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# Activation of the CpxRA System by Deletion of *cpxA* Impairs the Ability of *Haemophilus ducreyi* To Infect Humans[▽]

Stanley M. Spinola,[1,2,3,4]* Kate R. Fortney,[1] Beth Baker,[5] Diane M. Janowicz,[1] Beth Zwickl,[1] Barry P. Katz,[1] Robert J. Blick,[6]† and Robert S. Munson, Jr.[5,7]

*Departments of Medicine,[1] Microbiology and Immunology,[2] and Pathology and Laboratory Medicine[3] and Center for Immunobiology,[4] Indiana University, Indianapolis, Indiana 46202; The Research Institute at Nationwide Children's Hospital[5] and Department of Pediatrics,[7] Ohio State University, Columbus, Ohio 43205; and Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390[6]*

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***Haemophilus ducreyi*** **must adapt to the environment of the human host to establish and maintain infection in the skin. Bacteria generally utilize stress response systems, such as the CpxRA two-component system, to adapt to hostile environments. CpxRA is the only obvious two-component system contained in the *H. ducreyi* genome and negatively regulates the *lspB-lspA2* operon, which encodes proteins that enable the organism to resist phagocytosis. We constructed an unmarked, in-frame *H. ducreyi cpxA* deletion mutant, 35000HPΔ*cpxA*. In human inoculation experiments, 35000HPΔ*cpxA* formed papules at a rate and size that were significantly less than its parent and was unable to form pustules compared to the parent. CpxA usually has kinase and phosphatase activities for CpxR, and the deletion of CpxA leads to the accumulation of activated CpxR due to the loss of phosphatase activity and the ability of CpxR to accept phosphate groups from other donors. Using a reporter construct, the *lspB-lspA2* promoter was downregulated in 35000HPΔ*cpxA*, confirming that CpxR was activated. Deletion of *cpxA* downregulated DsrA, the major determinant of serum resistance in the organism, causing the mutant to become serum susceptible. Complementation in *trans* restored parental phenotypes. 35000HPΔ*cpxA* is the first *H. ducreyi* mutant that is impaired in its ability to form both papules and pustules in humans. Since a major function of CpxRA is to control the flow of protein traffic across the periplasm, uncontrolled activation of this system likely causes dysregulated expression of multiple virulence determinants and cripples the ability of the organism to adapt to the host.**

*Haemophilus ducreyi* causes chancroid, a sexually transmitted genital ulcer disease endemic in Africa, Asia, and the tropics (36). Chancroid is a public health problem because it facilitates the transmission and acquisition of human immunodeficiency virus type 1 (HIV-1) (36).

To determine how *H. ducreyi* causes infection, we developed a human inoculation model in which strain 35000HP (HP, human passaged) and its derivatives are inoculated into the skin of the arm of healthy adult volunteers via puncture wounds (24). Papules develop within 24 h and either spontaneously resolve or evolve into pustules within 2 to 5 days, simulating natural infection. Within 24 h, polymorphonuclear leukocytes (PMN) and macrophages traffic into the wounds on collagen and fibrin scaffolds, surrounding the organism (7). The phagocytes coalesce into an abscess that eventually erodes the epidermis (7, 8). Throughout experimental infection, *H. ducreyi* organisms are found in the abscess and associate with PMN and macrophages, both of which fail to ingest the organism (7). Similar relationships between *H. ducreyi* and host cells are maintained in natural ulcers (9). Thus, *H. ducreyi* must adapt to the hostile environment of an abscess to establish and maintain infection in the human host.

We recently used selective capture of transcribed sequences (SCOTS) (6) and microarrays of the SCOTS-derived sequences (unpublished) to identify genes important to the survival of *H. ducreyi* in pustules. We found that several hundred bacterial transcripts were differentially regulated in pustules relative to their expression levels *in vitro*, suggesting that *H. ducreyi* senses and responds to the host environment.

Gram-negative bacteria generally utilize multiple stress response systems to adapt to changing environments (32, 34). Of these systems, only genes encoding homologues of the alternative sigma factor RpoE and CpxRA are contained in the genome of 35000HP (GenBank accession no. AE017143). Although some Gram-negative bacteria contain over 30 two-component systems (43), CpxRA is the only obvious intact two-component system recognized in the *H. ducreyi* genome. Microarrays of the SCOTS-derived transcripts indicated that several *H. ducreyi* homologues of the *Escherichia coli* CpxRA regulon were upregulated in pustules (unpublished), suggesting that this system is functioning during human infection.

*H. ducreyi* expresses the antiphagocytic proteins LspA1 and LspA2, whose expression is required for virulence in human volunteers and whose secretion is mediated by LspB (23, 25). Recently, Labandeira-Rey et al. showed that a *cpxR* deletion mutant has increased expression of LspB and LspA2 relative to 35000HP and that recombinant CpxR binds to promoter sequences preceding the *lspB-lspA2* operon (25). These data suggest that CpxRA has a direct effect on the expression of LspB and LspA2 and is linked to pathogenesis (25).

In *E. coli*, CpxA is a sensor that spans the cytoplasmic mem-

* Corresponding author. Mailing address: 224 Medical Science Building, 635 Barnhill Drive, Indianapolis, IN 46202-5124. Phone: (317) 274-1427. Fax: (317) 274-1587. E-mail: sspinola@iupui.edu.

† Present address: Institute of Genome Sciences and Policy, Duke University, Durham, NC 27708.

