 1D).

Construction of *relA* and *relA spoT* deletion mutants. To understand the role of (p)ppGpp in *H. ducreyi* pathogenesis, we constructed an unmarked, in-frame *relA* single-deletion mutant and an unmarked, in-frame *relA spoT* double-deletion mutant. PCR and sequence analysis confirmed that both the *relA* and *spoT* genes were deleted in *H. ducreyi* 35000HPΔ*relA*Δ*spoT*, with the exception of the start codon and the last 21 bp of the *relA* and *spoT* ORFs.

***H. ducreyi* synthesizes (p)ppGpp, and deletion of *relA* and *spoT* results in loss of (p)ppGpp synthesis.** To determine if *H. ducreyi* synthesizes (p)ppGpp, *H. ducreyi* 35000HP,

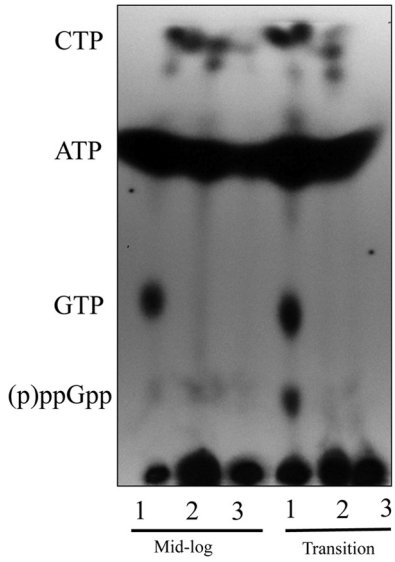

FIG 2 *De novo* synthesis of (p)ppGpp in *H. ducreyi*. Bacteria were labeled with $KH_2{}^{32}PO_4$ in phosphate-limited RPMI medium plus 5% FBS. (p)ppGpp was extracted and detected by polyethylenimine cellulose thin-layer chromatography and autoradiography. Synthesis of (p)ppGpp was detected in cells harvested from the mid-log and transition phases of growth. Lanes 1, *H. ducreyi* 35000HP; lanes 2, *H. ducreyi* 35000HPΔ*relA*; lanes 3, *H. ducreyi* 35000HPΔ*relA*Δ*spoT*. The blot is representative of three independent experiments.

35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* were grown to the mid-log, transition, and stationary phases, harvested, and labeled with $KH_2{}^{32}PO_4$; (p)ppGpp was detected using autoradiography. This assay detects *de novo* (p)ppGpp synthesis only in the absence of induced stress. (p)ppGpp synthesis was detected in strain 35000HP grown to the mid-log and transition phases; maximal synthesis was detected in cells harvested from the transition phase (Fig. 2). Synthesis of (p)ppGpp was detected only variably in 35000HP harvested from the stationary phase (data not shown). We detected only one species of the molecule and were unable to distinguish between ppGpp and pppGpp. In both *H. ducreyi* 35000HPΔ*relA* and *H. ducreyi* 35000HPΔ*relA*Δ*spoT* collected from all phases of growth, synthesis of (p)ppGpp was not detected. Thus, under these growth conditions, both the *relA* single mutant and the *relA spoT* double mutant had a $(p)ppGpp^0$ phenotype.