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5α | Strain 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 temperature-sensitive λ*c*I857 repressor | 26 |
| *H. ducreyi* | | |
| 35000HP | Human passaged variant of strain 35000 | 2 |
| 35000HPΔ*cpxR* | 35000HP*cpxR*::*cat* insertion/deletion mutant | 25 |
| 35000HPΔ*cpxA* | 35000HP*cpxA* unmarked, in-frame deletion mutant | This study |
| FX517 | 35000*dsrA*::*cat* insertion mutant | 16 |
| MF35000 | Spontaneous mutant of 35000HP that expresses upregulated OmpP2B | 31 |
| Plasmids | | |
| pRSM2072 | *H. ducreyi* suicide vector | 11 |
| pLS88 | *H. ducreyi* shuttle vector | 15 |
| pML141 | pLS88 derivative containing the *cpxA* ORF flanked by 265 bp upstream and 17 bp of downstream sequences cloned into a SacII site | M. Labandeira-Rey and E. Hansen |
| pRSM2947 | Plasmid containing the origin of replication and kanamycin resistance gene from pLS88 and the FLP recombinase gene from pFT-A | 38 |
| pRSM2975 | Derivative of pRSM2947 containing a point mutation conferring a temperature-sensitive phenotype in *H. ducreyi* | This study |
| pRB157 | pLS88 derivative containing a ΩAmp cartridge, followed by a BglII site for insertion of promoter sequences and a promoterless GFP cassette derived from pGreenTIR (29) | This study |
| pKF1 | pRB157 derivative containing the *lspB* promoter region | This study |
| pKF2 | pRB157 derivative containing the *dsrA* promoter region | This study |

brane and has autokinase, kinase, and phosphatase activities (32, 34, 41). In the absence of envelope stress, the default status of CpxA is as a phosphatase, and the system is inactive (41). CpxA autophosphorylates in response to membrane stress, and activated CpxA donates its phosphoryl group to CpxR, a response regulator, forming CpxR-P. CpxR-P binds to conserved DNA sequences preceding approximately 100 genes, enhancing or inhibiting their transcription (14). In changing environments, the phosphorylation status of CpxR is tightly modulated by CpxA to control membrane traffic and organism integrity (33). In *cpxA* deletion mutants, CpxR accepts phosphate groups from small molecules donors, such as acetyl phosphate (Ac-P), and cannot be dephosphorylated, leading to excess CpxR-P and activating the system (41).

In the present study, we constructed an *H. ducreyi cpxA* deletion mutant, which was unable to initiate infection in humans. Deletion of *cpxA* led to the downregulation of *lspB-lspA2*, suggesting that CpxR was activated in this mutant. Deletion of *cpxA* also resulted in the downregulation of DsrA, an outer membrane protein (OMP) that is the major determinant of serum resistance in this organism (16). The data suggest uncontrolled activation of CpxR downregulates several major virulence determinants of *H. ducreyi* and cripples the ability of the organism to survive *in vivo*.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, oligonucleotides, and culture conditions.** The bacterial strains and plasmids used in the present study are included in Table 1. Selected oligonucleotide primers are listed in Table 2. All *H. ducreyi* strains were grown on chocolate agar plates supplemented with 1% IsoVitaleX and incubated with 5% $CO_2$. For the human inoculation experiments, *H. ducreyi* samples were grown in a proteose peptone broth-based medium with 5% heat-inactivated fetal calf serum as described previously (23). In other experiments, *H. ducreyi* was grown in a Columbia broth-based medium with 2.5% heat-inactivated fetal calf serum as described previously (25). Except as indicated, all *H. ducreyi* cultures were grown at 33°C. When appropriate, the media were supplemented with chloramphenicol, spectinomycin, or kanamycin at 0.3, 200, or 20 μg/ml. *E. coli* strains were grown in Luria-Bertani broth or plates at 37°C, except for strain DY380, which was grown or maintained at 32°C and induced to express λ recombinase at 42°C.

**Construction and complementation of an unmarked, in frame *H. ducreyi cpxA* deletion mutant.** We developed tools to apply "recombineering" methodology (4) to make unmarked, in frame deletion mutants in *H. influenzae* (38) and applied this methodology to *H. ducreyi.* In brief, 70-bp primers were designed to facilitate construction of 35000HPΔ*cpxA.* The forward primer (P1) included 47 bp upstream of *cpxA* plus the ATG codon. The reverse primer (P2) included 21 bp at the 3′ end of *cpxA*, including the TAA codon plus 29 bp of the downstream region. The 3′ end of the primers contained 20 bp complementary to regions 5′ and 3′ of a spectinomycin (*spec*) resistance cassette flanked by flippase (FLP) recognition target (FRT) sites, which had been cloned into pRSM2832 (38). PCR of pRSM2832 with the 70-bp primers yielded a 2-kb amplicon that contained the *spec* cassette flanked by FRT sites and 50 bp of DNA homologous to regions 5′ of and 3′ of *H. ducreyi cpxA*.