***relA* and *spoT* contribute to *H. ducreyi* virulence in human volunteers.** To determine if RelA and SpoT contribute to virulence in humans, we inoculated volunteers in four iterations with *H. ducreyi* 35000HP and with the 35000HPΔ*relA*Δ*spoT* mutant. In the first iteration, one volunteer was inoculated at three sites with an EDD of 157 CFU of *H. ducreyi* 35000HP and at three sites with an EDD of 24, 48, and 96 CFU of *H. ducreyi* 35000HPΔ*relA*Δ*spoT*. In the second iteration, three volunteers were each inoculated at three sites with an EDD of 74 CFU of *H. ducreyi* 35000HP and with the 35000HPΔ*relA*Δ*spoT* mutant at three sites with EDDs of 33, 66, and 132 CFU. In the third iteration, one volunteer was inoculated with an EDD of 49 CFU of *H. ducreyi* 35000HP at three sites and at EDDs of 21, 41, and 81 CFU of the 35000HPΔ*relA*Δ*spoT* mutant at three sites. In the final iteration, one volunteer was inoculated with an EDD of 81 CFU of *H. ducreyi* 35000HP at three sites and at three sites with EDDs of 35, 71, and 141 of the 35000HPΔ*relA*Δ*spoT* mutant. Overall, papules formed at 94% (95% CI, 84.5% to 99.9%) of the parent-inoculated sites and at 94% (95% CI, 84.5% to 99.9%) of the mutant-inoculated sites ($P = 1.0$) (Table 2). After 24 h of infection, the mean surface areas of the papules were 23.7 ± 24.4 mm^2 at parent sites and 18.2 ± 22.4 mm^2 at mutant sites ($P = 0.36$). Pustules formed at 72.2% (95% CI, 48.3% to 96.2%) of parent sites and 27.8% (95% CI, 0.1% to 56.2%) of mutant sites ($P = <0.0001$).

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

Volunteer (sex)[a]	Observation period (days)	Strain[b]	Dose (CFU)[c]	No. of initial papules	Final papule outcome	
					No. resolved	No. of pustules
433 (F)	7	P	157	3	2	1
		M	24–96	3	3	0
436 (F)	8	P	74	3	0	3
		M	33–132	3	2	1
437 (F)	7	P	74	3	0	3
		M	33–132	2	1	1
438 (M)	7	P	74	3	0	3
		M	33–132	3	0	3
439 (M)	7	P	49	3	1	2
		M	21–81	3	3	0
440 (M)	9	P	81	2	1	1
		M	35–141	3	2	0

[a] Volunteer 433 was inoculated in the first iteration. Volunteers 436, 437, and 438 were inoculated in the second iteration. Volunteer 440 was inoculated in the third iteration. Volunteer 439 was inoculated in the fourth iteration. M, male; F, female.
[b] P, parent *H. ducreyi* strain 35000HP; M, mutant *H. ducreyi* strain 35000HPΔ*relA*Δ*spoT*.
[c] Doses inoculated at 3 sites, except 24–96 (one dose each of 24, 48, and 96 CFU), 33–132 (one dose each of 33, 66, and 132 CFU), 21–81 (one dose each of 21, 41, and 81 CFU), and 35–141 (one dose each of 35, 71, and 141 CFU).

Thus, the *relA spoT* mutant was partially attenuated for pustule formation in humans.

Three volunteers (436, 437, and 438) developed pustules at both mutant and parent sites. For each subject, one parent site and one mutant site were subjected to a biopsy procedure and the biopsy specimens were divided in half and semiquantitatively cultured or stained with hematoxylin-eosin and anti-CD3 antibodies as previously described (47). All samples contained micropustules in the epidermis and dermal CD3 cells (data not shown). Histopathologically, the pustules formed at mutant sites were indistinguishable from the pustules formed at parent sites.

All colonies recovered from the inocula, surface cultures, and biopsy specimens were tested for the presence of *relA*, *spoT*, and *dnaE* sequences by colony hybridization. The *dnaE* probe hybridized to all the colonies tested from both parent ($n = 144$) and mutant ($n = 140$) inocula, while the *relA* and *spoT* probes hybridized only to colonies from the parent inocula. At least one positive surface culture for *H. ducreyi* was obtained during follow-up visits from 39.9% of the parent inoculation sites and 16.7% of the mutant inoculation sites. The *dnaE* probe hybridized to colonies from both parent-inoculated sites ($n = 280$ colonies) and mutant-inoculated sites ($n = 32$ colonies), while the *relA* and *spoT* probes bound only to colonies from the parent sites. All 3 paired biopsy specimens of mutant and parent pustules yielded *H. ducreyi*. The *dnaE* probe hybridized to all parent ($n = 108$) and mutant ($n = 101$) colonies obtained from the biopsy specimens, while the *relA* and *spoT* probes hybridized only to colonies obtained from the parent biopsy specimens. There was no evidence of cross contamination between the inocula and mutant and parent inoculation sites.

Deletion of *relA* and *spoT* affects the growth and survival of *H. ducreyi*. To determine the effect of the loss of (p)ppGpp on the growth and survival of *H. ducreyi*, we compared the growth of the mutant strains to the growth of the parent strain in broth culture. By quantitative culture, the strain results determined by time interaction analysis were significant ($P = <0.0001$), indicating that the differences between the parent strain and either mutant were not consistent over time. The growth of each mutant strain was not different from that of the parent strain from 0 to 16 h (Fig. 3), but both mutants survived longer than the parent strain in broth culture from 24 to 36 h ($P = <0.0001$), suggesting that the lack of production of (p)ppGpp enhanced survival in the stationary phase.

Deletion of *relA* alters the OMP profile of *H. ducreyi*. As required by our clinical protocol, we investigated whether deletion of *relA* and *spoT* affected the expression of outer membrane components of *H. ducreyi*. LOS and OMP were prepared from *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* harvested from mid-log phase. The 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* strains had identical LOS profiles (data not shown). Analysis of OMP showed increased expression of a band that migrates between 40 to 45 kDa in both *H. ducreyi* 35000HPΔ*relA* (data not shown) and *H. ducreyi* 35000HPΔ*relA*Δ*spoT* compared to the parent strain (Fig. 4A). Preliminary tandem mass spectrometry experiments done on the excised band suggested that the overexpressed protein was OmpP2B, a putative porin previously described in *H. ducreyi* (data not shown) (43). Western blot analysis performed with an OmpP2B-specific antibody confirmed that the *relA spoT* mutant expressed more OmpP2B than the parent strain (Fig. 4B).

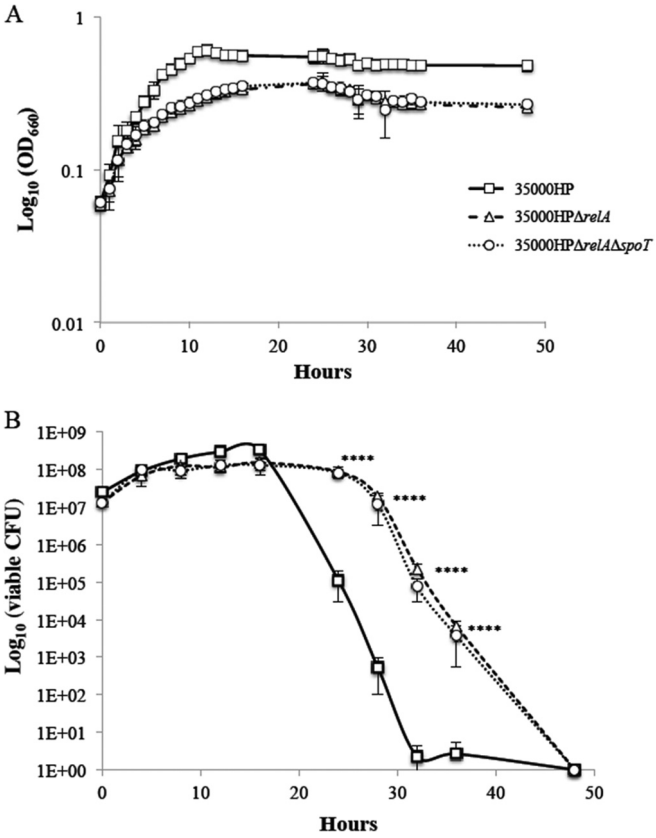

FIG 3 Growth of *H. ducreyi* (p)ppGpp mutants. (A) Growth of *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* measured by absorbance at OD_{660} over 48 h. The OD data are means ± standard deviations (SD) of the results of 3 independent experiments. (B) Growth of *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* measured as CFU over 48 h. The CFU data are geometric means ± SD of the results of three independent experiments. ****, $P \leq 0.0001$.