TABLE 2. Oligonucleotides used in this study

| Primer | 5′ to 3′ sequence[a] |
|---|---|
| P1 | .....ATCCCTCGCAAGTTCGGGGGATTTTCTCTTTTTAAA GTATTTAATATATG**ATTCCGGGGATCCGTCGACC** |
| P2 | .....ATAGACCGCTTTACCTAAAATGTATTAAATTATTCA AGCCAGAGTGGTAG**TGTAGGCTGGAGCTGCTTCG** |
| P3 | .....TATCTGCTCGGAGTTGCTTATCT |
| P4 | .....ACCGTTTCACCATTCTCGTT |
| P5 | .....AACGTTACCTTCAGCAAGCGGTTC |
| P6 | .....GCCGTTTGGGATCGTCGAGTGTATA |
| P7 | .....GCCATTCCTAATTTCGATGCGCGTTC |
| P8 | .....TTTGAGTGGCTACAGAAAGGCGAC |
| P9 | .....CCAAAGGTACCAAGCGTGTCTGTATCCCAC |
| P10 | .....GGGGATATCATTTAAATACAAGGTCAATGCTCGGC |
| P11 | .....GGAAGATCTCGCTACATCAGTTA |
| P12 | .....GGAAGATCTATTTGTTAAAGTGCTCAC |
| P13 | .....GGAAGATCTATTGGAGTGGACCAGGACAGCATT |
| P14 | .....GGAAGATCTAATCACCTCATTAAGTAAATAAT |
| P15 | .....CCTTCACCCTCTCCACTGACAG |
| P16 | .....TGGACTTGGTCTAATGAAGGCGGT |
| P17 | .....AAACGCCAGGAGCATATGTCACGA |

[a] Boldfaced text represents sequences with homology to the mutagenic cassette. Underlining indicates regions corresponding to restriction enzyme sites as described in text. All *H. ducreyi* sequences are from GenBank accession no. AE017143.

The *cpxRA* operon and ~1.8-kb of DNA 5′ and 3′ of these genes was amplified by PCR using chromosomal DNA as a template, Easy-A polymerase (Stratagene, La Jolla, CA), and primers 3 and 4. The amplicon was cloned into pGEM-T Easy and then transformed into *E. coli* DY380, which contains λ recombinase (26). Induction of λ recombinase generated plasmids where *cpxA* was replaced with the mutagenic cassette, except for the *cpxA* start codon and the terminal 21 bp of the *cpxA* open reading frame (ORF). A SpeI fragment containing the insertionally inactivated *cpxA* gene and flanking DNA was ligated into the suicide vector pRSM2072 (11). The construct was confirmed by sequencing and then electroporated into 35000HP. Cointegrates were selected on chocolate agar plates containing spectinomycin and then resolved by passage on plates containing spectinomycin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (11). Allelic exchange was confirmed by colony PCR.

pRSM2947 contains a temperature sensitive replicon derived from pLS88, a kanamycin resistance cassette and FLP recombinase under the control of a *tet* repressor (38). pRSM2975, a derivative of pRSM2947, whose origin was further altered by a point mutation of G to A at base 2151 of pLS88 (15), so that the plasmid was unable to replicate in *H. ducreyi* at 35°C, was transformed into the mutant. Transformants were selected at 32°C on chocolate agar containing kanamycin. Transformants were grown in broth containing kanamycin at 32°C to mid-log phase, and anhydrotetracycline (200 ng/ml) was added to induce expression of the FLP recombinase. After 2 h, the cultures were diluted on chocolate agar plates that were incubated at 35°C for 48 h to cure the plasmid. Colonies were replica plated on chocolate agar, as well as chocolate agar supplemented with spectinomycin or kanamycin to identify clones that lost both the temperature-sensitive plasmid and the *spec* cassette. The plasmid and the *spec* cassette were lost in 16 of 343 (4.3%) of the clones; 5 clones had a deletion of the expected size by colony PCR. One clone was designated 35000HPΔ*cpxA*. Sequence analysis confirmed that *cpxA* had been replaced by a short ORF that consisted of the start codon of *cpxA*, 81 bp encoding a FLP scar peptide, and the last 21 bp of *cpxA*, including its stop codon.

To complement 35000HPΔ*cpxA* in *trans*, we electroporated the mutant with pML141, which was kindly provided by Maria Labandeira-Rey and Eric Hansen (University of Texas, Southwestern). pML141 contains the *cpxA* ORF flanked by 265 bp upstream and 17 bp of downstream sequences cloned into the SacII site of pLS88. As controls, we also transformed 35000HP and 35000HPΔ*cpxA* with pLS88.

**Human inoculation experiments.** Dedicated stocks of 35000HP and 35000HPΔ*cpxA* were prepared according to FDA guidelines (BB-IND 13064). Five healthy adult volunteers (four females and one male; one Hispanic and four black; mean age ± the standard deviation [SD] 33 ± 14 years) over 21 years of age participated in the study. Subjects gave informed consent for participation and for HIV serology, in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services and the Institutional Review Board of Indiana University-Purdue University of Indianapolis. The experimental challenge protocol, preparation and inoculation of the bacteria, calculation of the estimated delivered dose (EDD), clinical observations, surface cultures, definitions of clinical endpoints, biopsies, and antibiotic treatment of the volunteers were carried out exactly as described previously (24). To account for the correlation among sites within the same individual, comparison of papule and pustule formation rates for the two strains were performed using a logistic regression model with generalized estimating equations (GEE), as described previously (37). The GEE sandwich estimate for the standard errors was used to calculate the 95% confidence intervals (95% CI) for these rates except when the rate was zero and the estimate did not exist. For this case, we calculated the exact binomial confidence interval based on the number of subjects rather than sites, as described previously (37).

To confirm that bacteria isolated from the inocula, surface cultures, or biopsies had the intended phenotype, colonies were replica plated and grown on nitrocellulose filters. The filters were probed with amplicons corresponding to *dnaE* or to the deleted sequences of *cpxA*. The *dnaE* probe was made using primers P5 and P6 and the *cpxA* probe was made by using primers P7 and P8. The probes were labeled with digoxigenin using a DIG DNA labeling kit (Roche Applied Sciences, Penzberg, Germany) and detected with the DIG Easy Hyb protocol (Roche Applied Sciences) according to the manufacturer's instructions.