By OMP profile analysis, the size of a band that migrates with the apparent molecular weight of DsrA, the major determinant of serum resistance, seemed to be decreased in both *H. ducreyi* 35000HPΔ*relA* (data not shown) and *H. ducreyi* 35000HPΔ*relA*Δ*spoT* relative to strain 35000HP (Fig. 4A). By Western blotting, the level of expression of DsrA in *H. ducreyi* 35000HPΔ*relA*Δ*spoT* was somewhat reduced compared to that in strain 35000HP (Fig. 4C). We harvested cells from the 3 strains collected at the mid-log, transition, and stationary phases of growth; Western blot and densitometry analysis showed that expression of DsrA was significantly reduced in both the single mutant ($P = 0.05$) and the double mutant ($P = 0.043$) compared to that in the parent strain in cells collected at mid-log phase but not in cells collected at the transition or stationary phase (all P values $>$ 0.24) (Fig. S1A in the supplemental material and data not shown). In bactericidal assays using 50% normal human serum, *H. ducreyi* 35000HPΔ*relA*Δ*spoT* was as resistant to killing as the parent strain (see Fig. S1B). Thus, the reduction in DsrA expression in the mutant during the mid-log phase may not be biologically significant.

RelA and SpoT do not contribute to heat stress resistance. In other organisms, (p)ppGpp induces expression of genes critical for responses to heat shock such as *rpoH* (48–50). *H. ducreyi* 35000HP grows optimally at 33°C (51). To determine if *relA*

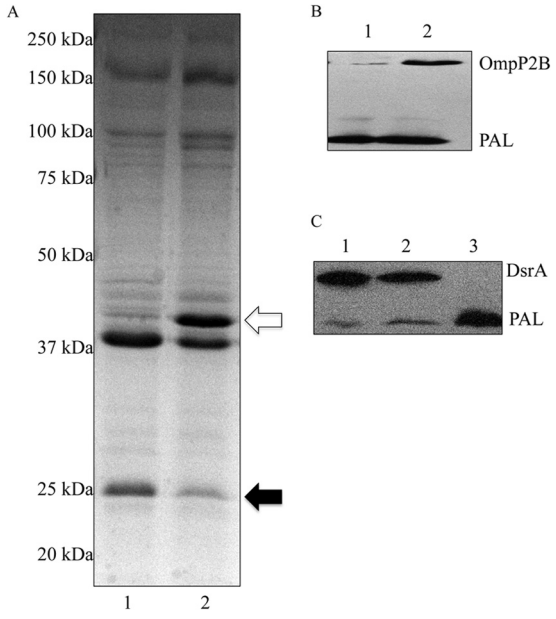

FIG 4 Outer membrane protein expression by *H. ducreyi* harvested from the mid-log phase. (A) Purified outer membranes were analyzed by SDS-PAGE and Coomassie staining. Arrows indicate protein bands selected for further analysis. Open arrow, OmpP2B; filled arrow, DsrA. Lane 1, *H. ducreyi* 35000HP; lane 2, *H. ducreyi* 35000HPΔ*relA*Δ*spoT*. (B) Western blot analysis of purified outer membrane proteins of *H. ducreyi* 35000HP and 35000HPΔ*relA*Δ*spoT*. Lysates were probed with OmpP2B-specific antibody. (C) Western blot analysis of purified outer membrane proteins of *H. ducreyi* 35000HP, *H. ducreyi* 35000HPΔ*relA*Δ*spoT*, and the *dsrA* mutant, FX517. Lysates were probed with DsrA-specific antibody. PAL monoclonal antibody (MAb) 3B9 was used to verify equivalent levels of loading. Lanes 1 and 2 are as described for panel A; lane 3, FX517.