**CpxR reporter constructs.** pRB157 is a pLS88 derivative that contains a BglII site for insertion of promoter sequences preceding a promoterless green fluorescent protein (GFP) cassette. pRB157 was constructed by amplifying the origin of replication and streptomycin resistance gene from pLS88 by PCR using the primers P9 and P10. These primers contain KpnI, EcoRV, and SwaI sites. The resulting 3.2-kb amplicon was then digested with KpnI and ligated to a KpnI/SmaI fragment from pGreenTIR (29) containing a promoterless *gfp* cartridge. To stop possible plasmid promoter-based transcription into the promoterless *gfp*, an ΩAmp cartridge from pKT254Ω-Ap (17) was inserted into the SwaI site upstream of the *gfp* ORF. Then, a BglII linker, consisting of dimers of the oligonucleotide 5′-CAGATCTG-3′ was ligated into the EcoRV site to yield pRB157, which contains the promoterless *gfp* construct in which DNA fragments with BglII-compatible ends could be inserted in front of the *gfp* ORF. The *lspB-lspA2* (301 bp) and the *dsrA* (178 bp) promoter regions were amplified by PCR and cloned into the BglII site to form pKF1 and pKF2, using the primer pairs P11-P12 and P13-P14, respectively. The orientation of the promoters with respect to the *gfp* cassette was confirmed by PCR by using each promoter-specific forward primer and a reverse primer (P15) that hybridized to the *gfp* sequences downstream of the BglII site. Each of the reporter constructs was electroporated into 35000HP, 35000HPΔ*cpxA*, and 35000HPΔ*cpxR*. Transformants of each strain harboring a specific reporter were grown in broth overnight, diluted into fresh media, and grown to mid log phase. Cells were harvested at various time points during exponential growth. Whole-cell lysates were analyzed by Western blots probed with an anti-GFP polyclonal serum (42) and with the peptidoglycan-associated lipoprotein (PAL)-specific monoclonal antibody, 3B9 (18). PAL is constitutively expressed by *H. ducreyi* and served as a control for protein loading. For each strain, the level of expression of GFP normalized to PAL was determined by densitometry using Adobe Photoshop CS4 (Adobe Systems, Inc., San Jose, CA).

**RNA isolation and real-time PCR.** Bacterial RNA was prepared from mid-log phase organisms by using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was treated twice with DNase I (Ambion, Austin, TX) for 1 h at 37°C and then purified by using the RNeasy system (Qiagen, Valencia, CA). The integrity of the samples was confirmed with an Agilent Bioanalyser (Agilent Technologies, Palo Alto, CA). After optimizing primers so that their efficiencies were >95%, we examined the level of transcript expression in RNA isolated from 35000HP and 35000HPΔ*cpxA*. Reactions utilizing bacterial RNA, the *dsrA* primers P16 and P17 and *dnaE* primers P5 and P6, and the one-step QuantiTect SYBR green reverse transcription-PCR (RT-PCR) kit (Qiagen) were performed in triplicate as described previously (19). The levels of expression were determined by using an ABI Prism 7000 sequence detector (Applied Biosystems, Carlsbad, CA). The data were expressed as the fold change of *dsrA* relative to the level of *dnaE* using the following equation: ratio = $(E_{dsrA})^{\Delta CTdsrA\ (35000HP\ -\ 35000HP\Delta cpxA)}/(E_{dnaE})^{\Delta CTdnaE\ (35000HP\ -\ 35000HP\Delta cpxA)}$, where $E$ is the amplification efficiency (equal to $10^{-1/\text{slope}}$) and $\Delta C_T$ is the change in cycle threshold (30).

**Phenotypic comparisons.** OMPs and lipooligosaccharides (LOS) and were prepared from 35000HP and 35000HPΔ*cpxA* and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (18). Western blots of whole-cell lysates of 35000HP(pLS88), 35000HPΔ*cpxA*(pML141), and 35000HPΔ*cpxA*(pLS88) and FX517, a *dsrA* insertion/deletion mutant, were probed with an anti-DsrA polyclonal serum (16), kindly provided by Christopher Elkins (University of North Carolina, Chapel Hill), or an anti-OmpP2B polyclonal serum (31), kindly provided by Anthony Campagnari (State University of New York at Buffalo). As a control for protein loading, each blot was probed with 3B9 (18).

**Bactericidal assays.** Bactericidal assays were performed exactly as described previously (1, 5), except a single healthy donor was the source of normal human serum (NHS). In the first set of experiments, we compared the survival in 50% NHS of plate-grown 35000HP, 35000HPΔ*cpxA*, and FX517, which is serum susceptible (16). In the second set of experiments, we compared the survival of 35000HP(pLS88), 35000HPΔ*cpxA*(pLS88), and 35000HPΔ*cpxA*(pML141). The data were reported as the percent survival in active NHS compared to that in heat-inactivated serum [(geometric mean CFU in active NHS/geometric mean CFU in heat-inactivated NHS) × 100]. Each experiment was repeated five times, and the arithmetic mean and SD of the percent survival were calculated. Comparison of the strains was performed by using paired Student *t* tests. With the Bonferroni adjustment for multiple comparisons, a $P$ value of <0.017 was considered significant for these assays.

## RESULTS

**An *H. ducreyi cpxA* deletion mutant is impaired in its ability to infect human volunteers.** In *H. ducreyi* 35000HP, *cpxR* and *cpxA* are located in an operon whose gene order is *mazG*, *cpxR*, *cpxA*, and *HD1471* (25). We constructed an unmarked, in-frame *cpxA* deletion mutant, 35000HPΔ*cpxA*, using recombineering methodology (4, 38). Sequence analysis showed that the *cpxA* gene was replaced by a short ORF that encoded a 34-amino-acid peptide and that *cpxR* and *HD1471* were in frame. As determined by quantitative RT-PCR, the expression

TABLE 3. Response to inoculation with live *H. ducreyi*

| Volunteer[a] (gender) | Observation period (days) | Strain[b] | Dose, (CFU)[c] | No. of initial papules | Final outcome of papule | |
|---|---|---|---|---|---|---|
| | | | | | No. resolved | No. of pustules |
| 361 (F) | 6 | P | 55 | 3 | | 3 |
| | | M | 38–150 | 3 | 3 | |
| 362 (M) | 5 | P | 55 | 3 | 3 | |
| | | M | 38–150 | 1 | 3 | |
| 363 (F) | 7 | P | 55 | 2 | 1 | 2 |
| | | M | 38–150 | 2 | 3 | |
| 364 (F) | 5 | P | 42 | 3 | 2 | 1 |
| | | M | 65–261 | 0 | 3 | |
| 366 (F) | 6 | P | 42 | 3 | 1 | 2 |
| | | M | 65–261 | 3 | 3 | |

[a] Volunteers 361, 362, and 363 were inoculated in the first group; volunteers 364 and 366 were inoculated in the second group. M, male; F, female.
[b] P, parent strain 35000HP; M, mutant strain 35000HPΔ*cpxA*.
[c] 38–150, one dose each of 38, 75, and 150 CFU; 65–261, one dose each of 65, 130, and 261 CFU.

level of *HD1471* was unchanged in strain 35000HPΔ*cpxA* compared to strain 35000HP. The growth rates of 35000HPΔ*cpxA* and 35000HP in broth were identical. Gram stains and colonial morphology suggested there were no differences in chaining or clumping of the mutant and the parent. The LOS profiles of the strains were identical (data not shown).

To test whether 35000HPΔ*cpxA* was virulent in humans, we inoculated two groups of volunteers. The first group of three subjects was inoculated with an EDD of 55 CFU of 35000HP at three sites on one arm and with 38, 75, and 150 CFU of 35000HPΔ*cpxA* at three sites on the other arm. The second group of two subjects was inoculated with 42 CFU of 35000HP at three sites on one arm and with 65, 130, and 261 CFU of 35000HPΔ*cpxA* at three sites on the other arm. Overall, papules formed at 93.3% (95% CI, 80.7 to 99.9%) of parent sites and 60% (95% CI, 35.2 to 84.8%) of mutant sites ($P = 0.039$) (Table 3). After 24 h of infection, the mean area of the papules at mutant-inoculated sites was $2.2 \pm 2.0$ mm$^2$, while the mean area of the papules at parent-inoculated sites was $15.1 \pm 10.1$ mm$^2$ ($P < 0.0001$). Pustules formed at 53.5% (95% CI, 28.1 to 78.6%) of sites inoculated with the parent and 0% (95% CI, 0 to 45.0%) of sites inoculated with the mutant ($P = 0.0003$). Thus, deletion of *cpxA* severely impaired the ability of *H. ducreyi* to initiate infection and survive *in vivo*.

Surface cultures obtained at follow-up visits grew *H. ducreyi* from 27% of the parent-inoculated and 0% of the mutant-inoculated sites. All colonies recovered from the parent sites ($n = 102$) and colonies from the parent ($n = 70$) and mutant ($n = 67$) inocula were tested for the presence of *cpxA* and *dnaE* sequences by colony hybridization. The *dnaE* probe hybridized to all of the colonies, whereas the *cpxA* probe hybridized only to the colonies obtained from the parent-inoculated sites or the parent inocula. Thus, there was no evidence of cross-contamination between mutant-inoculated and parent-inoculated sites.

**CpxR is activated in 35000HPΔ*cpxA*.** In the absence of CpxA, CpxR accepts phosphate groups from alternative donors such as Ac-P and cannot be dephosphorylated (41). Relative to the wild type, CpxR-P accumulates in *cpxA* deletion mutants and activates the Cpx regulon when organisms are grown in noninducing conditions, such as logarithmic growth (41).

CpxR downregulates the *lspB-lspA2* operon in *H. ducreyi* (25). To test whether CpxR was activated in 35000HPΔ*cpxA*, the *lspB* promoter region was cloned upstream of a reporter gene encoding GFP to form pKF1. We transformed pKF1 into 35000HP, 35000HPΔ*cpxA* and 35000HPΔ*cpxR* and grew the strains to mid log phase. As determined by Western blotting, the level of GFP expression was downregulated in 35000HPΔ*cpxA* relative to 35000HP and 35000HPΔ*cpxR* (Fig. 1), suggesting that CpxR was activated in 35000HPΔ*cpxA*.

**DsrA expression is repressed and OmpP2B expression is enhanced in 35000HPΔ*cpxA*.** A major function of CpxRA is to control protein traffic across the cytoplasmic membrane. Several OMPs, including DsrA, HgbA, NcaA, and PAL, are required for virulence in humans (24). To discern whether activation of CpxR affected the expression of OMPs, we compared the OMP profiles of 35000HPΔ*cpxA* and 35000HP. 35000HPΔ*cpxA* did not express a 28 kDa OMP, which is the apparent molecular mass of DsrA, a major determinant of serum resistance in the organism (16) (Fig. 2A). 35000HPΔ*cpxA* overexpressed a 43-kDa OMP, which is the apparent molecular mass OmpP2B, a porin expressed by *H. ducreyi*, which is dispensable for virulence in humans (24, 31) (Fig. 2A). Western blot analysis confirmed that DsrA expression was downregulated and OmpP2B expression was upregulated in 35000HPΔ*cpxA* (Fig. 2B and C), suggesting that CpxR regulates the expression of these OMPs.