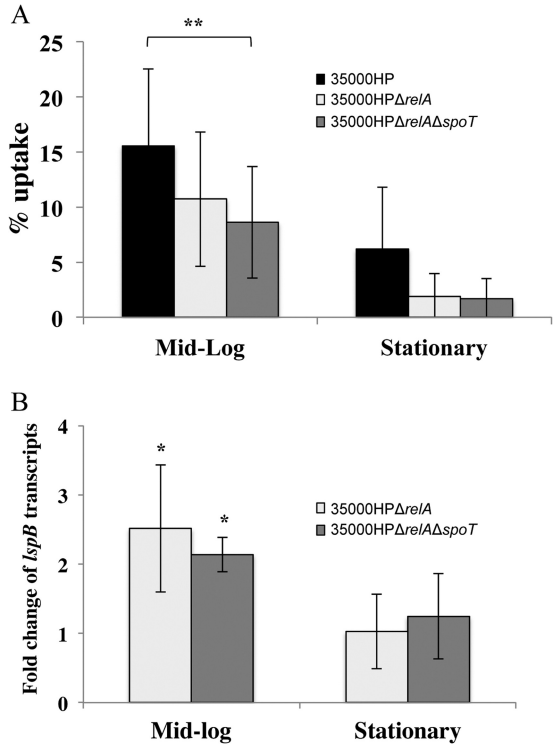

FIG 5 Uptake of *H. ducreyi* by human macrophages. (A) Percent uptake of *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* by human macrophages. Primary human CD14$^+$ cells were differentiated into monocyte-derived macrophages (MDMs). MDMs were infected with opsonized *H. ducreyi* at an MOI of 10:1. After 30 min of incubation, gentamicin was added to kill extracellular bacteria. Percent uptake was calculated as [(geometric mean CFU of gentamicin-protected bacteria at 1 h/geometric mean CFU of input bacteria) × 100]. Data are means ± SD of the results determined with five independent donors. (B) Fold change in the expression of *lspB* in *H. ducreyi* 35000HPΔ*relA* and 35000HPΔ*relA*Δ*spoT* relative to 35000HP. Expression levels of genes were normalized to *dnaE*. The data represent the means ± SD of the results from three independent RNA samples. *, $P \leq 0.05$; **, $P \leq 0.01$.

and *spoT* had a role in regulating responses to heat shock, we compared the survival of *H. ducreyi* 35000HPΔ*relA* and 35000HPΔ*relA*Δ*spoT* to that of strain 35000HP after incubation at 37°C for 1 h. Percent survival rates were not significantly different among the strains (data not shown). Thus, RelA and SpoT did not contribute to *H. ducreyi* survival following heat shock.

Deletion of *relA spoT* decreased uptake of mid-log-phase-grown *H. ducreyi* by macrophages. During both natural and experimental infection, *H. ducreyi* associates with neutrophils and macrophages but remains extracellular (18, 52). LspB is required for the secretion of LspA1 and LspA2, which are responsible for resistance to phagocytosis (53, 54). *lspB* and *lspA2* are cotranscribed; LspB is maximally expressed and LspA1 and LspA2 are maximally secreted during the stationary phase. To determine the role of (p)ppGpp in resistance to phagocytosis, we compared rates of uptake by primary human macrophages of *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* cells grown to the mid-log and stationary phases. No differences were found for the uptake of cells harvested at the stationary phase (Fig. 5A). However, compared to the parent strain, a significant reduction in the uptake of the Δ*relA*Δ*spoT* mutant ($P = 0.0076$) and a trend toward reduction in the uptake of the Δ*relA* mutant ($P = 0.052$) were shown for cells harvested at the mid-log phase. Since the uptake of the mutants was growth phase dependent, we assessed if the mutants had altered transcript levels of *lspB* relative to the parent. By qRT-PCR, *lspB* expression was increased in cells grown to the mid-log phase for both *H. ducreyi* 35000HPΔ*relA* (2.51 ± 0.92, $P = 0.027$) and 35000HPΔ*relA*Δ*spoT* (2.14 ± 0.54, $P = 0.029$) (Fig. 5B). No differences in *lspB* expression were found among the three strains grown to stationary phase (Fig. 5B). Thus, the increased expression of *lspB* in the mutants grown to the mid-log phase may explain their reduced uptake by macrophages.