The OMP profile of 35000HPΔ*cpxA* was very similar to that of a spontaneous mutant (MF35000) of 35000HP (31), which exhibits upregulated expression of OmpP2B. However, MF35000 expressed parental levels of DsrA in Western blot (data not shown). Sequence analysis of MF35000 showed that its *cpxA* and *cpxR* alleles, *ompP2B* promoter region, and *dsrA* promoter region were identical to that of 35000HP (data not shown). Thus, the increased expression of OmpP2B in MF35000 is not likely related to the CpxRA system.

***dsrA* transcription is regulated by activated CpxR.** Since the expression of DsrA was decreased in strain 35000HPΔ*cpxA*, we determined the nucleotide sequence of *dsrA* and its promoter region in 35000HPΔ*cpxA*. The sequence was identical to that of 35000HP, indicating that there were no mutations in the

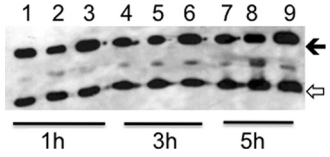


FIG. 1. Regulation of *lspB* promoter activity in the *cpxA* and *cpxR* deletion mutants during exponential growth. Whole-cell lysates of 35000HP(pKF1) (lanes 1, 4, and 7), 35000HPΔ*cpxA*(pKF1) (lanes 2, 5, and 8), and 35000HPΔ*cpxR*(pKF1) (lanes 3, 6, and 9) were probed with an anti-GFP polyclonal serum and with the PAL-specific monoclonal antibody, 3B9. Cells were obtained after 1 h (lanes 1 to 3), 3 h (lanes 4 to 6), and 5 h (lanes 7 to 9) of growth. The solid arrow indicates GFP, and the open arrow indicates PAL. Normalized to PAL, the relative levels of expression of GFP for the *cpxA* mutant were 55, 76, and 75% that of the parent, respectively, after 1, 3, and 5 h of growth. The relative levels of expression of GFP for the *cpxR* mutant were 103, 123, and 98% that of the parent, respectively, after 1, 3, and 5 h of growth. The blot is representative of two independent experiments.

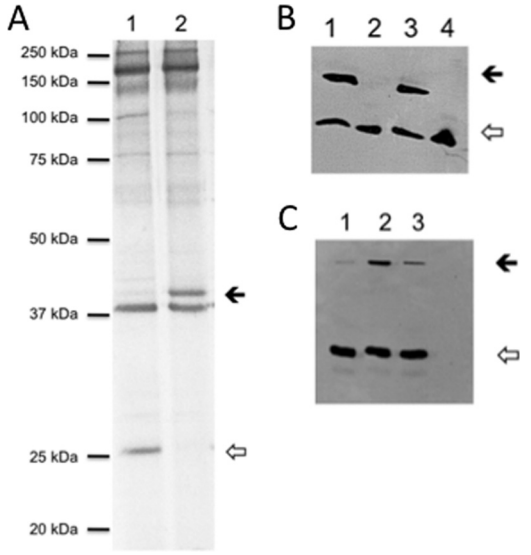


FIG. 2. Phenotypes of the *cpxA* deletion mutant. (A) OMP profiles of 35000HP (lane 1) and 35000HPΔ*cpxA* (lane 2). The closed arrow indicates the 43-kDa band, and the open arrow indicates the 28-kDa band. (B) Whole-cell lysates of 35000HP(pLS88) (lane 1), 35000HPΔ*cpxA*(pLS88) (lane 2), 35000HPΔ*cpxA*(pML1411) (lane 3), and the *dsrA* mutant FX517 (lane 4) probed with an anti-DsrA polyclonal serum and with the PAL-specific monoclonal antibody, 3B9. The closed arrow indicates DsrA, and the open arrow indicates PAL. In some experiments, a faint DsrA band was detectable in 35000HPΔ*cpxA*(pLS88). (C) Whole-cell lysates probed with an anti-OmpP2B polyclonal serum and with 3B9. Lanes 1 to 3 correspond to the first 3 lanes in panel B. The closed arrow indicates OmpP2B, and the open arrow indicates PAL.

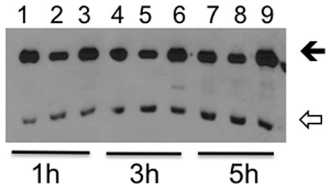


FIG. 3. Regulation of *dsrA* promoter activity in the *cpxA* and *cpxR* deletion mutants during exponential growth. Whole-cell lysates of 35000HP(pKF2) (lanes 1, 4, and 7), 35000HPΔ*cpxA*(pKF2) (lane 2, 5, and 8), and 35000HPΔ*cpxR*(pKF2) (lanes 3, 6, and 9) were probed with an anti-GFP polyclonal serum and with the PAL-specific monoclonal antibody, 3B9. Cells were obtained after 1 h (lanes 1 to 3), 3 h (lanes 4 to 6), and 5 h (lanes 7 to 9) of growth. The solid arrow indicates GFP, and the open arrow indicates PAL. Normalized to PAL, the relative levels of expression of GFP for the *cpxA* mutant were 51, 63, and 82% that of the parent, respectively, after 1, 3, and 5 h of growth. The relative levels of expression of GFP for the *cpxR* mutant were 107, 126, and 154% that of the parent, respectively, after 1, 3, and 5 h of growth. The blot is representative of two independent experiments.

*dsrA* gene or 5′ flanking region. There was a putative CpxR-P recognition sequence (GTAAATAATTGTCAA) at the −29 to −15 position preceding the *dsrA* ORF. As determined by quantitative RT-PCR, the mean ± the SD level of expression of *dsrA* transcripts in 35000HPΔ*cpxA* was (13.1 ± 0.6)-fold downregulated relative to that of 35000HP, after normalization to the housekeeping gene, *dnaE.* To confirm that activated CpxR controlled the expression of *dsrA*, we cloned the *dsrA* promoter region (178 bp) upstream of a GFP reporter gene to form pKF2 and transformed 35000HP, 35000HPΔ*cpxA*, and 35000HPΔ*cpxR* with pKF2. GFP expression was downregulated in the 35000HPΔ*cpxA* background relative to 35000HP and 35000HPΔ*cpxR* during logarithmic growth (Fig. 3), confirming that the transcription of *dsrA* is regulated by CpxR.

**35000HPΔ*cpxA* is serum sensitive.** We compared the survival of 35000HP, 35000HPΔ*cpxA*, and the DsrA-deficient mutant FX517 in 50% NHS, exactly as described previously (5). The mean percent survivals ± the SD were 83% ± 22% for 35000HP, 8% ± 9% for 35000HPΔ*cpxA*, and 2% ± 4% for FX517 (Fig. 4A). Thus, the downregulation of DsrA in 35000HPΔ*cpxA* correlated with increased serum susceptibility of the mutant.

**Complementation of 35000HPΔ*cpxA* restores parental expression levels of DsrA and OmpP2B and partially restores the serum resistance phenotype.** The *cpxA* ORF was cloned into the shuttle vector pLS88 to from pML141. Complementation of 35000HPΔ*cpxA* with pML141 in *trans* restored the expression of DsrA (Fig. 2B). Similarly, complementation restored OmpP2B expression to parental levels (Fig. 2C). We also compared the survival of 35000HP(pLS88), 35000HPΔ*cpxA*(pLS88), and 35000HPΔ*cpxA*(pML141) in 50% NHS. The mean percent survivals ± the SD were 94.8% ± 9.0% for the parent, 2.9% ± 1.7% for the *cpxA* mutant, and 16.2% ± 3.0% for the complemented mutant (Fig. 4B). Thus, complementation of 35000HPΔ*cpxA* partially restored serum resistance to the mutant.

## DISCUSSION

CpxRA is the only obvious intact two-component system contained in the genome of *H. ducreyi*. When tested in the human infection model, 35000HPΔ*cpxA* was highly impaired in its ability to cause disease. Deletion of the *cpxA* gene led to downregulation of a *lspB* reporter gene, strongly suggesting that CpxR was activated in 35000HPΔ*cpxA.* In addition to the downregulation of *lspB*, *dsrA* was profoundly downregulated in 35000HPΔ*cpxA*, and the loss of DsrA expression correlated with increased serum susceptibility of the *cpxA* mutant.

Of 24 *H. ducreyi* mutants previously evaluated in the human challenge model, 7 were classified as fully attenuated, meaning they were unable to form pustules at doses up to 10-fold that of the parent (24; unpublished results). All of the attenuated mutants, including the *dsrA* mutant FX517, formed papules at the same rate as the parent (10, 24). 35000HPΔ*cpxA* is the only mutant identified to date that is impaired in its ability to form both papules and pustules. Thus, the attenuation of 35000HPΔ*cpxA* is likely not solely attributable to the downregulation of DsrA.

Hansen and coworkers have shown that the OMP LspB is required for secretion of the antiphagocytic proteins LspA1 and LspA2 across the outer membrane (40). An *lspB* mutant is as impaired as an *lspA1 lspA2* double mutant in its ability to resist phagocytosis by human granulocytic and murine monocyte-macrophage cell lines (39). Although we did not evaluate 35000HPΔ*cpxA* for resistance to phagocytosis, it is highly likely that 35000HPΔ*cpxA* is impaired in this regard.

In *E. coli*, the conserved sequence for CpxR binding is GT $AAA(N)_5GTAAA$ (14). Using a weighted algorithm based on