RelA and SpoT are important for resistance to oxidative stress. (p)ppGpp expression is critical for the survival of bacteria exposed to oxidative stress (30, 55, 56). We compared the survival rates of *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* after incubation with either hydrogen peroxide or paraquat. When mid-log-phase or stationary-phase bacteria were incubated with 0.2 mM or 2 mM H_2O_2, both the *relA* and *relA spoT* mutants survived at significantly lower levels than the parent strain (Fig. 6A). When bacteria in either the mid-log phase or stationary phase were incubated with 0.2 mM or 2 mM paraquat, the *relA* and *relA spoT* mutants survived at significantly lower levels than the parent strain (Fig. 6C). Complementation of the *relA spoT* mutant in *trans* with plasmid pCH30 restored resistance to both 0.2 mM and 2 mM H_2O_2 (Fig. 6B) and 2 mM paraquat (Fig. 6D). Therefore, RelA and SpoT contribute to *H. ducreyi* survival under oxidative stress conditions.

Given that the *relA spoT* mutant was more sensitive to oxidative

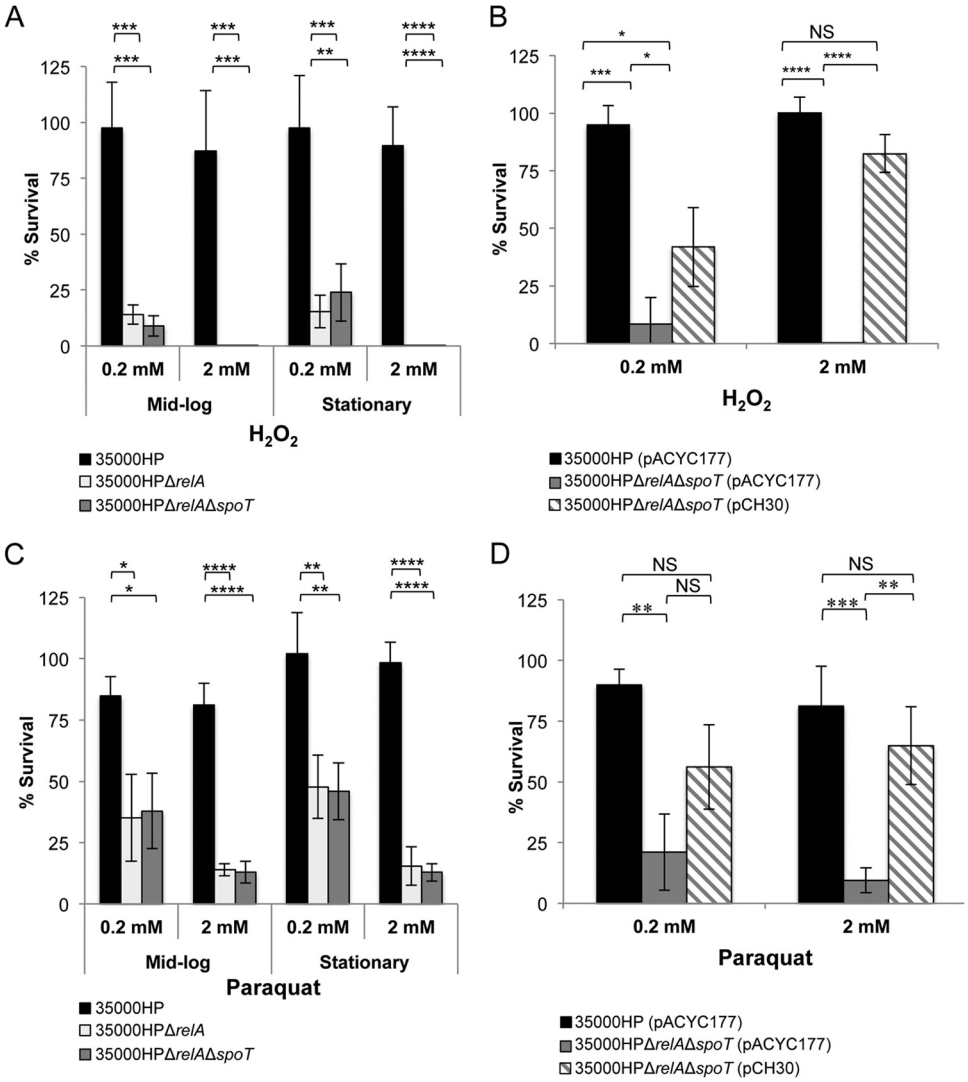

FIG 6 *H. ducreyi* survival after oxidative stress at the mid-log and stationary phases. (A) Percent survival of *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* following incubation with 0.2 mM or 2 mM H_2O_2 for 1 h. (B) Percent survival of mid-log-phase *H. ducreyi* 35000HP (pACYC177), 35000HPΔ*relA*Δ*spoT* (pACYC177), and the complemented 35000HPΔ*relA*Δ*spoT* strain (pCH30) following incubation with 0.2 mM or 2 mM H_2O_2 for 1 h. (C) Percent survival of *H. ducreyi* 35000HP, 35000HPΔ*relA*, and 35000HPΔ*relA*Δ*spoT* following 1 h of incubation with 0.2 mM or 2 mM paraquat. (D) Percent survival of mid-log-phase *H. ducreyi* 35000HP (pACYC177), 35000HPΔ*relA*Δ*spoT* (pACYC177), and 35000HPΔ*relA*Δ*spoT* (pCH30) following incubation with 0.2 mM or 2 mM paraquat for 1 h. All percent survival values were calculated as [(geometric mean CFU after treatment/geometric mean before treatment) × 100]. The data are means ± SD of the results of four independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. NS, not significant.

stress, we investigated whether (p)ppGpp regulated the expression of genes that encode putative oxidative stress-responsive proteins. The *oxyR* and *sodC* genes encode the hydrogen peroxide-inducible gene activator and periplasmic Cu-Zn superoxide dismutase, respectively. Compared to the parent strain, transcript levels of both *oxyR* and *sodC* were unchanged in the 35000HPΔ*relA*Δ*spoT* mutant (data not shown). Thus, the mechanism of (p)ppGpp-mediated resistance to oxidative stress is unclear.

Deletion of *relA* and *spoT* affects the expression of *dksA*. DksA is an important cofactor of (p)ppGpp that helps stabilize the interaction of (p)ppGpp with RNA polymerase (57). As overexpression of *dksA* has been shown to compensate for the loss of (p)ppGpp in other bacteria, we sought to determine if loss of (p)ppGpp affected transcript levels of *dksA*. qRT-PCR showed that, compared to the wild-type strain results, *dksA* was significantly upregulated in the 35000HPΔ*relA*Δ*spoT* mutant harvested from the mid-log phase (4.31 ± 0.87, $P = 0.0025$) and the transition phase (5.33 ± 2.01, $P = 0.03$) of growth. No difference in the levels of *dksA* expression was found between the 35000HPΔ*relA*Δ*spoT* and 35000HP strains grown to the stationary phase. Thus, loss of *relA* and *spoT* may be in part compensated by upregulation of *dksA*.

DISCUSSION

Many bacterial species sense and respond to nutrient stress by utilizing the stringent response, which is regulated by (p)ppGpp. During human infection, *H. ducreyi* is found in the hostile environment of an abscess. Whether *H. ducreyi* is nutritionally stressed in this environment is unknown. Nevertheless, here we showed that an *H. ducreyi* (p)ppGpp^0 mutant is partially attenuated for

virulence in human volunteers, suggesting that (p)ppGpp plays an important role in *H. ducreyi* pathogenesis.