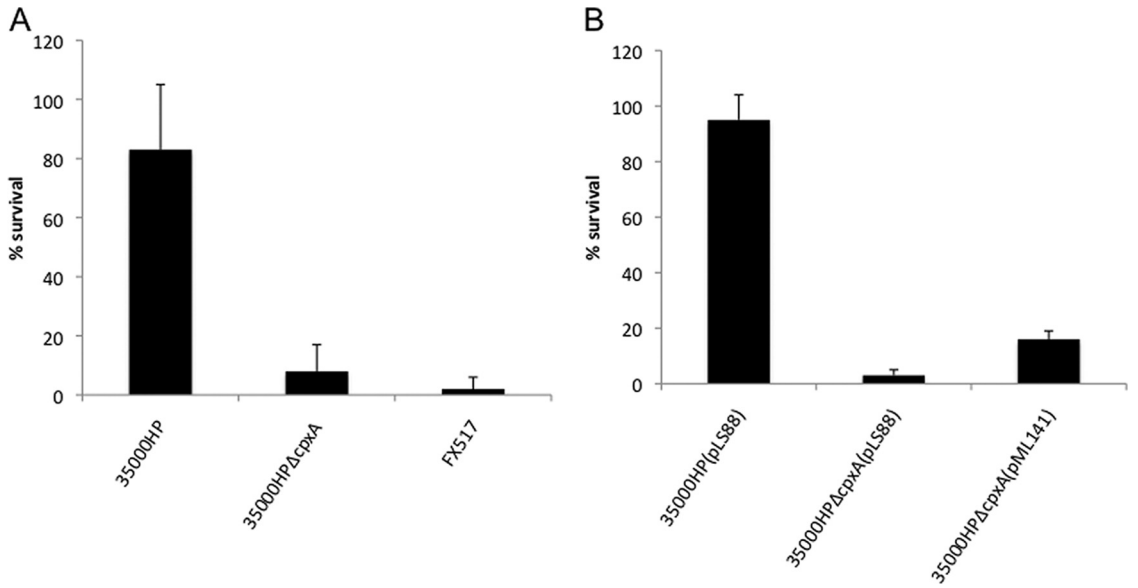


FIG. 4. Bactericidal assays. (A) Percent survival of 35000HP, 35000HPΔ*cpxA*, and FX517 in 50% NHS, calculated as follows: (geometric mean CFU in NHS/geometric mean CFU in heat-inactivated NHS) × 100. The *P* values were as follows: 35000HP versus 35000HPΔ*cpxA*, $P = 0.001$; 35000HP versus FX517, $P = 0.002$; and 35000HPΔ*cpxA* versus FX517, $P = 0.32$. (B) Percent survival of 35000HP(pLS88), 35000HPΔ*cpxA*(pLS88), and 35000HPΔ*cpxA* (pML141) in 50% NHS, calculated as in panel A. The *P* values were as follows: parent versus mutant, $P = 0.0003$; parent versus complemented mutant, $P = 0.0003$; and mutant versus complemented mutant, $P = 0.0023$. For both panels A and B, values are mean plus the SD from five independent experiments.

*E. coli* consensus sequences to search the genome of 35000HP (27), there were 192 putative CpxR targets in *H. ducreyi* (Y. Liu and S. M. Spinola, unpublished data). In addition to *dsrA* and *lspB*, several promoter regions of genes that encode proven virulence determinants in *H. ducreyi*, including *hgbA* and *sapA*, contained putative CpxR recognition sequences. Thus, the *cpxA* mutant is likely attenuated due to dysregulated expression of multiple gene products.

Labandeira-Rey et al. recently showed that *dsrA* and *ompP2B* transcripts are downregulated and upregulated, respectively, when *H. ducreyi* is grown in medium lacking serum versus medium containing serum (25). *H. ducreyi* has a higher growth rate in serum replete medium than in serum deplete medium, suggesting that the organism is relatively stressed in the latter condition, which could activate CpxRA (25). Our observation that *dsrA* was downregulated and *ompP2B* was upregulated in the *cpxA* mutant is consistent with these observations.

We inactivated the *cpxA* gene by use of a "recombineering" strategy, which is especially suitable for making unmarked, in-frame deletion mutants in genes contained in operons. Prior to testing a mutant in an operon in human volunteers, we are required to show that allelic exchange had occurred in the mutant and that the downstream genes are normally transcribed. We are precluded by several regulatory bodies that provide oversight of the human challenge experiments from testing *trans*-complemented mutants in humans. However, complementation of 35000HPΔ*cpxA* in *trans* restored parental levels of expression of DsrA and OmpP2B and partially restored serum resistance to the organism, suggesting that the reduced virulence of the mutant is likely due to deletion of *cpxA* and uncontrolled activation of CpxR.

CpxRA functions in adhesion, controls regulators of virulence, and acts to counter starvation in several organisms (13, 28, 32). However, CpxR is not required for *Salmonella enterica* serovar Typhimurium and *Y. enterocolitica* to infect mice (21, 22) or for *Vibrio cholerae* to colonize the murine intestine (35). Activation of CpxR in *cpxA* deletion mutants or *cpxA** mutants, which have constitutively active kinase activity, downregulates type III secretion translocators and effectors in *E. coli and Yersinia pseudotuberculosis* (12, 28), and both *cpxA** and *cpxA* deletion mutants of serovar Typhimurium are highly attenuated in mice (22). These data have led to a theme in the literature that activation of CpxR reduces bacterial pathogenicity by downregulation of virulence determinants (12, 28, 32). Since a major function of CpxRA is to reduce protein traffic to the periplasm in order to relieve cytoplasmic membrane stress, it is not surprising that virulence factors that traverse the periplasm are downregulated in *cpxA** and *cpxA* deletion mutants, as we observed for 35000HPΔ*cpxA*. In contrast, deletion of *cpxR* causes downregulation of components of the type IV secretion system in *Legionella pneumophila* and downregulation of *lrhA*, a positive regulator of virulence determinants in *Xenorhabdus nematophila* (3, 20). CpxR is required for the virulence of the latter organism in its insect host (20). Taken together, the data suggest that the CpxRA system has different contributions to pathogenesis in different organisms.

In summary, the *H. ducreyi cpxA* mutant was fully attenuated in its ability to cause disease in human volunteers. The mutant could have been attenuated because its CpxRA system was unable to sense and respond to the host, or because it formed too much CpxR-P and downregulated DsrA, LspB, and LspA2 and dysregulated the expression of multiple other virulence determinants that traverse the cytoplasmic membrane. Future studies will address defining the genes controlled by this regulon and evaluating a *cpxR* mutant in humans.

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