The *H. ducreyi* RelA and SpoT proteins are 59% and 69% identical to their respective homologs in *E. coli*. In *E. coli*, RelA is a monofunctional enzyme that catalyzes the synthesis of (p)ppGpp; SpoT is a bifunctional enzyme that primarily catalyzes hydrolysis of (p)ppGpp and has limited synthetic capabilities (58). We constructed a *relA* single-deletion mutant and a *relA spoT* double-deletion mutant in *H. ducreyi* but were unable to recover a *spoT* deletion mutant. In other organisms, expression of a (p)ppGpp synthase in the absence of a hydrolase leads to growth arrest and cell death; therefore, for many species, *spoT* is considered an essential gene (35, 59). In a radiolabeling-based *de novo* (p)ppGpp synthesis assay, both the *H. ducreyi relA* and *relA spoT* mutants were unable to synthesize (p)ppGpp. As there is no defined medium available for *H. ducreyi*, one limitation of our data is that we did not study (p)ppGpp synthesis in response to nutrient stress, which is usually used to enhance the synthetase activity of these enzymes. Given that deletion of *relA* alone was sufficient to abolish the ability of *H. ducreyi* to synthesize (p)ppGpp and that we were not able to construct a *spoT* mutant in the presence of *relA*, these data suggest that RelA is likely the primary (p)ppGpp synthetase and that SpoT is likely the primary (p)ppGpp hydrolase in *H. ducreyi*.

Recently, (p)ppGpp was shown to have a role in exponential growth (60). The *H. ducreyi relA spoT* mutant grown to the mid-log phase exhibited several phenotypes different from those of its parent that were not seen when the mutant was grown to stationary phase, including significantly reduced uptake by macrophages, increased transcript levels of *lspB*, increased transcript levels of *dksA*, reduction in the expression levels of DsrA, and upregulation in the expression levels of the porin OmpP2B. Given that many of the phenotypes associated with deletion of *relA spoT* were observed in cells grown to the mid-log phase but not in those grown to the stationary phase, these data suggest that (p)ppGpp likely plays a role in gene regulation during the exponential growth of *H. ducreyi*.

Since *H. ducreyi* colocalizes with macrophages and neutrophils during natural and experimental infection, the organism is likely exposed to reactive oxygen species from phagocytes (18, 61). In addition, *H. ducreyi* may be exposed to oxidative free radicals generated endogenously during metabolism. Both the *relA* single mutant and *relA spoT* double mutant showed increased sensitivity to H_2O_2- and paraquat-induced oxidative stress *in vitro*. Taken together, these data suggest that the attenuation of the (p)ppGpp^0 mutant in humans may be in part due to increased sensitivity of the (p)ppGpp^0 mutant to oxidative stress.

The *relA spoT* double mutant formed pustules at a rate significantly lower than the parent in human volunteers and met the criteria for partial attenuation in the model (17). Since OmpP2B is not required for pustule formation in humans (62), the increased expression of OmpP2B by the mutant likely had no effect on its virulence. The increased sensitivity to oxidative stress and the growth-phase-dependent reduction in the expression of DsrA favor attenuation of the mutant. However, the growth-phase-dependent upregulation of *lspB* transcripts, the decreased uptake by macrophages, and the increased longevity of the mutant in the stationary phase favor virulence. DksA phenotypically compensates for the loss of (p)ppGpp in a (p)ppGpp^0 mutant in *E. coli* (63), and the increased transcript levels of *dksA* may have partially compensated for the loss of (p)ppGpp in the mutant and contributed to the partial attenuation of the mutant *in vivo*.

Another limitation of our study is that we examined the (p)ppGpp^0 mutant for only a limited number of phenotypes that are usually associated with the virulence of *H. ducreyi* in human volunteers. Given the pleiotropic effects of ppGpp on gene transcription, it is likely that other factors played a role in the attenuation of the (p)ppGpp^0 mutant. Nevertheless, our findings suggest an important role for (p)ppGpp in *H. ducreyi* virulence in humans. Our findings also highlight the underappreciated roles of (p)ppGpp in regulating phenotypes associated with exponential growth. Transcriptome analysis is under way to delineate the mechanisms underlying (p)ppGpp-mediated functions and its potential pleiotropic effects in *H. ducreyi*. Given that upregulation of *dksA* may partially compensate for the loss of ppGpp, it will be interesting to understand the role of DksA in *H. ducreyi* pathogenesis.

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